THE DEVELOPMENT OF METHODOLOGIES TOWARDS THE ISOLATION OF TRANSCRIPTIONALLY ACTIVE CHROMATIN.

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CERTIFICATION OF SUPERVISOR

In terms of paragraph eight of "General regulations for the degree of PhD" I, as supervisor of the candidate Robin Warren, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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ABSTRACT.

The characterization of transcriptionally active chromatin and it's associated proteins, depends on the identification of unique targets to facilitate the specific enrichment of these domains by affinity chromatography. Three potential targets have been identified and evaluated. 1) The transcription complex. 2) The nascent RNA. 3) Nucleotide sequence.

The transcription complex: The transcription complex forms an integral component of transcriptionally active chromatin and thus was considered to be a unique characteristic of these domains. The affinity of RNA polymerase II, a component of the transcription complex, for the toxin α-amanitin was envisaged as a means of targeting an affinity ligand (biotin) onto the transcription complex. Biotinylated amanitin derivatives with varying spacer arms were synthesized and assayed to determine the efficiency of targeting the biotin binding protein, streptavidin, onto the transcription complex. The novel photoreactive derivative, Bio-32-NAP-amanitin, covalently biotinylated the transcription complexes and simultaneously facilitated the targeting of streptavidin.

The specific targeting of streptavidin onto the transcription complex has enabled the development of a new technique to localize these complexes within the nucleus at the molecular level, through the electron microscope.

Streptavidin affinity chromatography of the biotinylated transcription complexes was complicated by the finding that the biotinyl residue was inaccessible to conventionally immobilized streptavidin. Various methods were investigated to translocate the biotin or streptavidin to increase accessibility. The spacer arm bis-biotinyl-dextran was found to alleviate all sterical constraints and facilitated the specific retention of the transcription complexes on the solid phase. However, specific enrichment could not be demonstrated due to the absence of a release mechanism. With the development of future technologies in the field of immobilized streptavidin, it is envisaged that specific enrichment of the photochemically biotinylated transcription complexes will be possible.

Nascent RNA: Nascent RNA is linked to the transcriptionally active chromatin via the transcription complex and therefore was proposed as a potential target. The affinity ligand Hg was incorporated into the nascent RNA by *in vitro* transcription,

generating a Hg labelled soluble ternary transcription complex. However, sulfhydryl affinity chromatography was unable to retain these complexes, demonstrating that the Hg groups were either inaccessible or blocked. Further analysis has shown that the nuclear proteins bind to the Hg-RNA via ionic and sulfhydryl interactions. These interactions could only be reversed by partial protein denaturation, which in turn caused dissociation of the histones. This implies that the nascent RNA no longer constitutes a target for the isolation of transcriptionally active chromatin.

Nucleotide sequence: The nucleotide sequence is a unique characteristic of an individual gene and of transcriptionally active chromatin, if the respective gene is in the process of being transcribed. Nucleoprotein hybridization offers a method of specifically introducing an affinity ligand onto target chromatin domains. Various biotinylated DNA probes complementary to the 5' end of the histone H2B gene (*Parachinus Angulosis*) were constructed and their hybridization and streptavidin retention properties were evaluated.

Specific hybridization was demonstrated between the probe and the histone gene quintet chromatin fragments, which inturn were retained by immobilized streptavidin. Nuclease catalyzed release was shown to be complete, however, non-specific retention to the solid phase prevented solubilization of the target chromatin. Systematic analysis of commercially available streptavidin matrices showed that non-specific binding could be dramatically reduced. This enabled the specific enrichment of the target histone gene quintet chromatin. Thus, in principle this technique would enable the enrichment of transcriptionally active chromatin.

CONTENT INDEX.

OVERVIEW.

PRINCIPLES OF METHODOLOGY FOR THE ISOLATION OF ACTIVELY TRANSCRIBING CHROMATIN.

The eucaryotic genome of higher organisms contains in the order of 10^9-10^{10} base pairs, which translates to a DNA length of approximately 2-3 meters and is compacted into a 10 μm diameter nuclear sphere. This compaction is achieved by folding the DNA into a hierarchy of higher order structures. Initially 145 base pairs of DNA are folded around the histone octamer containing two histones each of H2A, H2B, H3 and H4. This forms the nucleosome core. The binding of histone H1 to the core and the internucleosomal DNA induces nucleosomal condensation into the 30 nm fibre, which in turn is packaged into higher order structures in the presence of scaffold proteins (van Holde, 1988).

Early electron micrographs of sectioned nuclei showed that there are two forms of chromatin condensation, namely heterochromatin a highly compact state and euchromatin a loosely compacted state (see review Franke *et al.*, 1978). A correlation between transcriptional activity and the degree of chromatin condensation suggested that the transcribed DNA was located within the euchromatin (Littau *et al.*, 1965). This open structured conformation facilitates, presumably, the access of the transcription machinery to the DNA template so that specific transcription may occur. This then leads to the question: What are the specific characteristics of euchromatin that allow transcription and how do their structures differ from that of heterochromatin? It has been suggested that the histones must play, because of their function as structural chromosomal proteins, a major role in the regulation of gene expression.

Nuclease sensitivity. In the past three decades various methods have been developed to separate active from inactive chromatin. The first methods developed used mechanical shearing to randomly shear the chromatin producing a soluble fraction (Fenster *et al.*, 1963), however, due to the indiscriminate nature of this method other fractionation procedures based on physicochemical properties of the euchromatin were required. These included, ECTHAM-cellulose chromatography (Reeck *et al.*, 1972), glycerol gradient centrifugation (Murphy et al., 1973), gel exclusion

chromatography (Janowski *et al.*, 1972) and buoyant density centrifugation (Rickwood *et al.*, 1974). However, enrichment of transcribed sequences was not observed with these methods (Kreig and Wells, 1976; McCarthy *et al.*, 1973).

Marushige and Bonner (1971) and Gottesfeld *et al.* (1974) used the nuclease DNAse II to digest the chromatin, proposing that this nuclease may recognize a unique feature of active chromatin. The resulting solubilized chromatin was then further fractionated under near physiological conditions by the addition of mono or divalent ions. Analysis of the chromatin fraction showed the co-isolation of endogenous RNA polymerase activity (Marushige and Bonner, 1971), and a 2-4 fold enrichment of the actively transcribed globin gene (Gottesfeld and Partington, 1977). The nucleosome repeat was similar to that of bulk chromatin, which prompted the authors to conclude that DNase II preferentially digested the open structured chromatin, (Gottesfield and Melton, 1978). However, this method to enrich active chromatin is controversial (see review Mathis, 1980).

Weintraub and Groudine (1976) used the nuclease DNAse I as a probe for chromatin conformation and showed that both the actively expressed and potentially active genes were hypersensitive in comparison to the non expressed genes. These results were interpreted to demonstrate that nucleosomes in active chromatin exhibit an altered conformation. Initially probing with micrococcal nuclease revealed no preferential digestion, which was attributed to the presence of nucleosomes inhibiting digestion. However, in a later study Bellard *et al.*, (1977), demonstrated that micrococcal nuclease released nucleosomes from transcribed genes at a faster rate than those of non transcribed genes and concluded that preferential recognition of the extended structure of active chromatin was responsible. This altered chromatin conformation was shown to be a feature of the active nucleosome core rather than the higher order folding induced by the linker binding histones (Villeponteaux *et al.*, 1978).

Bloom and Anderson (1978) used the selective activity of micrococcal nuclease to solubilize a mononucleosome fraction enriched 5-6 fold with the transcribed ovalbumin sequence. Similar results were shown by Levy-Wilson and Dixon (1978 and 1979a), using a combination of micrococccal nuclease digestion and differential salt solubility to obtain an enrichment of 7 fold for sequences complementary to poly-A containing RNA within the mononucleosome fraction. They further demonstrated that this fraction was enriched in the non histone protein H6 (analogous to HMG 14 and 17), and depleted in histone H1 (Levy-Wilson *et al.*, 1979b).

Non-Histone Proteins. The results achieved with nuclease digestion focused the direction of research on determining what structural features conferred the nuclease sensitivity within the active nucleosome. Reconstitution experiments, in which 0.35 M NaCl washed chromatin was reconstituted with the 0.35 M NaCl eluate, restored DNase I hypersensitivity to the expressed globin gene (Weisbrod and Weintraub, 1979). This suggested that the non-histone proteins, HMG 14 and 17 released by 0.35 M NaCl (Goodwin and Johns, 1973), were involved in conferring the nuclease sensitive structure (Wiesbrod and Weintraub, 1979; Gazit *et al.*, 1980). Similarly, reconstitution with purified HMG 14 and 17 specifically restored nuclease sensitivity to mononucleosomes containing actively transcribed genes, suggesting that a unique non-elutable feature resided on nucleosomes in the active chromatin (Wiesbrod *et al.*, 1980). These findings were conflicting, as HMG containing mononucleosomes electrophoretically fractionated showed no increase in DNase I sensitivity relative to non HMG containing nucleosomes and contained non-transcribed sequences. It was suggested this may have been due to a redistribution of the HMG proteins during nuclease digestion (Nicolas *et al.*, 1983), or that the HMG proteins are distributed on both transcribed and non-transcribed genes (Seale *et al.*, 1983).

Based on the concept of a specific recognition, an HMG affinity chromatography technique was developed to enrich active nucleosomes (Wiesbrod and Weintraub, 1981; Weisbrod, 1982). The results demonstrated the specific enrichment of transcribed sequences within the bound nucleosome fraction. Swerdlow and Varshavsky (1983), explained that the specific recognition of nucleosomes attached to transcribed DNA by the HMG proteins, resided in the length of the DNA tails resulting at the time of nuclease digestion rather than an altered nucleosome structure. In order to circumvent this problem of the recognition of HMG's on nucleosomes, a monoclonal antibody directed against HMG 17 was used in conjunction with immunoprecipitation to precipitate an oligonucleosome fraction (Dorbic and Wittig, 1986). Hybridization analysis revealed an enrichment factor of between 60 to 100 fold for active versus inactive chromatin, so confirming the association of HMG's with active chromatin. However, the authors point out that this enrichment decreases with increasing oligonucleosome length as a result of the distribution of HMG proteins on inactive chromatin, with a frequency of 1 per 20 nucleosomes. In contrast to this observation Blanco *et al.* (1985), using a similar technique, showed that HMG-T was associated with inactive genes.

Histone Acetylation. The postsynthetic acetylation of histones lowers their net positive charge, possibly resulting in an alteration of the nucleosome conformation. Allfrey *et al.* (1964), implicated this modification in the regulation of transcription. This hypothesis was further investigated by probing the chromatin structure of butyrate treated cells (which causes hyperacetylation (Riggs *et al.*, 1977)) with DNase I (Vidali *et al.*, 1978). The results demonstrated an enhanced nuclease sensitivity, correlated with the hyperacetylation of the histones H3 and H4. Similarly, chromatin fractionation techniques based on the selective salt solubility of active chromatin, indicated the enrichment of acetylated histones within the active fraction (Davie and Candido, 1978; Levy-Wilson *et al.*, 1979c; Nelson *et al.*, 1980). This preferential solubility has been directly linked to acetylation promoting the decondensation of chromatin, (Perry and Chalkley, 1981; Nelson *et al.*, 1986), however, Ridsdale and Davie (1987) concluded that the depletion of histone H1 is the main contributing factor. A further correlation between histone acetylation and active chromatin was shown for nucleosomes isolated by HMG affinity chromatography (Weisbrod, 1982) or HMG immunoaffinity chromatography. (Malik *et al.*, 1984).

Johnson *et al.* (1987), showed that the histone H3 was hyperacetylated in the unfolded nucleosomes, present on the ribosomal genes of *Physarum*, and proposed that this modification functioned to open the nucleosome conformation. Allegra *et al.* (1987), reported a method to selectively purify the unfolded nucleosomes, based on an open structure exposing the sulfhydryl group of histone H3 and thereby facilitating the binding to a mercurated solid phase. Hybridization analysis of the bound nucleosomes indicated a 3-4 fold enrichment in transcribed sequences, and that the associated histones H3 and H4 were hyperacetylated. Further analysis into the mode of binding to the mercurated solid phase revealed that two factors were responsible, namely SH (sulfhydryl) containing non histones and the SH of histone H3 (Walker *et al.*, 1990). Both fractions were enriched in transcribed sequences and hyperacetylated histones. More recent experiments using mutant yeast strains revealed that hyperacetylation can not fully account for the nucleosome conformation formed in active chromatin and the authors propose that other factors may be involved. (Chen *et al.*, 1991). In support of this, a number of groups have shown that hyperacetylation alone does not disrupt the nucleosome core, but that it decreases the intranucleosomal forces, and in the presence of a destabilizing factor, an unfolded nucleosome results. (Oliva *et al.*, 1990 and review therein). Positive supercoiling ahead of the transcription complex has been proposed as a mechanism to unfold the nucleosomes into a structure which is subsequently stabilized by a combination of non histone binding and histone modification (Lee and Garrad, 1991).

A direct link between histone acetylation and transcriptionally active chromatin was demonstrated by Hebbs *et al.* (1988). It was shown that antibodies directed against acetylated H4, used in conjunction with immunoprecipitation, selected a hyperacetylated chromatin fraction. Hybridization analysis showed an enrichment of up to 30 fold for the active globin gene, confirming the association between acetylation of histones and transcription. Ip *et al.* (1988), fractionated chromatin by density gradient ultracentrifugation after crosslinking with formaldehyde, and showed that differential density of the active chromatin was dependent on the frequency of transcription. The histones associated with the active fraction were hyperacetylated and showed a higher turnover of acetate relative to the bulk chromatin. The precise function of acetylation with respect to transcriptional activity has yet to be determined. (see review Turner, 1991).

Histone Ubiquitination. The ubiquitination of histones has been correlated with chromatin decondensation (Matsui *et al.*, 1982), and proposed to be associated with transcriptionally active genes (Levenger *et al.*, 1982). Ridsdale and Davie (1987), reported that polynucleosomes isolated by salt solubility and gel exclusion chromatography were enriched 50 fold in the actively transcribed globin gene. Comparison of the histone content revealed that ubiquitinated histones H2A and H2B were enriched within the active fraction. In contrast Dawson *et al.* (1991), using a combination of nick translation and affinity chromatography to enrich transcribing genes (Dawson *et al.*, 1989), showed an equal distribution of ubiquitinated H2A within the bound and unbound fractions. As both these methods resulted in incomplete fractionation, Nickel *et al.* (1989), investigated the distribution of ubiquitinated histones within the transcriptionally active macro and inactive micro nuclei of *Tetrahymena*. Their results show that ubiquitinated H2B is preferentially associated with the transcriptional active domains, while ubiquitinated H2A is found in both the expressed chromatin and repressed chromatin. They conclude that this modification has a role in maintaining the structure of transcriptionally active chromatin.

Lability of H2A-H2B dimers. The altered nucleosomal conformation is proposed to relieve the inhibitory effect of nucleosomes, allowing transcription to proceed. Baer and Rhodes (1983), questioned whether RNA polymerase II could recognize this unique feature. Reconstitution of RNA polymerase II with nucleosome cores produced a complex enriched 10 fold in transcribed genes, confirming a specific

recognition. Protein analysis of the complex revealed the loss of an H2A-H2B dimer from the bound octamer, suggesting that this may be a feature of the active nucleosome. In support of this model, *in vitro* transcription on reconstituted nucleosomes templates lacking one H2A-H2B dimer showed an increase in the efficiency of transcription, indicating that the depleted core may be a intermediate of *in vivo* transcription (Gonzalez and Palacian, 1989). Furthermore, in sea urchin embryos , which exhibit a very high transcription rate, the H2B-H2A dimer is more rapidly turned over than the H3-H4 dimer. Suggesting a preferential loss and replacement of the dimer pair within the core particle during transcription (Schwager *et al.*, 1985).

Reconstitution of these hexamer cores with HMG 14 and 17 failed to increase the transcription rate, however structural stabilization was observed (Gonzalez and Palacian, 1990). Suggesting that HMG's may be a component of transcriptionally active chromatin. No evidence has been sought for the existence of such H2A-H2B depleted nucleosomes in the active chromatin fraction by other enrichment procedures.

Gene Sequence. The nucleotide sequence of a individual gene or gene family is a unique characteristic and therefore a target for the isolation of transcriptionally active chromatin. Workman and Langmore (1985b), developed the nucleoprotein hybridization technique to enrich a specific subset of active genes. This method involved the hybridization of an mercurated DNA probe complimentary to an exposed sequence within the target gene, followed by sulfhydryl affinity chromatography. Vincenz *et al.* (1991) using a similar technique showed that the target gene could be enriched by a factor >700 fold. However, the yield was low making it difficult to characterize the chromatin proteins. It can be envisaged that this technique, with increased yields will provide an insight into the structure of active chromatin.

Future methodologies. The criteria that have been applied for the development of techniques to enrich active chromatin have largely depended upon characteristics that are not unique to actively transcribing chromatin. This implies that the purification observed was not absolute and therefore the conclusions drawn as to the properties of active chromatin remain controversial. Future enrichment methods will have to be based on known unique components of the transcribing chromatin. The transcription machinery which includes RNA polymerase II, nascent RNA and other associated

proteins can be described as unique complexes, specifically bound to the transcribing chromatin. These characteristics have not yet been fully investigated as targets for the development of enrichment methodologies.

CHAPTER 1

α-AMANITIN DIRECTED BIOTINYLATION OF RNA POLYMERASE II TRANSCRIPTION COMPLEXES.

INTRODUCTION:

The cytotoxin α-amanitin is a bicyclic octapeptide occurring in high concentrations in the toadstool *Amanita Phalloides* (see review Wieland, 1986). The toxicity is primarily based on the inhibition of mRNA transcription catalyzed by RNA polymerase II in eucaryotic cells. α-Amanitin binds to the 140 kD B3 subunit of RNA polymerase II forming a 1:1 complex with a dissociation constant of 10^{-9} M (Cochet-Meilhac and Chambon, 1974; Brodner and Wieland, 1976). This binding is both temperature and salt concentration dependent and has a half life of 2 days. The mechanism of inhibition is the arrest of the enzymatic translocation of RNA polymerase II along the DNA template, phosphodiester bond formation is not inhibited, as was proposed in an earlier study (Vaisius and Wieland, 1982). Furthermore, the binding of α-amanitin does not prevent nucleotide triphosphate binding or promote dissociation of the DNA-RNA polymerase II complex. In fact, the binding of α-amanitin has been suggested to stabilizes the ternary transcription complex (Vaisius and Wieland, 1982).

The sequential chemical modification of the individual amino acids of α-amanitin has indicated their relative contribution to the binding constant. These data have been used to orientate the molecule within the binding pocket (Wieland, 1986). According to a model based on such data, the hydroxytryptophan indole nucleus is positioned on the non-binding surface of the toxin and protrudes into the solvent. Hence, this residue can be modified without dramatically influencing the binding (Faulstich *et al.*, 1981; Falk-Pedersen *et al.*, 1982). Two types of substitution have been investigated for the indole nucleus; 1) Diazo coupling with diazonium salt (Falk-Pedersen *et al.*, 1982), because the azo linkage is sensitive to biological reducing agents this method has limited application. 2) Etherization of the 6'-OH group with alkyl halides, after transformation into the phenolate ion (Faulstich *et al.*, 1981). This modification is stable and allows for the introduction of a variety of functional spacer moieties, while retaining 30% of the inhibition capacity.

Traditionally, the specific inhibitory characteristics of α-amanitin have been applied to determine the level of RNA polymerase II activity during *in vitro* transcription. Chemical derivatization has increased the functional application of α-amanitin as a tool in molecular biology. The areas of research that have been focused on are: 1) Cell toxicity and 2) RNA polymerase II.

Cell toxicity: The degree of α-amanitin toxicity is dependent on specific uptake mechanisms, characteristic of the cell type. Derivatization of α-amanitin has been proposed to increase the non-specific uptake of the toxin into resistant cells. Faulstich *et al.* (1981), showed that lipophillic derivatives enhanced the toxicity three fold, however, this effect is not related to the carbon chain length and therefore may be mediated via protein interactions. Similar results were observed for protein conjugates (Faulstich and Fiume, 1985). IgG conjugates have been used to target α-amanitin towards specific cell types to use them as "magic bullets" (Davis and Preston, 1981). Bermbach and Faulstich (1990), prepared a epidermal growth factor (EGF)-poly ornithine amanitin complex, which showed a 5-10 fold enhanced cytotoxicity to cells bearing the EGF receptor. The authors propose that complexes of this nature may have therapeutic value.

RNA polymerase II: The location of RNA polymerase II during the cell cycle was determined using a fluorescent α-amanitin derivative (Wulf *et al.*, 1980). The results showed, at interphase that the polymerase was exclusively located in the nucleoplasm and during mitosis RNA polymerase II was evenly distributed within the cytoplasm with no specific binding to the chromosomes. However, during metaphase and anaphase fluorescence was associated with the centrioles, which was explained by the presence of other α-amanitin binding proteins.

α-Amanitin-Sepharose 4B was the first affinity chromatography matrix used to purify RNA polymerase II from crude extracts, resulting in a 100 fold purification (Lutter *et al.*, 1984). This methodology was improved with the synthesis of a soluble biotinyl-amanitin derivative, which was used in conjunction with an avidin matrix to purify RNA polymerase II from nuclei extracts (Vaisius and Faulstich, 1986). The native RNA polymerase II eluate showed the co-isolation of actin, however, the mechanism of the actin retention has not been fully investigated. The mild conditions of this technique may favor the co-isolation of RNA polymerase II binding proteins.

The compartmentalization of the chromatin within the eucaryotic nucleus has been proposed to be spatially ordered, facilitating transcription, RNA processing and RNA

transport (review Jackson, 1991; Carter and Lawrence, 1991). Hutchison and Weintraub (1985), applied the technique of nick translation with biotinyl-UTP to localize the active chromatin within the interphase nucleus. Their results indicated a peripheral location of DNAse I sensitive chromatin situated along the interchromatin channels that communicate with the nuclear pores. This technique was adapted for visualization by immunoelectron microscopy using a combination of anti-biotin and IgG colloidal gold (Thiry, 1991a and 1991b), and a similar peripheral location was observed. In contrast, Frankan *et al.* (1980) showed by autoradiography that radiolabelled nascent RNA was distributed throughout the interior of the nucleus. Furthermore, integration of the transcriptionally active Epstein-Bar Virus genome into lymphocytes indicated positioning in the inner nuclearsphere, suggesting that in some cases active genes are located in the interior (Lawrence *et al.*, 1988; Carter and Lawrence, 1991). Recent evidence shows that transcription is focused into a number of domains within the nucleus (Jackson *et al.*, 1993). The RNA transcripts located within these domains appear to be nuclear matrix bound (Xing and Lawrence, 1991; Jackson *et al.*, 1993). Intron splicing occurs as a function RNA migration away from the site of transcription, suggesting that the RNA processing machinery is spatially organized along the RNA track (Xing *et al.*, 1993).

The criteria for determining the location of active chromatin or newly transcribed RNA within the nucleus have been based on: 1) Nuclease sensitivity. 2) "In situ" hybridization. 3) *In vitro* transcription. However, the localization of the transcription machinery in the nucleus has not yet been demonstrated through the positive identification of a specific protein of that machinery.

I envisage a soluble biotinyl-amanitin derivative (Vaisius and Faulstich, 1986), to be a probe for the transcription complexes within the eucaryotic nucleus. The bifunctional binding properties of this biotinyl-amanitin imply that biotin binding proteins could be targeted to the transcription complex facilitating labelling.

METHODS AND MATERIALS:

Distillation and drying of organic solvents.
(according to Vogel, 1970)

Preparation of molecular sieve 3Å.

Molecular sieve 3Å was washed with ethanol followed by extensive washing with H_2O to remove all traces of organic material and fines. The beads were then dehydrated by baking at 300° C for 48 hours and stored under vacuum in a desiccator.

Dimethylformamide and dimethylsulfoxide.

Dimethylformamide or dimethylsulfoxide (AR grade; Merck) was distilled under vacuum, with a constant flow of dry nitrogen, at between 60 at 65° C. The initial 20 % of the distillate was discarded to remove all impurities and H_2O and the following fraction was collected. The purified dimethylformamide or dimethylsulfoxide was then stored over molecular sieve, under nitrogen and used within 5 days to ensure the absence of breakdown products.

Ethanol.

Ethanol (AR grade; Merck) was initially dried with the addition of molecular sieve 3Å (25 % v/v), followed by an incubation period of 24 hours. Cleaned magnesium fillings (1.25 g) and a spatula tip of iodine crystals were added to 50 ml dry ethanol and gently refluxed until most of the magnesium had been converted to magnesium ethylate. 200 ml of dry ethanol was then added and refluxing was continued for 1 hour to covert all traces of H_2O to magnesium hydroxide. The ultra dry ethanol was then distilled at 78° C with an anhydrous $CaCl_2$ vent. The initial distillate (50 ml) was discarded and the following 50 ml was collected. Molecular sieve 3Å was immediately added (15 ml) and the container was flushed with dry nitrogen and stored for 24 hours before use.

Triethylamine and N-methylmorpholine.

Ninhydrin (0.1 g) and potassium hydroxide (1 g) were added to either triethylamine or N-methylmorpholine (100 ml) and mixed at 24° C for 2 hours. The triethylamine or N-methylmorpholine was then distilled at 89° C and 116° C, respectively, with a $CaCl_2$ vent. The initial distillate (20 ml) was discarded and the following 40 ml was collected and stored in the dark.

Tetrahydrofuran.

Freshly pressed sodium wire was added to 500 ml tetrahydrofuran (AR grade; Merck) and incubated for 24 hours. The tetrahydrofuran was then distilled at 66° C with a $CaCl_2$ vent. The initial distillate (100 ml) was discarded and the following 200 ml was collected and stored under dry nitrogen.

Synthesis of biotinylated α-amanitin derivatives.

Biotin-N-hyroxysuccinimide ester.
(according to Jasiewiewicz *et al.*, 1976).

Briefly, biotin (1 g; 4.09 mmol) and N-hydroxysuccinimide (515 mg; 4.48 mmol) were dissolved in 33 ml dry dimethylformamide. Dicyclohexylcarbodiimde (844 mg; 4.09 mmol) in 11 ml dry dimethylformamide was then slowly added over 30 min and the reaction was shaken at 55° C for 16 hours. The acyl urea was precipitated by cooling to 4° C and collected by filtration. The solvent was removed from the filtrate by rotary evaporation at 65° C and the resulting residue was dissolved in dry isopropanol by refluxing for 30 min. The biotin-N-hydroxysuccinimide ester crystallized on cooling and was collected by filtration and dried in vacuo.

Biotinaminocaproyl-N-hydroxysuccinimide ester.
(adapted with modifications from to Costello *et al.*, 1979).

Biotin-N-hydroxysuccinimide (340 mg; 1 mmol) dissolved in 8 ml dry dimethylformamide was added to ε-aminocaproic acid (131 mg; 1 mmol) in 8 ml 100

mM $NaHCO_3$ pH 8.0 and stirred at 21°C for 4 hours. The solution was acidified to pH 2.0 by the addition of dilute HCl, which resulted in the formation of a heavy precipitate. This precipitate was collected by filtration and washed with cold 10 % citric acid followed by washing with cold H_2O. The product biotinaminocaproic acid was dried in vacuo and then against phosphorous pentoxide at 37°C for 72 hours. A single cinnemaldehyde positive spot was observed when assayed by thin-layer chromatography on silica F_{254} plates developed with benzene/methanol (7:3), with an $R_f = 0.13$.

Biotinaminocaproic acid (305 mg; 0.86 mmol) and N-hydroxysuccinimide (118 mg; 1.03 mmol) were dissolved in 15 ml dry dimethylformamide. Dicylohexylcarbodiimide (195 mg; 0.946 mmol) in 5 ml dry dimethylformamide was then slowly added over 30 min and the reaction was shaken at 55° C for 16 hours. The acyl urea was precipitated by cooling to 4° C and collected by filtration and the solvent was then removed from the filtrate by rotary evaporation at 55° C. The residue was washed 3 times with dry diethylether and then dissolved in 75 ml dry isopropanol by refluxed for 45 min. The biotinaminocaproyl-N-hyroxysuccinimide ester crystallized on cooling and was collected by low speed centrifugation and dried in vacuo. A single cinnemaldehyde positive spot was observed when assayed by thin-layer chromatography on silica F_{254} plates developed with benzene/methanol (7:3), with an $R_f = 0.3$.

αN-biotinaminocaproyl-εN-(Boc)-lysyl-N-hyroxysuccinimide ester.

Biotinaminocaproyl-N-hydroxysuccinimide ester (96 mg; 0.2 mmol) in 2 ml dimethylformamide was added to εN-Boc-lysine (50 mg; 0.2 mmol) dissolved in 2 ml 100mM $NaHCO_3$ pH 8.0 (requires gentle warming) and mixed at 21° C for 16 hour. The solvent was removed by repeated freeze drying and the residue dissolved in a minimum volume of H_2O and acidified to pH 2.0 with dilute HCl. The resulting precipitate was collected by low speed centrifugation and washed 3 times with cold 0.1M HCl and 3 times with cold H_20 followed by freeze drying. The product αN-biotinaminocaproyl-εN-(Boc)-lysine showed a single cinnemaldehyde positive spot with an $R_f = 0.21$ when assayed by thin-layer chromatography on silica F_{254} plates when developed with chloroform/methanol (1:1).

αN-biotinaminocaproyl-εN-(Boc)-lysine (50 mg; 86 μmol) was dissolved in 4 ml dry dimethylformamide by heating to 70° C. N-hyroxysuccinimide (11.9 mg; 103μmol)

together with dicylohexylcarbodiimide (17.7 mg; 86 μmol), dissolved in 2 ml dry dimethylformamide were then added and the reactants were incubated at 21° C for 16 hours. The acyl urea was precipitated by cooling to 4° C and removed by low speed centrifugation. The solvent was removed from the filtrate by rotary evaporation at 55° C and the resulting oily residue was washed twice with diethylether. This residue was aliquoted, dried in vacuo and stored in a desiccator under vacuum over silica gel at -20° C. No further purification was performed. The ester was shown to be active, as incubation with ethylenediamine quenched the ninhydrin reaction.

Biotin-hexaglycyl-N-hydroxysuccinimide ester.
(according to Meier and Ruoho, 1978, with modifications.)

Hexaglycine (100 mg; 0.26 mmol) and Na_2CO_3 (56 mg; 0.52 mmol) were dissolved in 5 ml H_2O by gentle warming to 50° C and added to biotin-N-hydroxysuccinimide (101 mg; 0.28 mmol) in 2.5 ml dry dimethyformamide, and kept with slow agitation at 21° C for 16 hours. The solution was then acidified to pH 2.0 with the addition of dilute HCl, resulting in the formation of a heavy precipitate. This precipitate (biotinyl-hexaglycine) was collected by low speed centrifugation, washed 3 times with cold 0.1 M HCl, 3 times with cold H_20 and dried in vacuo over phosphorous pentoxide at 37° C for 72 hours.

Biotinylhexaglycine (100 mg; 0.17 mmol) was dissolved in 100 ml dry dimethylsulfoxide by heating to 90° C. N-hydroxysuccinimide (24 mg; 0.2 mmol) together with dicyclohexylcarbodiimide (35 mg; 0.17 mmol) dissolved in 10 ml dry dimethylsulfoxide were then added and the reaction was mixed at 21° C for 16 hours. The acyl urea was precipitated by cooling to 4° C and removed by low speed centrifugation. The solvent was removed by rotary evaporation at 65° C, the residue was washed twice with dry diethylether and dried in vacuo. No further purification was performed and the active ester was demonstrated as described above.

αN-biotin-hexaglycyl-εN-(Boc)-lysyl-N-hydroxysuccinimide ester.

Biotinylhexaglycine (100 mg; 0.17 mmol) was dissolved in 50 ml dry dimethylsulfoxide by heating to 90° C. Solid 1,1-carbonyldiimidazole (30.5 mg; 0.188 mmol) was then added and the reaction was allowed to slowly cool to 21° C followed by mixing for a further 3 hours. εN-Boc-lysine (0.2mmol) together with

triethylamine (0.4 mmol) in 30 ml dry dimethylsulfoxide was then added and the reaction was incubated for a further 16 hours. The solvent was removed in vacuo, followed by repeated freeze drying. The product αN-biotinyl-hexaglycyl-εN-(Boc)-lysine was purified by reverse phase chromatography on a Waters μBonda-Pak C18 column, eluted with a linear acetonitrile/H_2O (containing 0.1 % trifluoroactic acid) gradient. The major peak (see Fig 1) was collected, freeze dried and further dried in vacuo against phosphorous pentoxide at 37° C for 72 hours. Thin layer chromatography on silica F_{254} plates developed with n-propanol/NH_4OH (1:1), yielded one product with an $R_f = 0.7$. The biotin moiety was indicated by developing with cinnemaldehyde and the ε amino group of the lysine was shown by ninhydrin after treatment with trifluoroacetic acid. Amino acid analysis confirmed the ratio of glycine:lysine to be 6.6:1.

αN-Biotinyl-hexaglycyl-εN-(Boc)-lysine (48 mg; 59 μmol) was dissolved in 15 ml dry dimethylsulfoxide. Solid 1,1-carbonyldiimidazole (11.5 mg; 70.7 μmol) was added and the solution was mixed at 21° C for 3 hours. N-hydroxysuccinimide (8.2 mg; 70.7 μmol) was then added and mixing continued for a further 16 hours. The solvent was removed by rotary evaporation at 65° C and the residue was washed 3 times with dry diethylether and dried in vacuo. The crude ester was dissolved in dry dimethylsulfoxide, aliquoted and redried in vacuo and kept in a desiccator under vacuum, over silica gel, at -20° C. No further purification was performed and the presence of N-hydroxysuccinimide ester was shown as described above.

N-Boc-ethylenediamine.
(according to Geiger *et al.*, 1971).

Boc-hydrazide (2.644 g; 20 mmol) was dissolved in 12 ml dry peroxide free dioxane, 4 ml 5 M HCl (20 mmol) was then added and temperature was maintained at 0° C. $NaNO_2$ (1.394 g; 20.2 mmole) in 3.6 ml H_2O was slowly added over 10-15 min at 5° C, followed by a further 15 min incubation at 21° C. Ethylenediamine (1.33 g; 10 mmol) together with triethylamine (10.1 mmol) in 3 ml dry dioxane was added and the solution incubated at 50° C for 24 hours with vigorous mixing. The solvent was removed by rotary evaporation and the residue dissolved in a minimum volume of H_20. The bis-Boc-ethylenediamine crystallized on the addition of isopropanol, was collected by centrifugation and dried in vacuo.

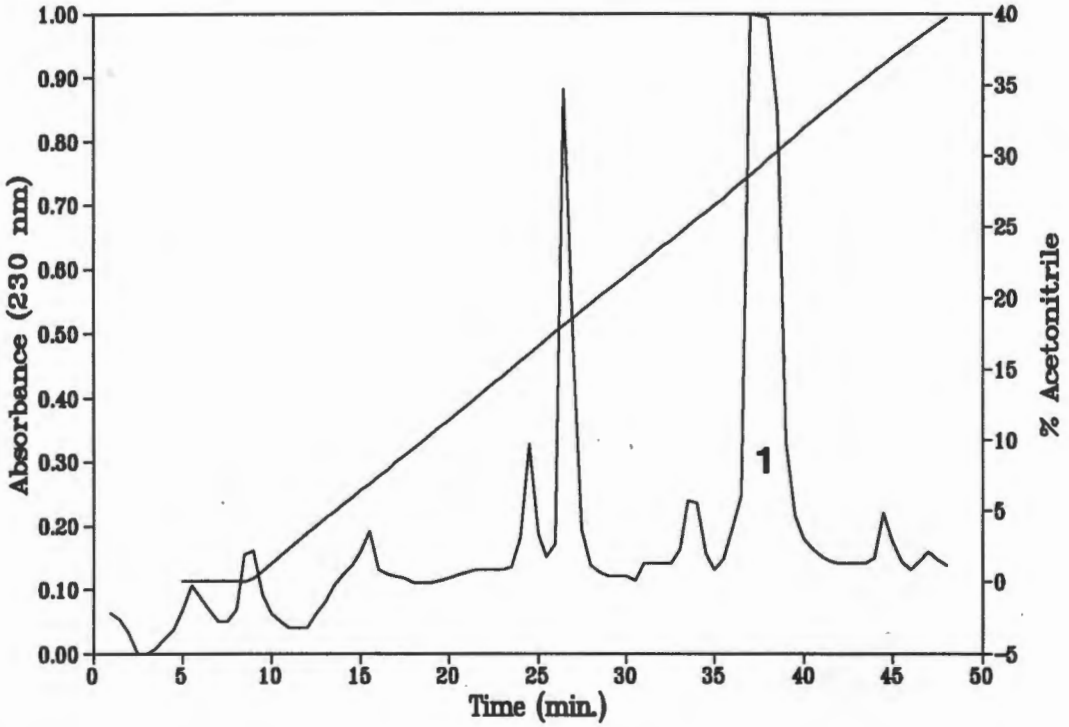


Figure 1. Elution profile of the products formed when biotinylhexaglycine was coupled to εN-(Boc)-lysine, via activation with carbodiimidazole.

The reaction products formed when biotinylhexaglycine was coupled to εN-(Boc)-lysine via activation with carbodiimidazole (see methods and material), were dissolved in 0.05 % TFA and applied to a μBonda Pak C18 reverse phase HPLC column equilibrated in 0.1 % TFA. The products were eluted with an acetonitrile gradient (0-40 %), containing 0.1 % TFA. The αN-biotinyl-hexaglycyl-εN-(Boc)-lysine peak (peak 1) was identified by a positive reaction with both ninhydrin and acidic cinnemaldehyde after treatment with trifluroacetic acid.

N-Boc-ethylenediamine was prepared by dissolving the bis-Boc-ethylenediamine in dry 2 M HCl/ether and incubating at 21° C for 30 min, with gentle stirring. The resulting precipitate was collected by centrifugation, washed with an excess of dry diethylether and then dried in vacuo. A single ninhydrin positive product with an R_f = 0.55 was observed when chromatographed on silica F_{254} thin-layer plates developed with butanol/acetic acid/H2O (3:1:1).

N-Boc-N-(6 Bromocaproyl)-ethylenediamine.
(according to Faulstich *et al.*, 1981).

6-Bromocaproic acid (97.5 mg; 0.5 mmol) and triethylamine (0.5 mmol) were dissolved in 3.5 ml dry tetrahydrofuran and cooled to -15° C. Ethoxycarbonyl chloride ((0.5 mmol) was then added and incubated for 5 min. N-Boc-ethylenediamine (98 mg, 0.05 mmol) together with triethylamine (0.05 mmol) in 5 ml dry tetrahydrofuran were then added and the reaction allowed to come to 0° C, followed by a further 10 min incubation at 0° C. 0.5 ml H_2O was added and incubated for 10 min at 20° C. The solvent was removed by rotary evaporation and the residue re-dissolved in ethyl acetate, washed 3 times with cold citric acid (5 % w/v), 3 times with potassium hydrogen carbonate (10 % w/v), and 3 times with sodium chloride (10 % w/v). The solvent was removed by rotary evaporation and the N-Boc-N-(6 bromocaproyl) ethylenediamine crystallized from methanol/ ethylacetate/benzine (0.03:0.5:1.0). Elemental analysis showed: C, 46.1 %; H, 7.6 %; N, 8.35 %. The structure was confirmed by NMR analysis (data not shown).

O-[5-[[(aminoethyl) amino]-carbonyl]-pent-1-yl]-α-amanitin trifluoroacetate. (NH_2-10-amanitin).
(according to Faulstich *et al.*, 1981, with modifications).

The α-amanitin (1mg; 1.1 µmol) was dried over phosphorous pentoxide at 37° C in vacuo and then dissolved in 95.6 µl dry ethanol containing 1.1 mole equivalents sodium ethylate (the sodium ethylate was prepared by dissolving a freshly cut block of sodium in dry ethanol and the concentration was determined by titration against HCl) and immediately dried in a centrifugal evaporator at 30° C. The resulting yellowish residue was dissolved in 25 µl dimethylformamide containing N-Boc-N-(6-bromocaproyl)-ethylenediamine (1.484 mg; 4.4 µmol). The reaction was allowed to proceed at 20° C for 24 hours after which the solvent was removed in vacuo. The

brown residue was dissolved in 500 µl 10 % acetonitrile/H_20 and fractionated by reverse phase chromatography on a Waters µBonda Pak C18 column (Fig 2A). The products were eluted with an acetonitrile/H_20 gradient ranging from 0-70 % (containing 0.1 % trifluroacetic acid). The eluate was monitored at 219 or 305 nm and major peaks (see Fig 2A) were collected and freeze dried. The ether derivative could be identified by the absence of a phenolate shift at pH 12.0 (Fig 3), and was shown to be homogeneous when chromatographed on silica F_{254} thin-layer plates, developed with chloroform/methanol/H20 (65:25:4). α-Amanitin showed an R_f = 0.038 and NH_2-10-amanitin an R_f = 0.03. The Boc group was cleaved by dissolving the dried peak in 100 µl 100 % trifluroacetic acid and incubating for 2 min. Excess trifluroacetic acid was removed under a stream of nitrogen followed by repeated washes with peroxide free dry diethylether. Alternatively, the Boc groups were cleaved prior to fractionation. The total dried coupling reaction mixture, as described above, was incubated in 100 µl trifluoroacetic acid for 2 min and the excess trifluoroacetic was removed under a gentle steam of nitrogen. The residue was dissolved H_2O and neutralized with N-mehtylmorpholine followed by repeated drying in vacuo (excess residual trifluoroacetic acid alters the HPLC elution times). The products were then fractionated as described above using a Waters Nova-Pak C18 or Spherisorb ODS2 (3.9 mm x 150 mm) column (Fig 2B).

O-[5-[[[(biotinyl-amino)-ethyl]-amino] carbonyl]-pent-1-yl]-α-amanitin. (Bio-11-amanitin).

Biotin-N-hydroxysuccinamide ester (2 mg; 5.7 µmol) was dissolved in 50 µl dry dimethylformamide and added to NH_2-10-amanitin (approximately 500 µg) in 150 µl 100 mM $NaHCO_3$ pH 8.0 and incubated at 21° C for 16 hours. The solvent was removed in vacuo, the products were fractionated by reverse phase chromatography as previously described (see Fig 4A). The Bio-11-amanitin was shown to be homogeneous when chromatographed on silica F_{254} thin-layer plates developed with chloroform/methanol/H_2O (65:25:4), with an R_f = 0.074. Biotinylation was confirmed with acidic cinnemaldehyde and a solid phase streptavidin quenching assay (see below).

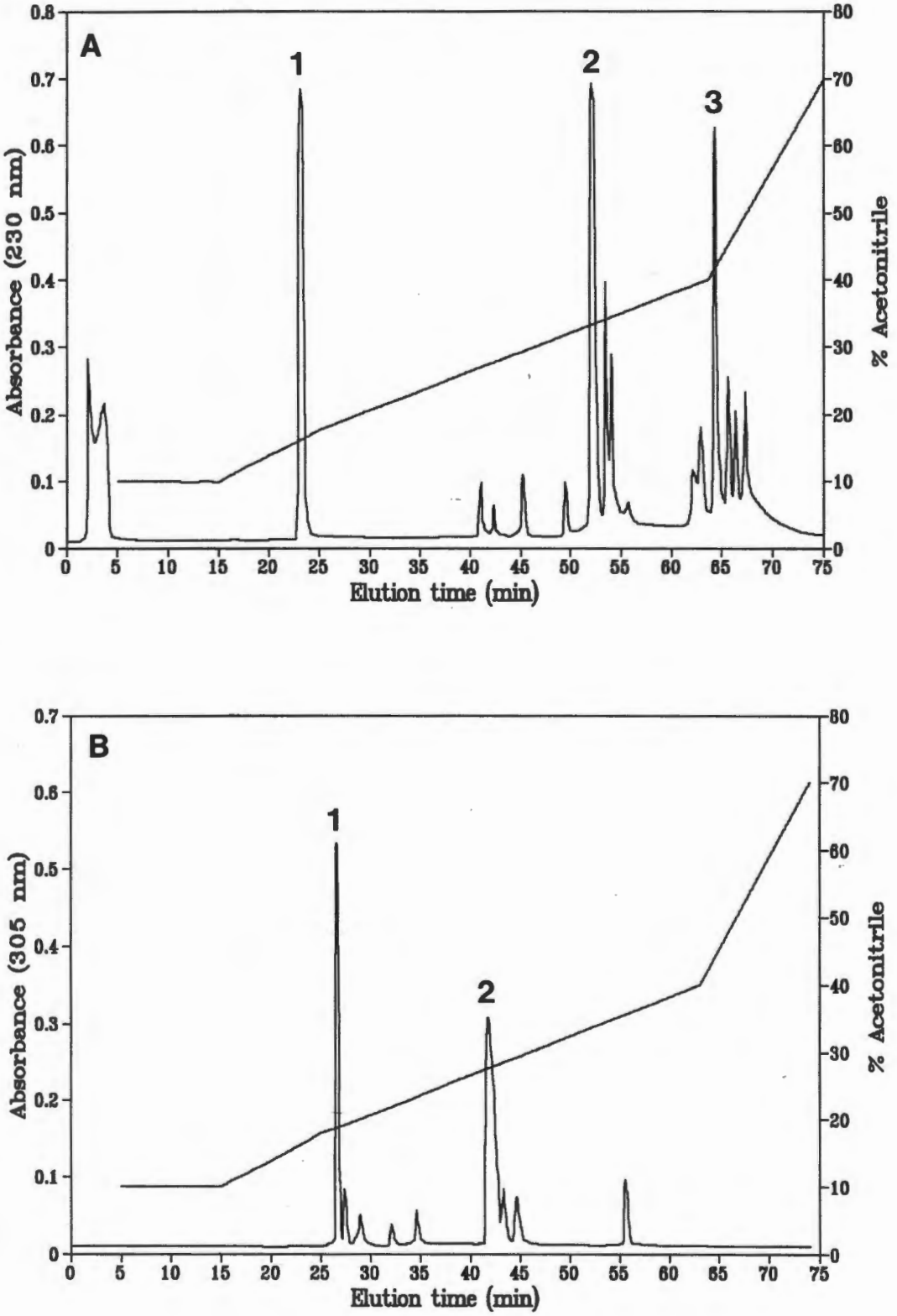


Figure 2. Elution profiles of the products formed by nucleophillic substitution, between the α-amanitin phenolate ion and N-Boc-N-(6-bromocaproyl)-ethylenediamine.

A) The coupling reaction mixture (see methods and materials) was dried in vacuo to remove the solvents, redissolved in 10 % acetonitrile/H_2O and applied to a μBonda

Pak C18 equilibrated in 0.1 % TFA. The products were eluted with an acetonitrile/H_2O gradient ranging from 0-70 % (containing 0.1 % TFA). The eluate was monitored at 230 nm to detect only amanitin derivatives. The three major peaks were collected and assayed for the presence of a phenolate shift at pH 12.

Peak 1. α-amanitin (positive phenolate shift).
Peak 2. Boc-NH-amanitin (negative phenolate shift).
Peak 3. $(Boc\text{-}NH)_2$-amanitin (negative phenolate shift).

B) The coupling reaction mixture was dried in vacuo, digested with 100 % TFA for 2 min, redried under nitrogen and neutralized with aqueous N-methylmorpholine, followed by redrying in vacuo. The de-Boced products were dissolved as described above and applied to a Waters Nova Pak C18 column equilibrated in 10 % acetonitrile/ H_2O containing 0.1 % TFA. The products were eluted with an acetonitrile gradient as described above.

Peak 1. α-amanitin.
Peak 2. NH_2-10-amanitin.

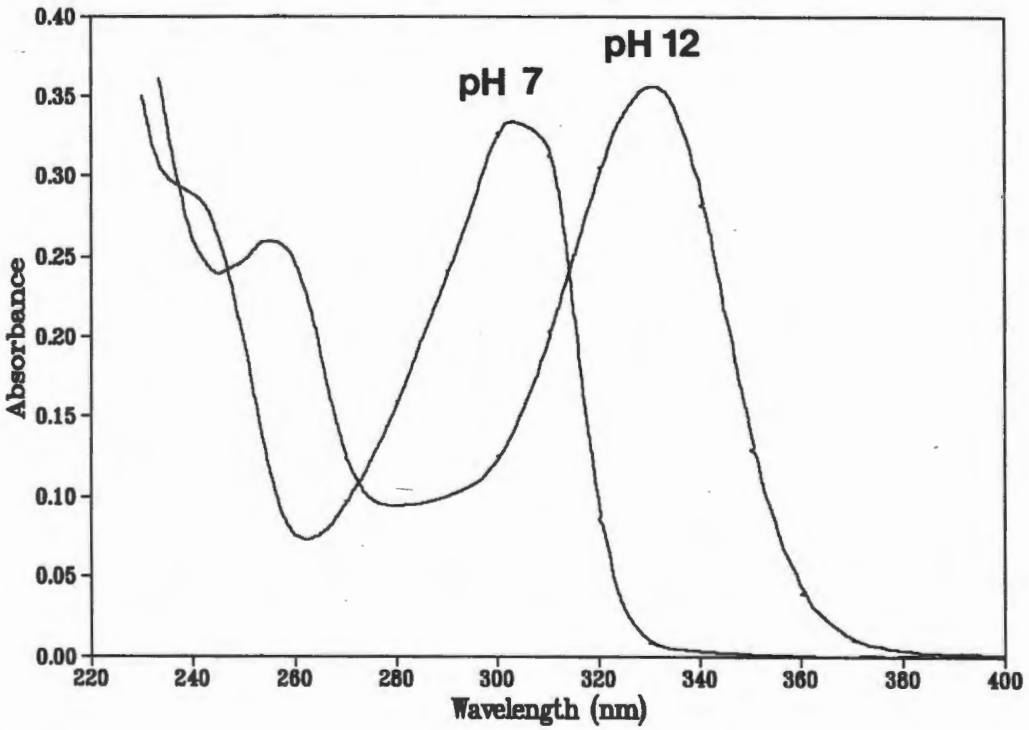


Figure 3. Absorbance spectrum of α-amanitin at neutral (pH 7.0) and alkaline pH (pH 12).

O-[5-[[[(biotinamincaproyl-amino)-ethyl] amino]-carbonyl]-pent-1-yl]-α-amanitin. (Bio-18-amanitin).

Biotinaminocaproyl-N-hydroxysuccinimide ester (2 mg; 4.4 μmol) was dissolved in 50 μl dry dimethylformamide and added to NH_2-10-amanitin (approximately 500 μg) in 150 μl 100 mM $NaHCO_3$ pH 8.0 and incubated at 21° C for 16 hours after which the solvent was removed in vacuo. The Bio-18-amanitin was purified by reverse phase HPLC (Fig 4B), and assayed as described above. The R_f was shown to be 0.074.

O-[5-[[[(biotinyl-hexaglycyl-amino)-ethyl] amino]-carbonyl]-pent-1-yl]-α-amanitin. (Bio-29-amanitin).

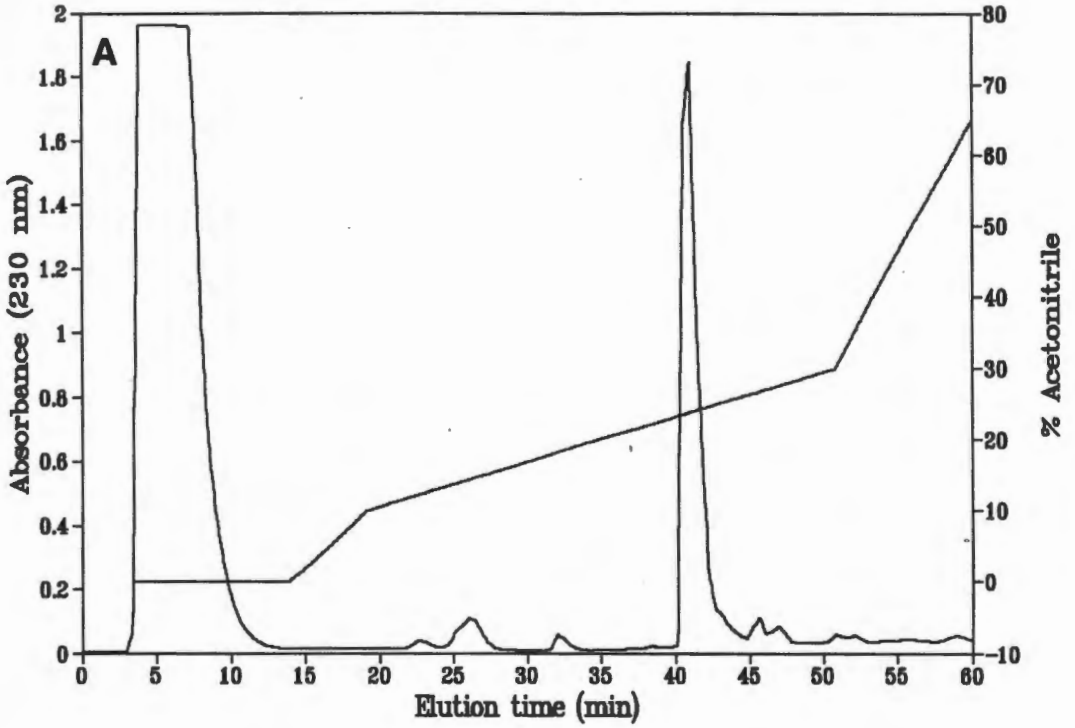
Excess crude biotinylhexaglycyl-N-hydroxysuccinimide (approximately 5 mg) was dissolved in 400 μl dry dimethylsulfoxide by warming to 65° C and added to NH_2-10-amanitin (approximately 500 μg) dissolved in 400 μl 100 mM $NaHCO_3$ pH 8.0. The coupling reaction was allowed to proceed at 21° C for 16 hours, after which the solvent was removed in vacuo. The Bio-29-amanitin was purified by reverse phase HPLC (see Fig 4D), assayed as described above and the R_f was shown to be 0.035.

O-[5-[[[(αN-biotinaminocaproyl-εN-(nitro-azido-phenyl)-lysyl)-aminoethyl]-amino]-carbonyl]-pent-1-yl] α-amanitin. (Bio-21-NAP-amanitin).

The reaction conditions were essentially the same as those described for Bio-32-NAP-α-amanitin (see below), only αN-biotinaminocaproyl-εN-(Boc)-lysyl-N-hydroxysuccinimide was substituted for αN-biotinyl-hexaglycyl-εN-(Boc)-lysyl-N-hydroxysuccinimide (see Fig 5A and 5B for HPLC elution profiles).

O-[5-[[[(αN-biotinyl-hexaglycyl-εN-(nitro-azido-phenyl)-lysyl-amino) ethyl]-amino]-carbonyl]-pent-1-yl]-α-amanitin. (Bio-32-NAP-amanitin).

An aliquot of αN-biotinyl-hexaglycyl-εN-(Boc)-lysyl-N-hydroxysuccinimide (approximately 5 mg) was dissolved in dry dimethylsulfoxide by gently warming to 70° C and added to NH_2-10-amanitin (approximately 500 μg) together with 2 μl N-methylmorpholine in 200 μl dimethylsulfoxide.


A
Absorbance (230 nm)
% Acetonitrile
Elution time (min)
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0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
-10
0
10
20
30
40
50
60
70
80
0
5
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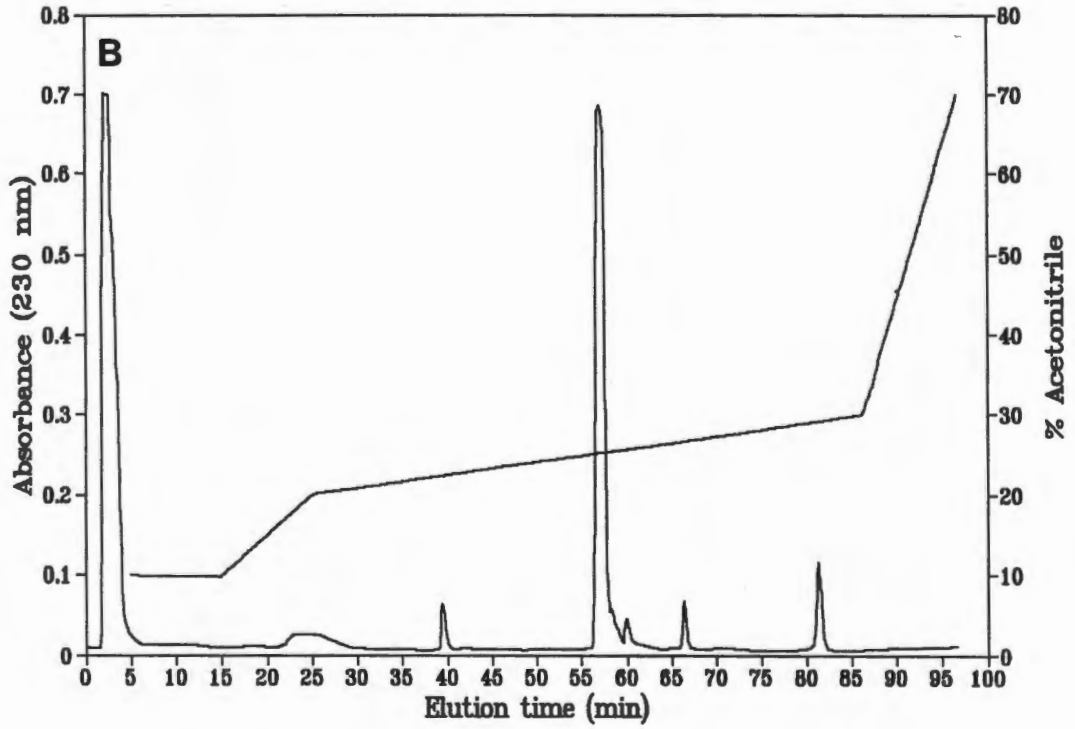
15
20
25
30
35
40
45
50
55
60


B
Absorbance (230 nm)
% Acetonitrile
Elution time (min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
50
60
70
80
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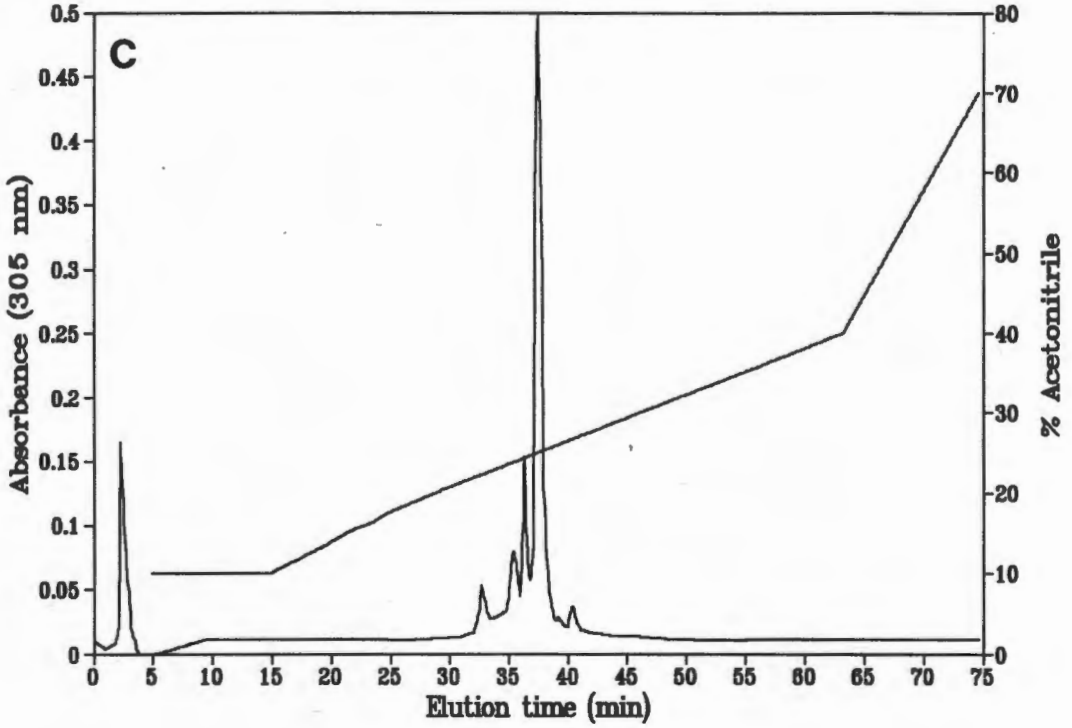


Figure 4. Elution profiles of biotinylated amanitin derivatives fractionated by reverse phase HPLC chromatography.

NH_2-amanitin was incubated with various biotinyl-N-hydroxysuccinimde derivatives as described in methods and materials. The resulting products were fractionated by reverse phase chromatography using a 10 to 70 % acetonitrile gradient containing 0.1 % TFA (see figure for gradient profile). The eluate was monitored at 305 nm to detect the amanitin indole nucleus and the biotinyl residue was shown by a solid phase streptavidin quenching assay and a positive reaction with acidic cinnemaldehyde.

A) Bio-11-amanitin, fractionated on a Waters μBona Pak C18 column.
B) Bio-18-amanitin, fractionated on a Spherisorb ODS2 column.
C) Bio-29-amanitin, fractionated on a Spherisorb ODS2 column.

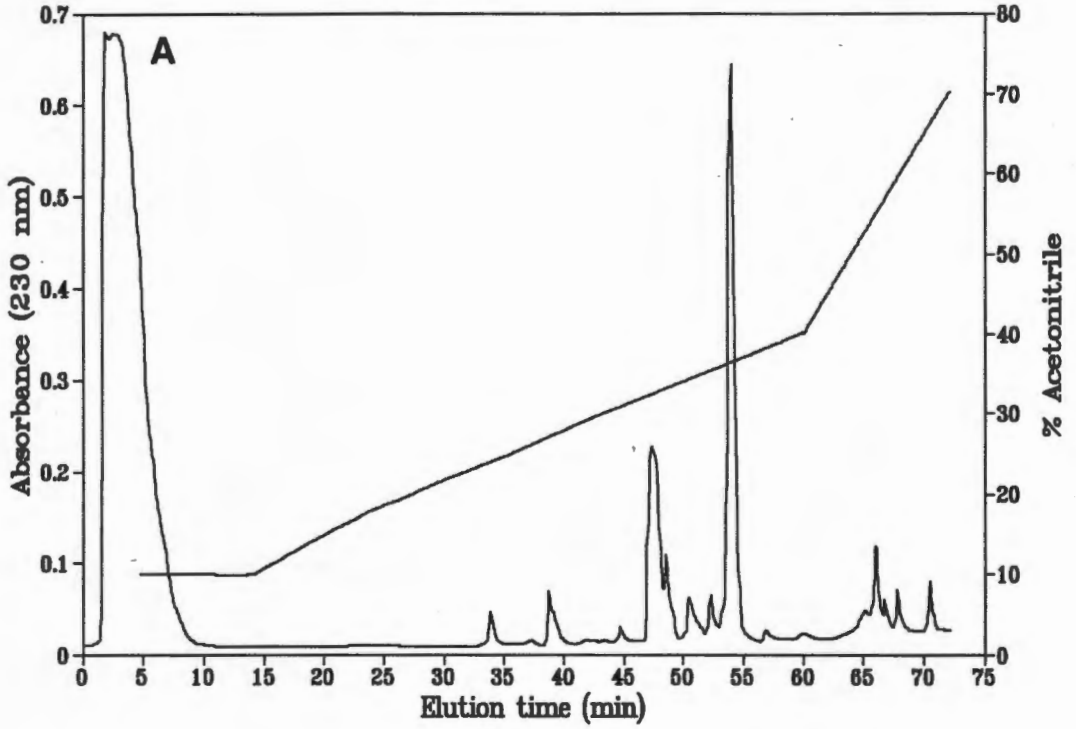
The reaction was incubated at 21° C for 16 hours, after which the solvent was removed in vacuo. The residue was dissolved in 100 µl trifluoroacetic acid to cleave off the Boc groups and trifluoroacetic acid was removed as described above. The Bio-32-LYS-amanitin was purified by reverse phase chromatography (Fig 5C), as described above and identified by the characteristic absorbance spectrum of α-amanitin with a λ_{max}=305 nm. The biotin moiety was shown using a solid phase streptavidin quenching assay and the ε amino group was demonstrated by a positive reaction with ninhydrin.

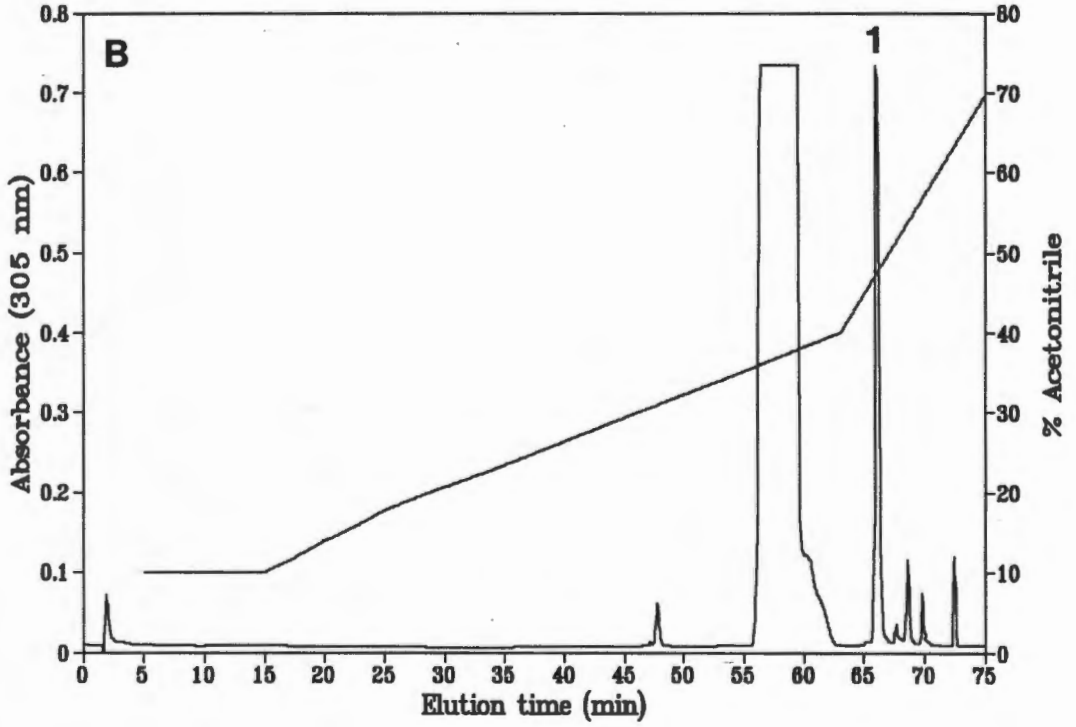
4-Fluoro-3-nitrophenyl azide (3.75 mg; 20 µmole) together with triethylamine (21 µmole) were dissolved in 30µl dry dimethylformamide and added to Bio-32-LYS-amanitin (approximately 400 µg) dissolved in 200 µl dry dimethlyformamide. The coupling reaction was allowed to proceed in the dark at 21° C for 16 hours after which the solvent was removed in vacuo. The residue was redissolved in 10-20 % acetonitrile/H_20 and fractionated by reverse phase chromatography (see Fig 5D). The product Bio-32-NAP-amanitin was identified by the characteristic absorbance spectrum of the coupled nitrophenyl azide
group which has a λ_{max}= 475 (Fig 6). Due to the low yield the concentration of the product was determined using the solid phase streptavidin quenching assay as described above. The Bio-32-NAP-amanitin was aliquoted and stored in light sealed containers at -70° C.

Solid phase streptavidin quenching assay.

Streptavidin-Sepharose 6B was prepared using the CNBr activation method (Cautrecasas, 1970). Briefly, 10 g moist Sepharose 6B was activated with 3 g CNBr at 20° C for 10 min while maintaining the pH at 11.0. The CNBr was remove by extensive washing with cold H_2O and cold 100 mM sodium borate pH 9.5. Streptavidin (1-2 mg) in 10 ml 100 mM sodium borate pH 9.5 was then added to the activated Sepharose 6B and incubated at 4° C for 24 hours. The excess active groups were then quenched with ethanolamine (1 mmol) after which the streptavidin-Sepharose was washed and stored in 100 mM NaCl; 10 mM Tris-HCl pH 8.0; 1 mM EDTA at 4° C. The biotin binding capacity was titrated using ^{14}C-biotin (specific activity 114.4 dpm/pmol).

The concentration of small biotinylated molecules was determined by incubating an aliquot with an aliquot of streptavidin Sepharose 6B (known biotin binding


A
Absorbance (230 nm)
% Acetonitrile
Elution time (min)


B
1
Absorbance (305 nm)
% Acetonitrile
Elution time (min)

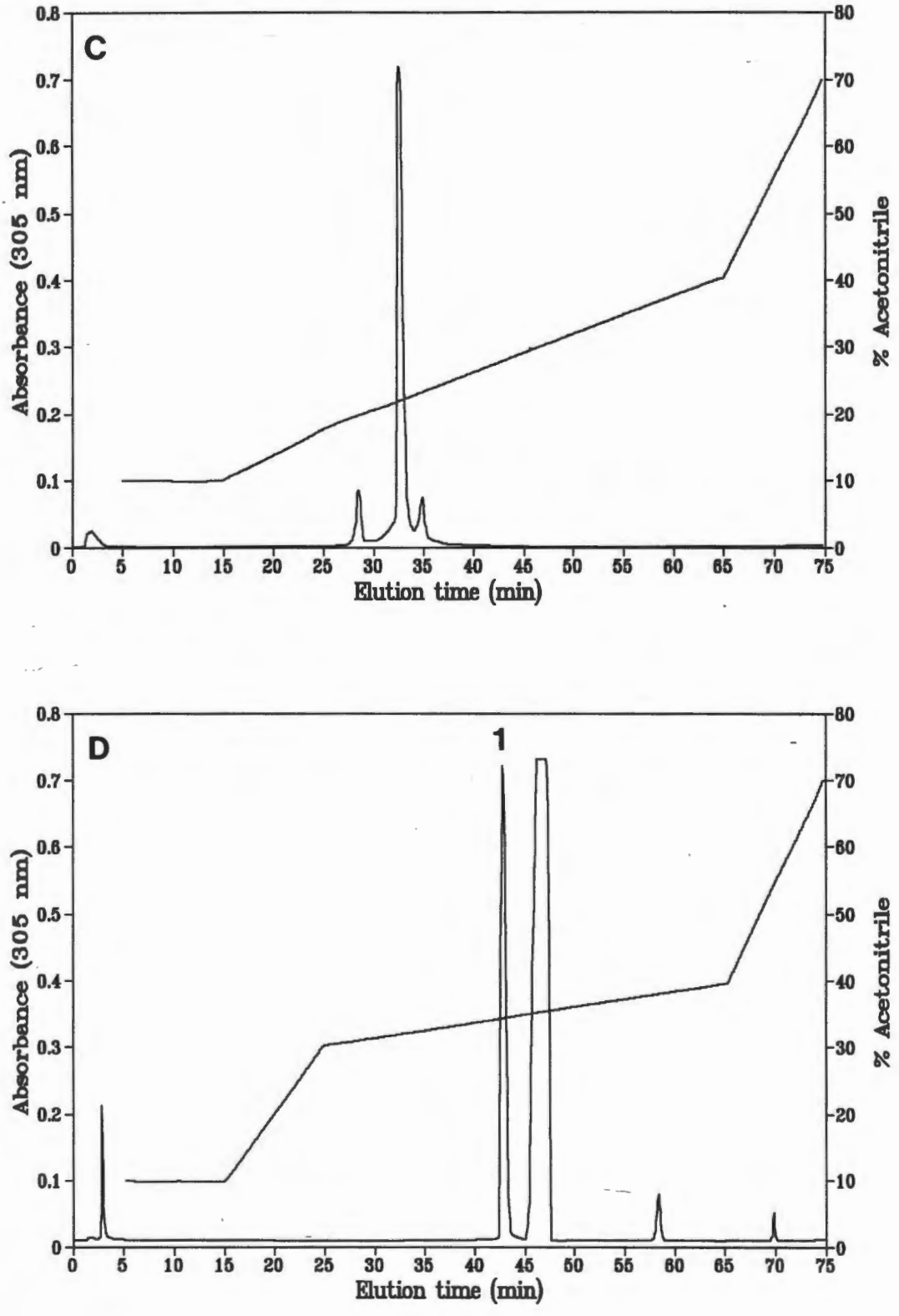


Figure 5. Elution profiles of biotinylated amanitin derivatives containing spacer arm side chain groups, fractionated by reverse phase chromatography.

NH_2-amanitin was incubated with either αN-biotinylaminocaproyl-εN-(Boc)-lysyl-N-hyrodxysuccinimde or αN-biotinyl-hexaglycyl-εN-(Boc)-lysyl-N-hydroxysuccinimde as described in methods and materials. The respective products, αN-Bio-21-εN-(Boc)-LYS-amanitin (A), and αN-Bio-32-εN-(Boc)-LYS-amanitin (C), were digested with TFA to remove the Boc groups and purified reverse phase chromatography. These amanitin derivatives were then incubated with 4-Fluoro-3-Nitrophenyl azide as described in methods and materials and the respective products Bio-21-NAP-amanitin (B), and Bio-32-NAP-amanitin (D), were purified by reverse phase chromatography. The eluates were monitored at 305 nm to detect the amanitin indole nucleus without photoactivation of the side chain nitroazidophenyl residue. The nitroazidophenyl residue was shown by the characteristic absorbance spectrum with a λ_{max} at 486 nm (see figure 6).

A) Bio-21-LYS-amanitin, fractionated on a Spherisorb ODS-2 column.
B) Bio-21-NAP-amanitin (peak 1), fractionated on a Spherisorb ODS-2 column.
C) Bio-32-LYS-amanitin, fractionated on a Waters Nova Pak C18 column.
D) Bio-32-NAP-amanitin (peak 1), fractionated on a Waters Nova Pak C18 column.

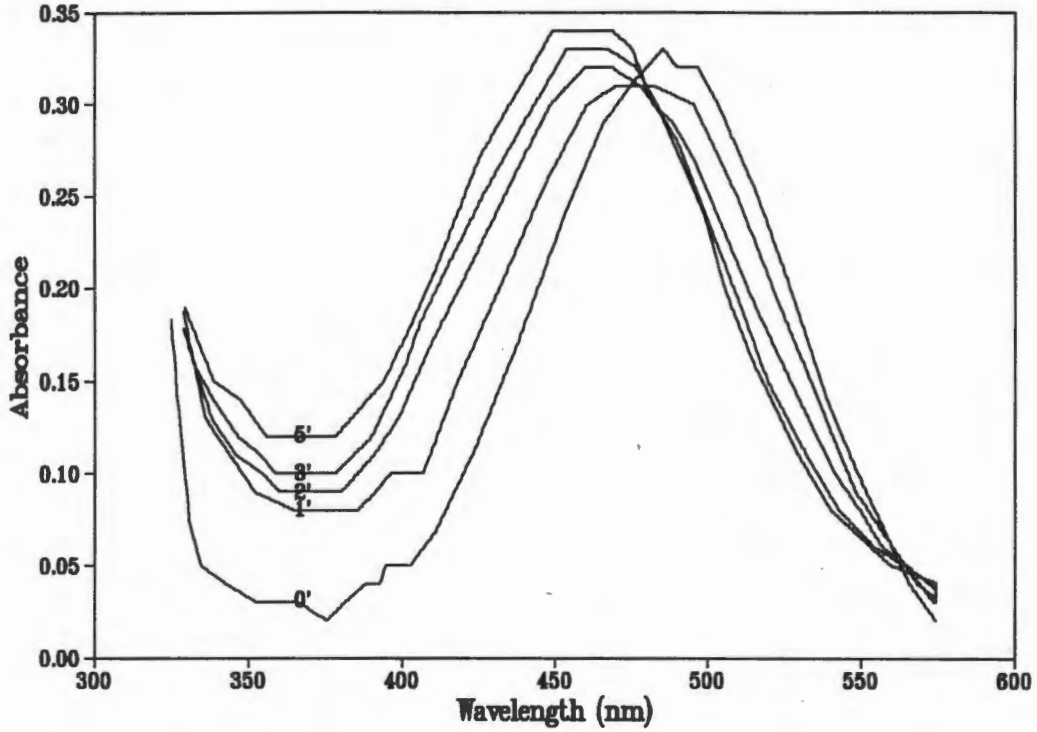


Figure 6. Absorbance spectrum of Bio-32-NAP-amanitin as a function of photolysis.

Bio-32-NAP-amanitin was diluted in NSB and the absorbance spectrum was determined (t = 0). The solution was then exposed to a quartz halogen light source for various time intervals (t = 1,2,3 and 5 min) to induce photolysis and the absorbance spectrum was redetermined. Exposure for more that 5 min failed to further alter the absorbance spectrum, demonstrating that photolysis was complete.

capacity) at 21° C for 30 min. An excess of ^{14}C-biotin was then added and incubation continued for a further 30 min. The matrix was then washed extensively and the bound radioactivity determined by scintillation counting.

$[dpm_{(unquenched)} - dpm_{(quenched)}]/114.4 = pmol\ biotin$

Where: 1) unquenched: total binding capacity.
2) quenched: binding of excess ^{14}C-biotin after matrix has been partially quenched by unknown sample.

Iodination of streptavidin.

Bolton and Hunter.

Streptavidin (1 µg) was dissolved in 10 µl 100 mM $NaHCO_3$ pH 8.5 and incubated with 200 µCi dried diiodo Bolton and Hunter reagent (specific activity 4000 Ci/mmol), for 30 min at 21° C. This was repeated five times with new aliquots of reagent, after which 50 µl 1 M ethanolamine was added to quench any non incorporated ester. The ^{125}I-streptavidin was then desalted using a Centracon 10 ultrafiltration membrane. An average of three iodine atoms were coupled to each streptavidin giving a specific activity of 6000 Ci/mmol.

^{125}I-Biotinyl-glycyl-tyrosine.

Biotinyl-glycyl-tyrosine was prepared according to Smith *et al.* 1987. The iodinated derivative was prepared by incubating biotinyl-glycyl-tyrosine (0.4 µg) together with 1 mCi ^{125}I in 50 µl 10 mM Tris-HCl pH 8.0 and 1 mM EDTA in an iodogen tube for 10 min at 21° C. The ^{125}I-biotinyl-glycyl-tyrosine was purified by reverse phase chromatography on a Waters Nova-Pak C18 column, eluted with an acetonitrile/H_2O gradient (containing 0.1 % trifluoroacetic acid). The specific activity was determined by the solid phase streptavidin quenching assay and was routinely found to be 1500 Ci/mmol.

^{125}I-Biotinyl-streptavidin.

^{125}I-biotinyl-streptavidin was prepared by titrating of 50-75 % of one binding site with ^{125}I-biotinyl-glycyl-tyrosine. Briefly, streptavidin (1 µg; 21.3 pmol (MW 47 KDa) was dissolved in H_2O (500 µl). ^{125}I-biotinyl-glycyl-tyrosine (ranging form 10 to 16 pmol) dissolved in 500 µl H_2O was then slowly added to the streptavidin with continual rapid mixing and the solvent was removed by freeze drying. The ^{125}I-biotinyl-streptavidin was then redissolved in the required buffer.

Isolation of 14 hour sea urchin embryo nuclei.

(Modification of methods developed by Morris and Marzluff (1983) and Hewish and Burgoyne (1973)).

Sea Urchins (*Parachinus Angulosus*) were collected from the Atlantic shore and spawning was induced by injection of 0.5 M KCl. The eggs were washed and resuspended to a final concentration of 4 % (v/v) in filtered sea water containing 100 mg penicillin and 50 mg streptomycin per liter. The eggs were fertilized with the addition of 0.1 ml sperm per liter and the efficiency of fertilization and subsequent synchrony monitored by light microscopy. The embryos were then grown to a specific stage of development at 21° C, with constant rotary shaking at 130 rpm.

The embryos were collected by centrifugation at 2000 xg and washed repeatedly with 0.55 M KCl until supernatant was clear. The pelleted embryos were resuspended in 2-3 volumes 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, and immediately centrifuged 12000 xg for 3 min. The swollen embryos were then lysed by homogenization using a tight glass dounce in 5 volumes 0.32 M Sucrose in Buffer A (15 mM NaCl, 65 mM KCl, 15 mM Tris-HCl pH 8.0, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM EGTA and 0.2 mM EDTA) containing 0.2 mM PMSF and 10 mM ß-mecaptoethanol. 10-15 volumes of 2 M Sucrose in Buffer A containing 0.2 mM PMSF and 10 mM ß-mecaptoethanol was then added to the homogenate and mixed well. The nuclei were pelleted by centrifugation at 48000 xg for 60 min and resuspended in 50 ml nuclei storage buffer (NSB) (25 % Glycerol in Buffer A) containing 0.2 mM PMSF and 10 mM ß-mecaptoethanol by gentle vortexing. The nuclei were flash frozen in liquid nitrogen and stored at -70° C. These conditions allowed for storage of up to 6 months without loss of transcriptional activity.

***In vitro* transcription.**

(According to Morris and Marzluff, 1983, with modifications.)

Nuclei.

14 hour sea urchin embryo nuclei were added to two volumes of transcription buffer (102.5 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0, 0.6 mM ATP, 0.6 mM GTP or 0.015 mM α-^{32}P-GTP (10 μCi/nmol), 0.6 mM CTP, 0.6 mM UTP or 0.02 mM ^{3}H-UTP (5 μCi/nmol), 15 mM ß-mercaptoethanol and 300 U RNAsin/ml) and incubated at 22° C. Aliquots were removed at various time intervals and stopped with the addition of 5 volumes stop buffer (1 % SDS and 10 mM EDTA pH 8.0). The incorporation ^{3}H-UMP or ^{32}P-GMP was determined by spotting the aliquots on GFC filter membranes and washing in 10 ml volumes of 10 % TCA (10 min), 5 % TCA (2 min) twice, 96 % ethanol (2 min) twice and the dried at 50° C. The acid insoluble counts were then determined by liquid scintillation.

Soluble chromatin.

14 hour sea urchin embryo nuclei were digested by incubating the nuclei with micrococcal nuclease (160 units/mg DNA) in nuclei storage buffer containing 1 mM $CaCl_2$ on ice for 10 min. The digestion was stopped by chelation of the calcium ions with the addition of EDTA (final concentration 2 mM) and the soluble chromatin was extracted by dialysis against 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1 mM PMSF at 4° C.

Run on *in vitro* transcription was initiated with the addition of 2 volumes of transcription buffer (135 mM KCl, 7.5 mM NaCl, 25 mM Tris-HCL pH 8.0, 15.5 mM $MgCl_2$, 0.075 mM spermine, 0.25 mM spermidine, 0.1 mM EDTA, 0.1 mM EGTA, 12.5 % glycerol, 0.6 mM ATP, 0.6 mM CTP, 0.6 mM UTP or ^{3}H-UTP (5 μCi/nmol), 6 mM GTP or 0.015 mM ^{32}P GTP (10 μCi/nmol) and 6 mM ß-mercaptoethanol) to 1 volume of soluble chromatin, followed by incubation at 22° C. The incorporation of either ^{3}H-UMP or ^{32}P-GMP into RNA was determined as described above.

The efficiency of α-amanitin or α-amanitin ether derivatives as inhibitors of RNA polymerase II transcription was determined by pre-incubation of the nuclei or

solubilized chromatin with various concentrations of the inhibitor at 0° C for 1 to 4 hours before *in vitro* transcription was then initiated.

Biotinylation of RNA polymerase II in permeabilized 14 hour sea urchin embryo nuclei.

Frozen 14 hour sea urchin embryo nuclei were thawed on ice and washed twice NSB containing 0.2 mM PMSF to remove the reducing reagents. The nuclei (3-4 mg DNA, as measured by OD_{260}) were resuspended to a total volume of 200 µl, Bio-32-NAP-amanitin ($2x10^{-10}$ mole; final concentration $1x10^{-6}$ M) was added and the nuclei were incubated in the dark at 0° C for 4 hours. (Controls included: 1) Incubation without any amanitin derivatives. 2) Pre-incubated for 1 hour with α-amanitin ($1x10^{-8}$ mole; final concentration $5x10^{-5}$ M) to saturate the binding sites before the addition of Bio-32-NAP-amanitin). The nuclei were then washed five times with NSB containing 0.2 mM PMSF and 0.05 % Nonidet LE to remove unbound Bio-32-NAP-amanitin, thereby preventing non-specific crosslinking. The Nonidet LE treatment, in addition, aided the subsequent entry of streptavidin, used for probing (see following section).

The Bio-32-NAP-amanitin was covalently crosslinked to the RNA polymerase II by illuminating the nuclei with a 200 Watt quartz halogen lamp at a distance of 15 cm for 30 min. The temperature of the nuclei was maintained at 0° C by circulating, from a suitably adjusted thermostat, an ice cold solution of 1 M sodium nitrite in 50 % ethanol around the sample (this is very important as the RNA polymerase II molecules are rapidly denatured by temperatures above 20° C and become transcriptionally inactive). This cooling solution in turn acted as a UV filter preventing chromatin and protein damage. The nuclei were then washed once with NSB containing 0.2 mM PMSF and 0.05 % Nonidet LE and resuspended in 500 µl of the same buffer.

The specificity of the covalent biotinylation of RNA polymerase II subunits was determined by Western blotting the nuclear proteins fractionated by 7.5 % SDS polyacrylamide gel electrophoresis (Laemlli, 1970; Current Protocols, 1987). The biotin moiety was detected using a streptavidin-alkaline phosphatase conjugate and developed in 1 M diethanolamine pH 9.6, 0.5 mM $MgCl_2$ and 0.1 M NaCl containing 0.165 µg/ml BCIP and 0.33 µg/ml NBT.

Probing for biotinylated RNA polymerase II.

The biotinylated RNA polymerase II transcription complexes were labelled "in situ" by incubating the permeabilized nuclei with either the ^{125}I-biotinyl-streptavidin complex or ^{125}I-streptavidin for 1-2 hours at 4° C. Unbound streptavidin was removed by washing four times with NSB containing 0.05 % Nonidet LE and 0.2 mM PMSF or crosslinking buffer (25 % glycerol in TKM buffer (65 mM KCl, 10 mM Tris-HCl pH 8.0 and 5 mM $MgCl_2$) containing 0.2 mM PMSF and 0.05 % Nonidet LE), and the radioactivity within the nuclei was determined.

For EM autoradiography the nuclei were fixed with 1 % formaldehyde and 1 % gluteraldehyde in crosslinking buffer for 1 hour at 4° C. The crosslinking reagents were removed by extensive washing after which the nuclei were gradually dehydrated by sequential washing in increasing concentrations of ethanol and finally washed with propylene oxide. The nuclei were then equilibrated into Epon 812 (medium hard) and allowed to set at 60° C for 48 hours (Hayat, 1990). Ultra thin sections (gold; 90-150 nm) were cut and transferred to formvar coated nickel grids or glass slides and prestained with lead citrate (Renoylds stain). The sections were coated with a 10-50 Å carbon film followed by a thin layer of EM-1 emulsion (according to Amersham). Autoradiography was allowed to proceed at 4° C for up to 1 month for the light microscopy slides and 3 months for the EM sections before developing (D19 (Kodak) at 20° C for 3 min; H_2O for 3 min, repeat wash twice; 30 % NaS_2O_5 for 3 min; H_2O for 3 min, repeat wash twice). The light microscopy slide were stained with methylene green and the EM sections were stained with 0.7 % urinal acetate in 30 % ethanol for 25 min to enhance the contrast.

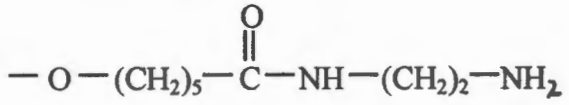
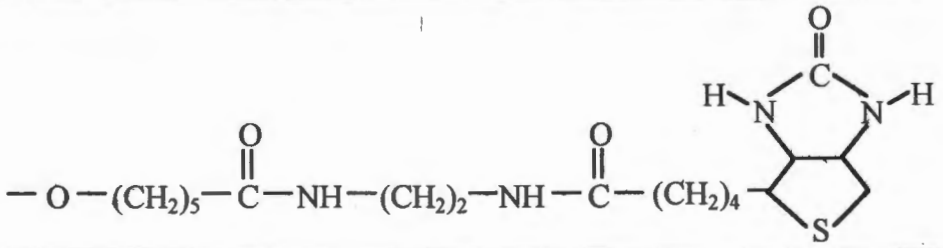
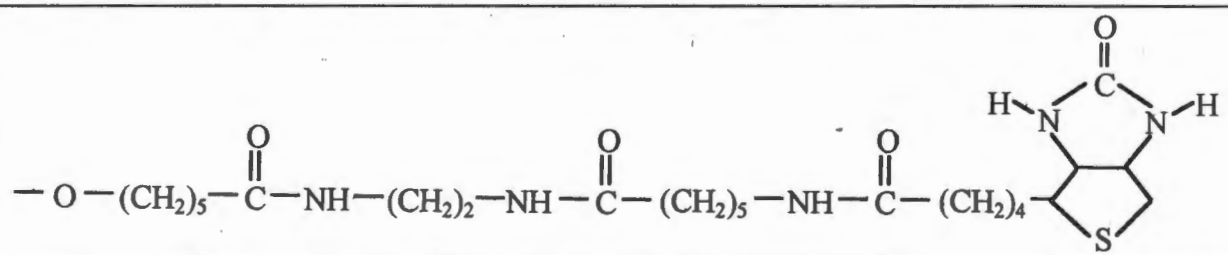
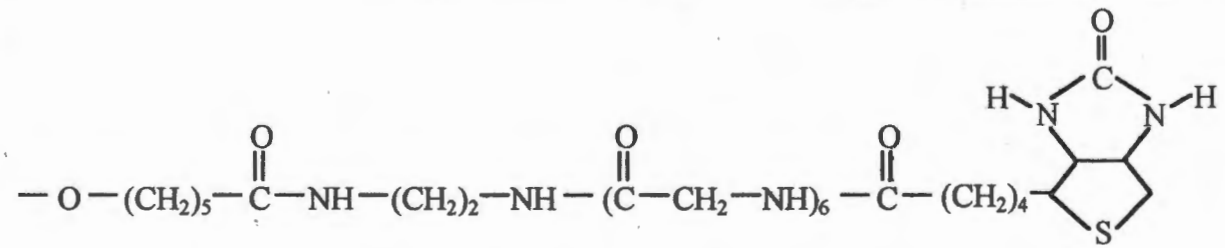
RESULTS AND DISCUSSION:

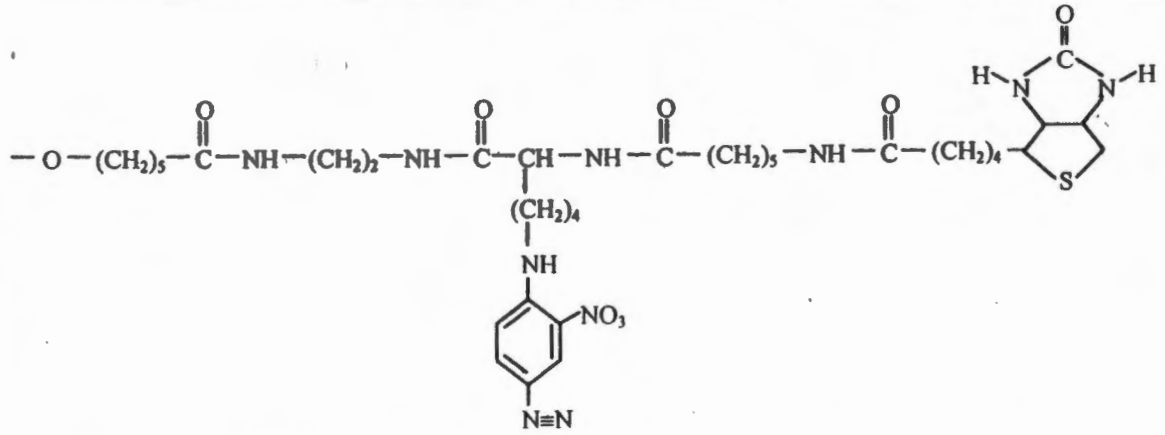
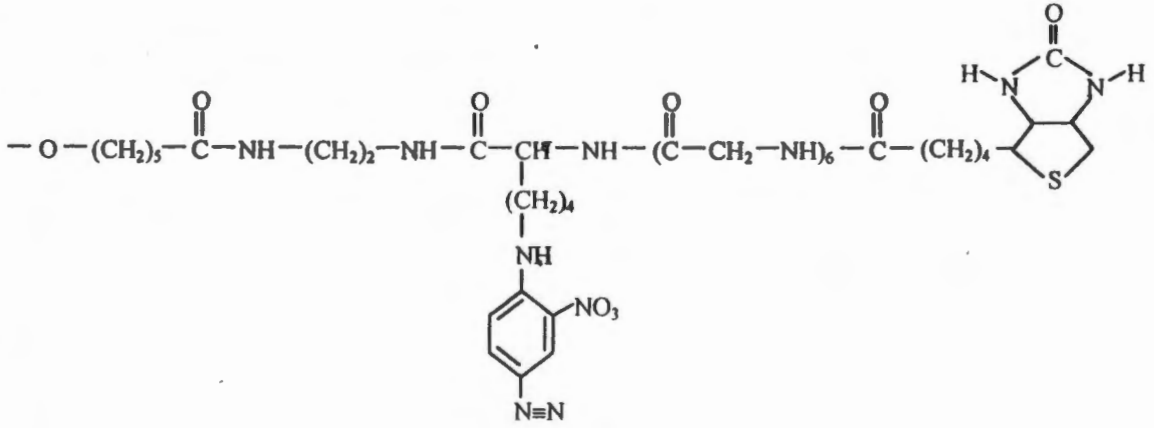
The chemical modification of α-amanitin by nucleophillic substitution of the 6'-hydroxyindole nucleus with alkyl-halides has been previously reported (Faulstich *et al.*, 1981). It is postulated that the indole nucleus projects away from the RNA polymerase II binding site and is not involved in binding, but rather functions to maintain the molecular conformation of α-amanitin (Wieland *et al.*, 1981; and Kostansek *et al.*, 1978). Therefore this method of etherisation facilitates the covalent coupling of functional spacer groups to the hydroxy-tryptophan residue without dramatically altering the inhibitory capacity (Faulstich *et al.*, 1981). Biotinylation of the coupled spacer moiety produces a soluble bifunctional probe specific for RNA polymerase II, which has been applied for the affinity purification of soluble RNA polymerase II (Vaisius and Faulstich, 1986). This conjugate is of interest, as it has the potential to biotinylate transcription complexes. By definition these complexes are associated with chromatin regions that are conformationally altered to allow the process of transcription. Thus, RNA polymerase II should be a unique handle for the isolation of both the transcription complex and the associated transcriptionally active chromatin. Furthermore, the specific labelling of the transcription complex "in situ" would enable the spatial localization of these complexes within the nucleus.

Synthesis of Bio-11-amanitin.

Bio-11-amanitin (Table 1) was synthesized and purified as described in methods and materials, with the aim to biotinylate RNA polymerase II transcription complexes "in situ". The spacer arm length is 13.4 Å, calculated by the expression (1.25*n*-0.3 Å) where *n* is the number of bonds between the carboxyl group of biotin and the oxygen of the indole nucleus for a fully extended chain (Green *et al.*, 1971)). The inhibitory characteristics of Bio-11-amanitin were determined using a sea urchin embryo nuclei or soluble chromatin transcription systems (see methods and materials and Fig 7). These results showed that the Bio-11-amanitin was a strong inhibitor of RNA polymerase II, totally inhibiting transcriptional activity at a concentration of 10^{-5} M with an estimated Ki of $2x10^{-7}$ M (Fig. 8). This was an order of magnitude higher than α-amanitin which has a Ki of $1.5x10^{-8}$ M (Fig. 8). (Using the same methodology ^{3}H-O-methyl-amanitin was synthesized as a radioactive amanitin derivative required in various procedures. It showed similar inhibitory characteristics to that of Bio-11-amanitin.)

Table 1. Chemical structures of the spacer arms coupled to α-amanitin via etherisation on the hydroxytryptophan residue.

Derivative.	Spacer arm structure.
NH_2-10-amanitin.	
Bio-11-amanitin.	
Bio-18-amanitin.	
Bio-29-amanitin.	

Derivative.	Spacer arm structure.
Bio-21-NAP-amanitin.	
Bio-32-NAP-amanitin.	

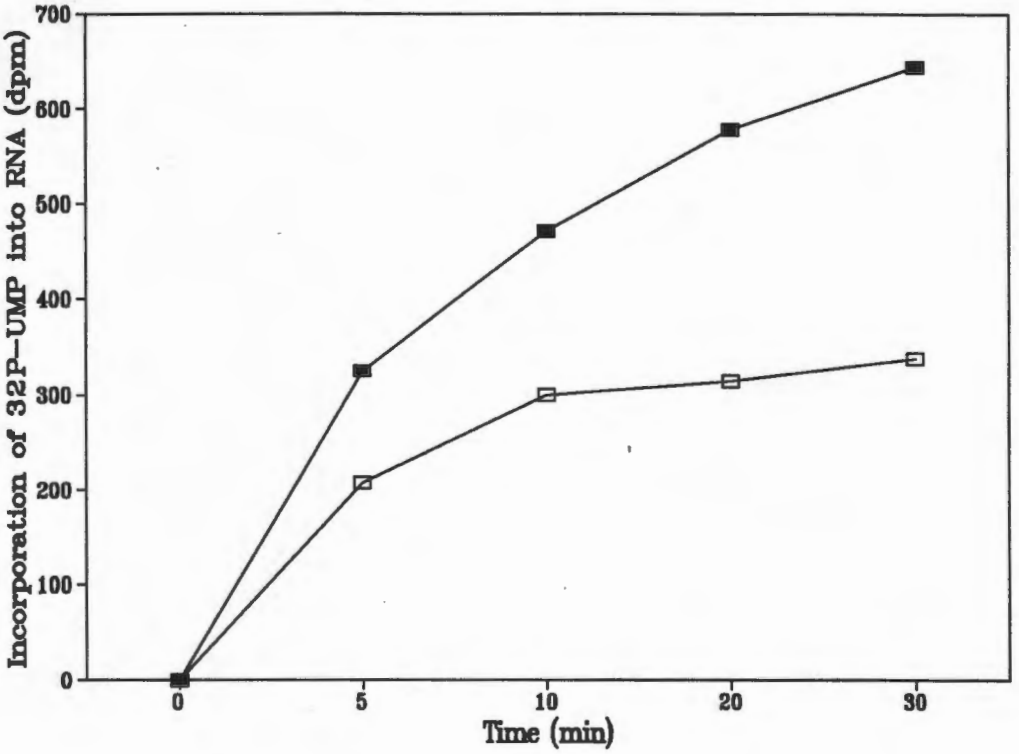


Figure 7. Run on *in vitro* transcription.

14 Hour sea urchin embryo nuclei and soluble chromatin were prepared as described in methods and materials. *In vitro* transcription was initiated with the addition of 2 volumes of transcription buffer followed by incubation at 22° C. Aliquots were removed at various time intervals and the transcription was stopped with the addition of 5 volumes of stop buffer (1 % SDS and 10 mM EDTA pH 8.0), spotted onto GFC filters and TCA precipitated as described in methods and materials. The incorporation of ^{3}H-UMP (specific activity 2.4 Ci/mmol) into RNA, transcribed from the nuclear (*filled squares*) or soluble chromatin (*open squares)* templates was determined by liquid scintillation counting and normalized to 1 μg input DNA.

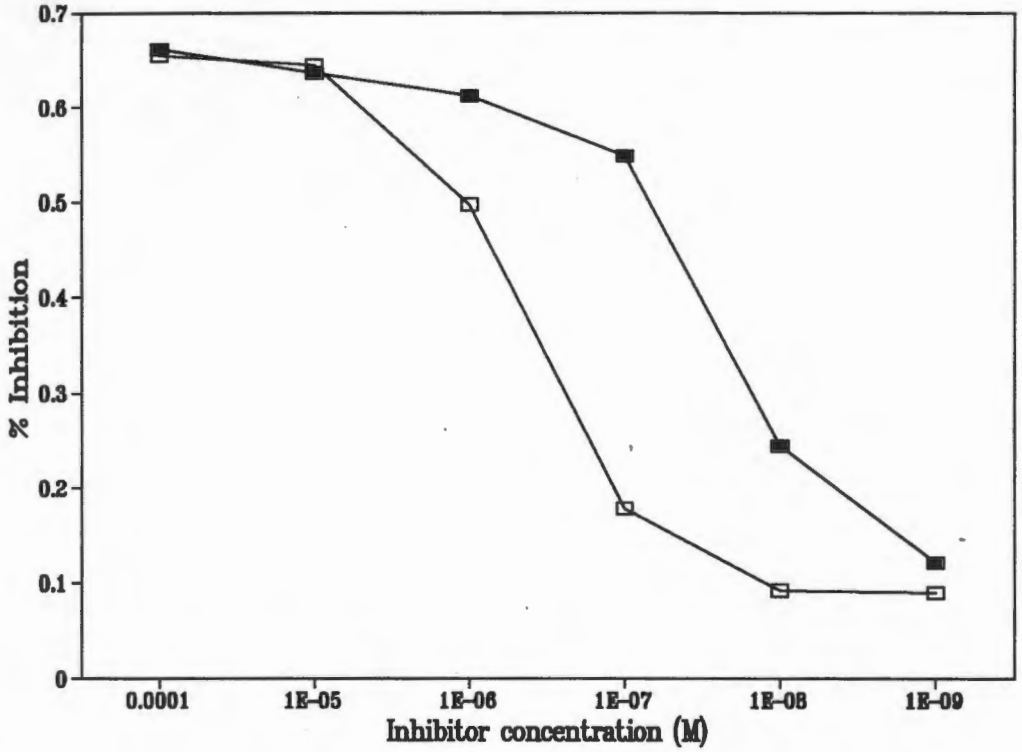


Figure 8. Inhibition of nuclear RNA polymerase II transcription activity, as a function of α-amanitin or Bio-11-amanitin concentration.

14 hour sea urchin embryo nuclei (*Parachinus Angulosis*) were incubated at 4° C for 1 hour with increasing concentrations of α-amanitin (*filled squares*) or Bio-11-amanitin (*open squares*) (ranging from $1x10^{-9}$ M to $1x10^{4}$ M) prior to the initiation of *in vitro* transcription. *In vitro* transcription was initiated with the addition of transcription buffer, followed by incubation at 22° C for 20 min. 5 volumes of stop buffer (1 % SDS and 10 mM EDTA pH8.0) was then added, aliquots were spotted on GFC filters (in duplicate) and TCA precipitated. Incorporation of ^{32}P-GMP into RNA was determined by liquid scintillation counting for 1 min.

The inhibition of 60-70 % of the transcriptional activity by α-amanitin ($1x10^{-6}$ M) or amanitin derivatives ($1x10^{-5}$ M), represents 100 % inhibition of RNA polymerase II transcription.

The stability of the [Bio-11-amanitin-RNA polymerase II] complex was determined using a competition assay. Nuclear RNA polymerase II was initially labelled with Bio-11-amanitin at a concentration of 10^{-5} M for 30 min. Unbound Bio-11-amanitin was removed by washing and the RNA polymerase II was labelled by incubation with excess ^{3}H-O-methyl-amanitin as a function of time. The half life of the [Bio-11-amanitin-RNA polymerase II] complex was found to be in the order of 24 hours relative (Table 2). The high affinity of Bio-11-amanitin for RNA polymerase II suggests that this derivative may be applied to target streptavidin to the transcription complex.

RNA polymerase II accounts for >60 % of the transcriptional activity in sea urchin embryo nuclei (early gastrula, 14 hour) (Fig. 8). Mild micrococcal nuclease digestion and solubilization of the chromatin at low ionic strength, resulted in a digestion dependent solubilization of up to 50 % of the RNA polymerase II activity, indicating the release of transcriptionally engaged RNA polymerase II (Fig. 9). However, when Bio-11-amanitin targeted, partially digested chromatin was incubated with streptavidin-Sepharose no retention of ^{3}H thymidine labelled chromatin could be shown. This indicated that either the RNA polymerase II bound Bio-11-amanitin is inaccessible for streptavidin, for steric reasons, or that the interaction of the biotinyl residue with streptavidin weakens the [amanitin-RNA polymerase II] link, thus leading to a breakdown of the latter complex.

To test the accessibility of the biotin moiety, Bio-11-amanitin targeted chromatin was incubated with streptavidin followed by a second incubation with excess ^{125}I-biotin to fill binding sites in streptavidin not occupied by the Bio-11-amanitin. The unbound ^{125}I-biotin and streptavidin were removed by filtration through nitrocellulose (Cochet-Meihac and Chambon, 1974). No specific binding of streptavidin to the biotin targeted chromatin was observed. Alternatively, the binding of streptavidin to the targeted biotin moiety may cause the release of amanitin from the RNA polymerase II binding site. However, titration with ^{3}H-O-methyl-amanitin of the available binding sites in Bio-11-amanitin targeted chromatin, after incubation with streptavidin, did not reveal any binding sites for ^{3}H-O-methyl-amanitin. Thus, streptavidin had not extracted the Bio-11-amanitin from the RNA polymerase II. Taken together these results demonstrated that the streptavidin could not see the amanitin-biotinyl residue when complexed with the RNA polymerase II transcription complex. These findings differ from Vaisius and Faulstich (1986), who reported that solid phase avidin bound to the biotin residue allowing for the purification of soluble RNA polymerase II. The observed inaccessibility could not be ascribed to

Table 2. Stability of the complex formed between α-amanitin or Bio-11-amanitin and RNA polymerase II.

Time (hours)	% RNA polymerase II quenched	
	α-amanitin	Bio-11-amanitin
2.0	99	80
4.0	96	74
24.0	94	59

Nuclei were incubated with either α-amanitin or Bio-11-amanitin at a concentration of $1x10^{-6}$ M or $1x10^{-5}$ M, respectively. Unbound amanitins were removed by repeatedly washing the nuclei with 1 ml aliquots of washing buffer (10 % glycerol, 1x buffer A, 10 mM ß-mercaptoethanol and 0.1 mM PMSF). The nuclei were then resuspended in 1 ml washing buffer and 0.1 µg ^{3}H-0-methyl-amanitin was added followed by a further incubation at 4° C. Aliqouts were removed at various time intervals and washed as described above. The quantity of ^{3}H-O-methyl-amanitin bound was determined by liquid scintillation counting of the nuclear pellet. The total dpms of ^{3}H-O-methyl-amanitin bound per unquenched nuclei aliquot was 40000 and the specific activity of ^{3}H-O-methyl-amanitin was 22000 dpm/pmol.

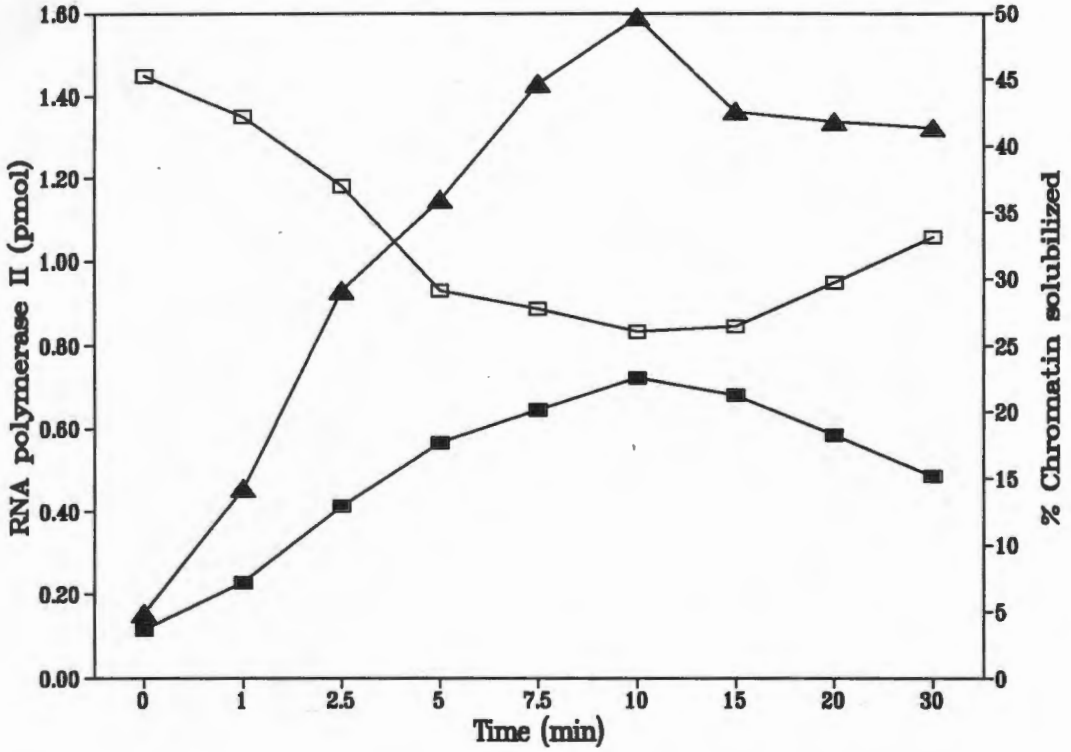


Figure 9. Solubilization of RNA polymerase II as a function of micrococcal nuclease digestion.

RNA polymerase II was labelled by incubating nuclei with ^{3}H-O-methyl-amanitin ($1x10^{-7}$ M, specific activity 22000 dpms/pmol) on ice for 1 hour. Excess amanitin was removed by repeatedly washing the nuclei with washing buffer (10 % glycerol, 1x buffer A, 10 mM ß-mercaptoethanol and 0.1 mM PMSF). The chromatin was then digested with micrococcal nuclease (150 units/mg DNA) in the presence of 1 mM $CaCl_2$ at 4° C for various time intervals. Aliquots were removed at various time intervals and digestion was stopped with the addition of EDTA (5 mM). The nuclei were pelleted by brief centrifugation and the soluble chromatin was extracted in 10 mM Tris-HCl pH 8.0, 1 mM EDTA (*filled triangles*). The partitioning of ^{3}H-amanitin labelled RNA polymerase II between the soluble (*filled squares*) and insoluble (*open squares*) fractions was determined by liquid scintillation of the respective fractions.

the dimensions of the respective streptavidin and avidin molecules as they are both very similar (55Å x 55Å x 41Å, Green *et al.*, 1971; and Wilchek and Bayer, 1990). Furthermore, the depth of the biotin binding pockets for both molecules are the same, each requiring a minimum spacer length of 6 bonds to position the biotinylated ligand on the protein surface, thus a sterical clash between the streptavidin surface and the RNA polymerase cannot account for absence of binding. Alternatively, it could be suggested that the conformation of the RNA polymerase II molecule may differ when forming the transcription complex, compared to the soluble form, or that other proteins of the transcription complex in the more immediate neighborhood of the polymerase limit access of streptavidin.

Synthesis of Bio-18-amanitin.

In order to overcome the observed sterical hindrance a second amanitin derivative, Bio-18-amanitin (see Table 1) was synthesized (see methods and materials for synthesis). The spacer length was increased by 8.4 Å, by the inclusion of aminocaproic acid into the spacer arm to give, if fully extended, a spacer length of 22.2 Å. Bio-18-amanitin totally inhibited RNA polymerase II transcriptional activity at a concentration of 10^{-5} M (Fig. 10A). This suggests that the length of the spacer arm has no effect on the binding of the amanitin moiety to RNA polymerase II. However, when complexed with avidin or streptavidin, prior to reaching the polymerase, the inhibitory capacity was reduced to the extent that a concentration of $>10^{-4}$ M was required for total inhibition of RNA polymerase II activity (Fig. 10B). Though the increase in the spacer length facilitates the binding of streptavidin, the binding of streptavidin or avidin causes a lowering of the inhibition constant. This implies that this derivative would be unfavorable for targeting streptavidin onto the transcription complex, as the equilibrium of the amanitin-RNA polymerase interaction would be shifted towards dissociation.

Synthesis of Bio-21-NAP-amanitin.

Chemical biotinylation under physiological conditions (ie. the formation of a covalent bond between the biotinyl-amanitin and the polymerase), would be the optimal method for specifically labelling RNA polymerase II, as this would overcome the observed instability of the [α-amanitin-RNA polymerase II] interaction, as a

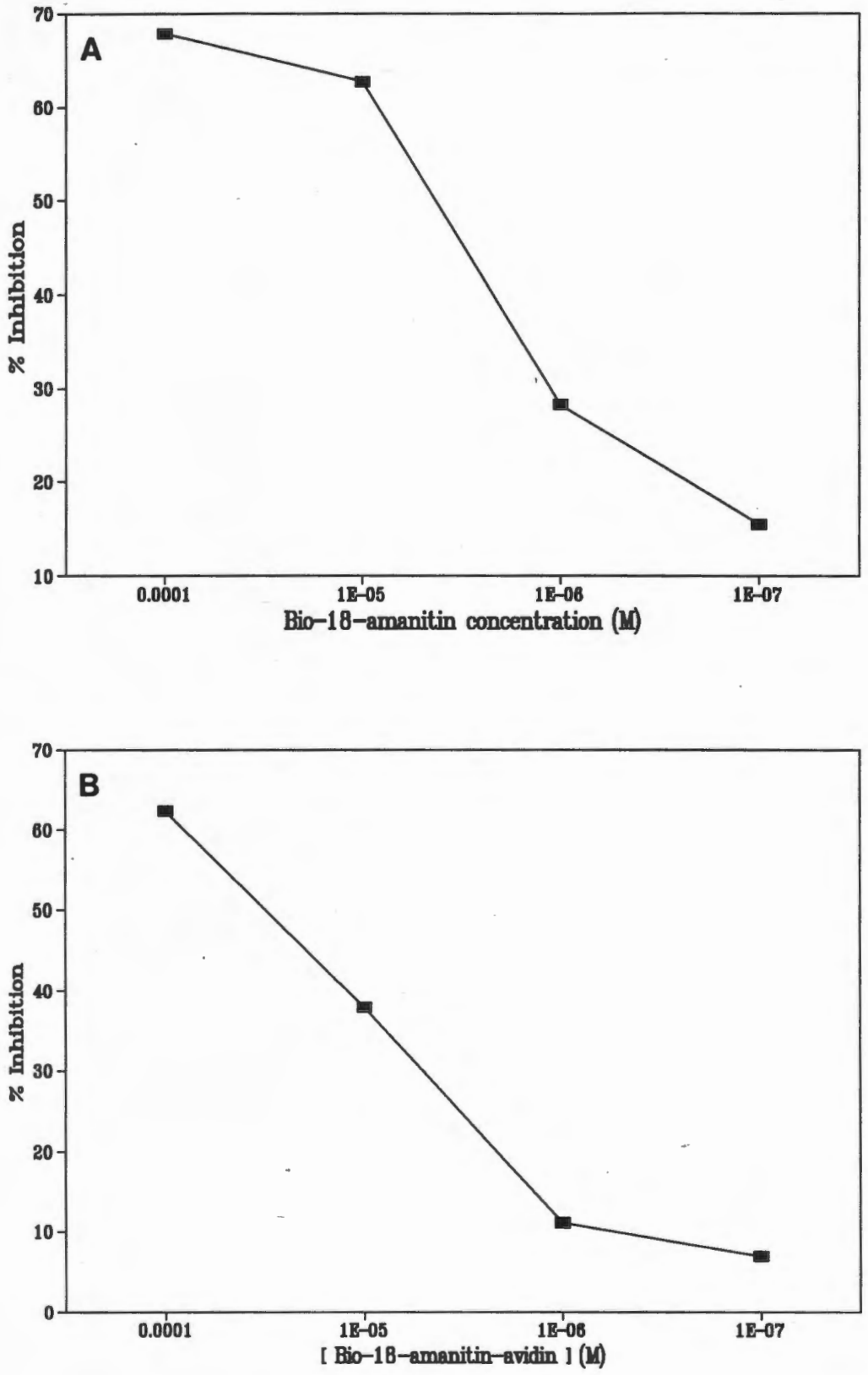


Figure 10. Inhibition of RNA polymerase II transcriptional activity as a function of Bio-18-amanitin concentration.

A) 14 hour sea urchin embryo nuclei were incubated with increasing concentrations of Bio-18-amanitin (ranging from $1x10^{-7}$ M to $1x10^{-4}$ M) at 4° C for 1 hour, prior the

initiation of *in vitro* transcription. Nuclear *In vitro* transcription was performed as described in methods and materials (see also figure 8 legend).

B) Solubilized chromatin was incubated with increasing concentrations of the complex [Bio-18-amanitin-avidin (molar ratio 1:1)] (ranging from $1x10^{-7}$ M to $1x10^{-4}$ M) at 4° C for 1 hour, prior to the initiation of *in vitro* transcription. Transcription was initiated with the addition of 2 volumes of transcription buffer, followed by incubation at 22° C for 10 min. The incorporation of ^{3}H-UMP in RNA was determined as described in methods and materials.

function of streptavidin binding. The method of photochemical crosslinking has been previously applied to covalently link substrate molecules to the enzyme catalytic sites in physiological buffers (Bayley, 1983). Following this principle, I propose that the high affinity of α-amanitin for RNA polymerase II can be used to specifically position both the biotinyl residue and a chemical crosslinking group within the transcription complex, thereby facilitating "in situ", covalent biotinylation of RNA polymerase II .

To achieve this aim a spacer arm was synthesized with the inclusion of lysine positioned 11 bonds (13.4 Å) from the indole nucleus of α-amanitin and 8 bonds (9.7 Å) from the biotin carboxyl group (overall spacer length 21 bonds, 25.9 Å). The nucleophillic ε-amino group of the lysine residue allowed for the covalent coupling of the photoreactive 4-Fluoro-3-Nitrophenyl azide group ((Bio-21-NAP-amanitin, Table 1) (for synthesis see methods and materials)). This photoreactive group was chosen because the activation spectrum of its azido group shows a relatively high quantum yield at a wavelength of 486 nm (Fig 6). This is important, as other available photoreactive groups are activated below 400 nm. Exposure of the α-amanitin to a wavelength of 305 nm has been reported to cause irreversible destruction of the indole nucleus, which results in the dissociation of the α-amanitin from the RNA polymerase II (Lutter, 1982).

The inhibitory capacity of Bio-21-LYS-amanitin was similar to previously synthesized derivatives, showing total inhibition of RNA polymerase II transcriptional activity at a concentration of 10^{-5} M (Fig. 11). Thus, it was presumed that Bio-21-NAP-amanitin would show a similar inhibitory capacity (inhibition experiments with Bio-21-NAP-amanitin could not be performed due to the limited amount of derivative synthesized).

Specific photochemical crosslinking of Bio-21-NAP-amanitin to a high molecular weight protein (approximate 140 kD) was shown by Western blot analysis of the total nuclear protein fractionated by SDS polyacrylamide electrophoresis (Fig. 12). This biotinylated protein was presumed to be the α-amanitin binding subunit of RNA polymerase II as the molecular weight corresponds to that of a previous report (Brodner and Wieland, 1976).

"In situ" labelling of the Bio-21-NAP-amanitin biotinylated RNA polymerase II in permeabilized sea urchin embryo nuclei with the ^{125}I-biotin-streptavidin

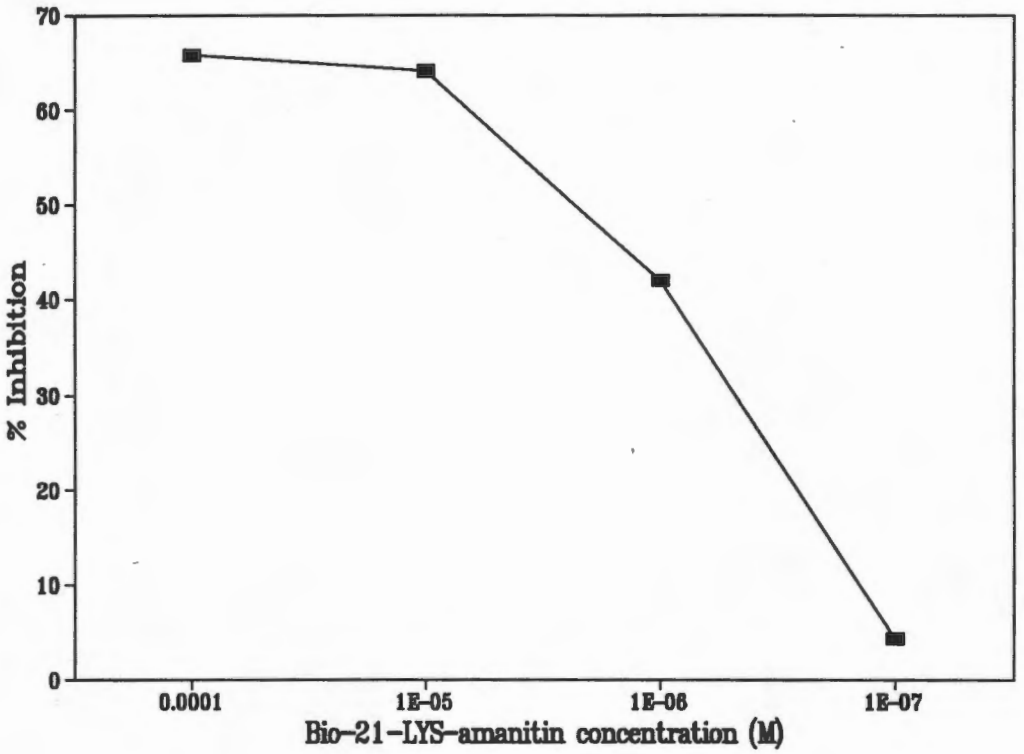


Figure 11. Inhibition of nuclear RNA polymerase II transcriptional activity as a function of Bio-21-LYS-amanitin concentration.

14 hour sea urchin embryo nuclei were incubated with increasing concentrations of Bio-21-LYS-amanitin (ranging from $1x10^{-7}$ M to $1x10^{-4}$ M) at 4° C for 1 hour, prior to the initiation of *in vitro* transcription. *In vitro* transcription was performed as described in methods and materials (see also figure 8 legend).

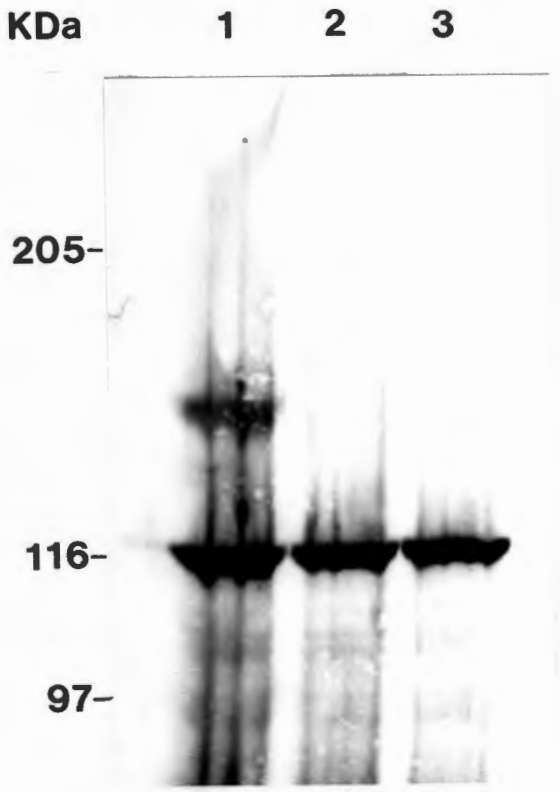


Figure 12. Western blot of nuclear RNA polymerase II photochemically biotinylated "in situ" with Bio-21-NAP-amanitin.

RNA polymerase II transcription complexes were photochemically biotinylated "in situ" with Bio-21-NAP-amanitin as described in methods and materials. The chromatin was then digested with micrococcal nuclease (150 units/mg DNA) and DNAse I (5 µg/mg DNA) in the presence of $CaCl_2$ (1 mM) and $MgCl_2$ (5 mM) at 4° C for 1 hour to reduce the viscosity. Digestion was stopped with the addition of an equal volume of 2x SDS application buffer and heated to 95° C for 2 min. Aliquots of the respective samples were then fractionated by electrophoresis in 7.5 % polyacrylamide and Western blotted onto Hybond C. The biotinylated proteins were visualized by developing with streptavidin-alkaline phosphatase in combination with BCIP and NBT.

Lane 1. Nuclei were incubated with Bio-21-NAP-amanitin followed by photoactivation.
Lane 2. Nuclei were pre-incubated with α-amanitin to quench the RNA polymerase II binding sites, followed by a second incubation with Bio-21-NAP-amanitin and photoactivation.
Lane 3. Control nuclei (incubated in the absence of amanitins).

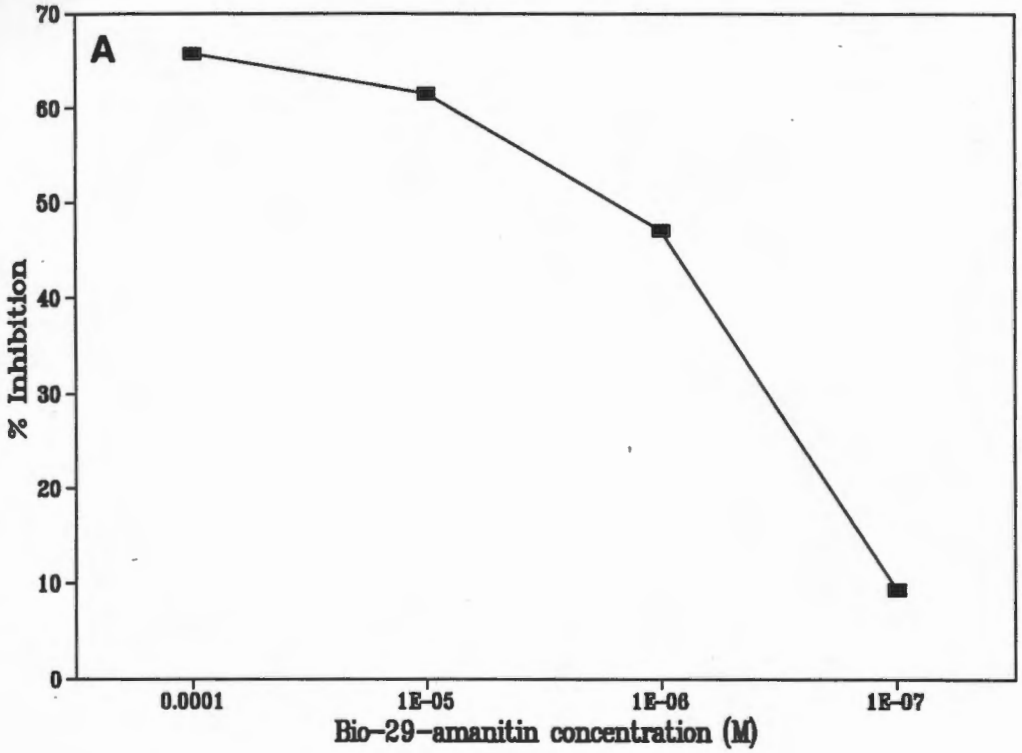
complex (molar ratio 0.5:1), failed to show any specific binding of streptavidin (data not shown). This result can in all likelihood be attributed to the length of the spacer arm. The azido crosslink could effectively reduce the original spacer length by a factor of approximately 2 due to its position within the spacer arm. Thus, when crosslinked to RNA polymerase II sterical hindrance might be re-introduced preventing the binding of streptavidin. This phenomena is only observed for RNA polymerase II in the native conformation and only when denatured (during SDS electrophoresis) was the biotin residue accessible for streptavidin binding, as seen from the Western blots analysis (Fig 12).

Synthesis of Bio-29-amanitin.

The above results indicate that the spatial separation between the biotin residue and the α-amanitin indole nucleus is crucial for the formation of a stable complex [streptavidin-biotinyl-amanitin-RNA polymerase II]. To increase the spacer length a further biotinylated α-amanitin derivative was synthesized, in which hexaglycine was substituted for aminocaproic acid ((Bio-29-amanitin, Table. 1) (see methods and materials for synthesis)). The effective extended length of the spacer arm was increased to 35.9 Å.

Characterization of the inhibitory properties showed that Bio-29-amanitin totally inhibited RNA polymerase II activity at a concentration of 10^{-5} M (Fig. 13A). When complexed with avidin prior to reaction with polymerase, 90 % of the inhibitory capacity was maintained at this concentration (Fig. 13B). This suggests that the increase in arm length positions the avidin or streptavidin in such a way that sterical hindrance only minimally effects the stability of the amanitin-RNA polymerase II interaction. This was supported further, by the fact that the addition of streptavidin to the targeted biotinyl residue failed to cause rapid dissociation of the amanitin-polymerase interaction (see Table 3).

"In situ" labelling of the nuclear [Bio-29-amanitin-RNA polymerase II] complex with ^{125}I-biotin-streptavidin clearly demonstrated that the biotin residue is accessible to streptavidin (Table 4). Furthermore this labelling is quenched by pre-incubation with α-amanitin demonstrating that the biotinylation of RNA polymerase II is dependent on the binding of the amanitin moiety.



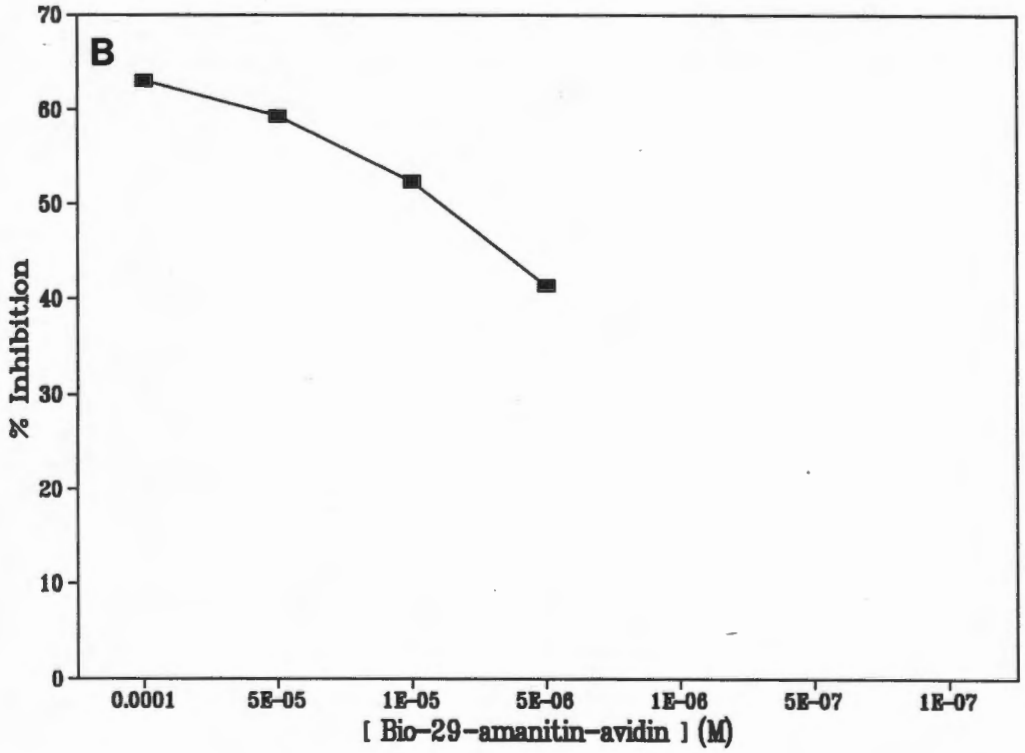


Figure 13. Inhibition of nuclear RNA polymerase II transcriptional activity as a function of increasing concentrations of Bio-29-amanitin.

A) 14 hour sea urchin embryo nuclei were incubated with increasing concentrations of Bio-29-amanitin (ranging from $1x10^{-7}$ M to $1x10^{-4}$M) at 4° C for 1 hour, prior to the initiation of *in vitro* transcription. *In vitro* transcription was performed as described in methods and materials (see also figure 8 legend).

B) Solubilized chromatin was incubated with increasing concentrations of the complex [Bio-29-amanitin-avidin (molar ratio 1:1)] (ranging from $5x10^{-6}$ M to $1x10^{-4}$ M) at 4° C for 1 hour, prior to the initiation of *in vitro* transcription. Transcription was initiated with the addition of 2 volumes of transcription buffer followed by incubation at 22° C for 10 min. The incorporation of ^{3}H-UMP in RNA was determined as described in methods and materials.

Table 3. Stability of the complex [streptavidin-Bio-29-amanitin-RNA polymerase II] as a function of time.

	% Transcriptional activity inhibited.	
Time (hours)	Bio-29-amanitin	Bio-29-amanitin-streptavidin
0.0	60.8 %	63.4 %
1.0	66.8 %	61.3 %
2.0	64.9 %	59.6 %

The complex [Bio-29-amanitin-RNA polymerase II] was formed "in situ" by incubating 14 hour sea urchin embryo nuclei with Bio-29-amanitin ($1.1x10^{-5}$ M) at 4° C for 1 hour. Unbound Bio-29-amanitin was removed by repeatedly washing the nuclei with nuclei storage buffer. The chromatin was solubilized by digestion with micrococcal nuclease as described in methods and materials. Aliquots of the solubilized chromatin were then incubated at 4° C in the absence or presence of excess streptavidin. *In vitro* transcription was initiated with the addition of transcription salts at various time intervals and allowed to proceed at 22° C for 10 min followed by the addition of stop buffer. The efficiency of ^{3}H-UMP incorporation into RNA was determined by liquid scintillation after TCA precipitation onto GFC filters.

Table 4. "In situ" labelling of nuclear RNA polymerase II with a ^{125}I-biotin-streptavidin complex via interaction with targeted Bio-29-amanitin

	^{125}I-biotinyl-streptavidin[d]	
Nuclei preparation	(dpm)	(pmol)
Control[a]	29562	0.09
Control[b]	33037	0.10
Experiment[c]	299477	0.93

a) Nuclei were incubated in the absence of α-amanitin or Bio-29-amanitin prior to labelling with ^{125}I-biotinyl-streptavidin.
b) Nuclei were pre-incubated with α-amanitin ($3x10^{-6}$ M) for 1 hour, followed by a second incubation with Bio-29-amanitin ($5x10^{-6}$ M) for 4 hours. Unbound amanitins were remove by washing, followed by photoactivation and labelling with ^{125}I-biotinyl-streptavidin.
c) Nuclei were incubated with Bio-29-amanitin ($5x10^{-6}$ M) for 4 hours, followed by photoactivation and labelling with ^{125}I-biotinyl-streptavidin.
d) Specific activity of ^{125}I-biotinyl-streptavidin was $3.22x10^5$ dpm/pmol and the molar ratio of biotin:streptavidin was 0.75:1.

Synthesis of Bio-32-NAP-amanitin.

The [streptavidin-Bio-29-amanitin-RNA polymerase II] complex appears to be relatively stable, allowing numerous washing steps once bound to the nuclei. However, it was uncertain as to whether this complex would remain intact when subjected to the high frictional drag forces of affinity purification. To counteract this possible problem the photoreactive 3-Nitrophenyl azide group was incorporated into the spacer arm on the ε amino group of lysine positioned 11 bonds (13.4 Å) from the amanitin indole nucleus and 19 bonds (23.4 Å) from the biotin carboxyl group. The product Bio-32-NAP-amanitin had an overall spacer length of 39.7 Å (Table 1) (see methods and materials for synthesis). The inhibitory capacity of Bio-32-NAP-amanitin was presumed to be the same as observed for Bio-29-amanitin as the spacer arm length of previous derivatives did not alter the Ki.

Amanitin directed, photochemical, biotinylation of the RNA polymerase II subunits by Bio-32-NAP-amanitin was shown by Western blot analysis of the total nuclear protein, developed using a streptavidin-alkaline phosphatase conjugate (Fig. 14). Two polypeptides with approximate molecular weights 220 Kd and 200 Kd, were shown to be specifically biotinylated. The 220 Kd polypeptide corresponds to the largest subunit of RNA polymerase II and the 200 Kd could be proposed to be either a dephosphorylated or proteolytically cleaved product of the largest subunit (Sawadogo, 1990). The crosslinking of Bio-32-NAP-amanitin to the largest subunit differs from Bio-21-NAP-amanitin which crosslinks to the 140 kD subunit. This suggests that the secondary structure of the spacer arms may position the respective photoreactive groups at different locations within the binding pocket. Furthermore, these results indicate that the largest subunit interacts closely with the α-amanitin binding site on the 140 kD subunit. This is consistent with a report that shows that the two major subunits interact strongly with each other (Bateman and Nicholson, 1984). Furthermore, the conformation of the α-amanitin binding site appears to be dependent on this interaction, as a single mutation in the largest subunit can confer amanitin resistance (Greenleaf, 1983).

Pre-incubation of RNA polymerase II with α-amanitin prior to the addition of Bio-32-NAP-amanitin and photochemical crosslinking demonstrated the specificity of biotinylation (Fig 14, lane 2). Thus, proving that Bio-32-NAP-amanitin only targeted the biotinylation of RNA polymerase II transcription complexes. However, Western blot analysis of total nuclear proteins from control nuclei (incubated in the absence of amanitin derivatives) demonstrated the presence of streptavidin-alkaline

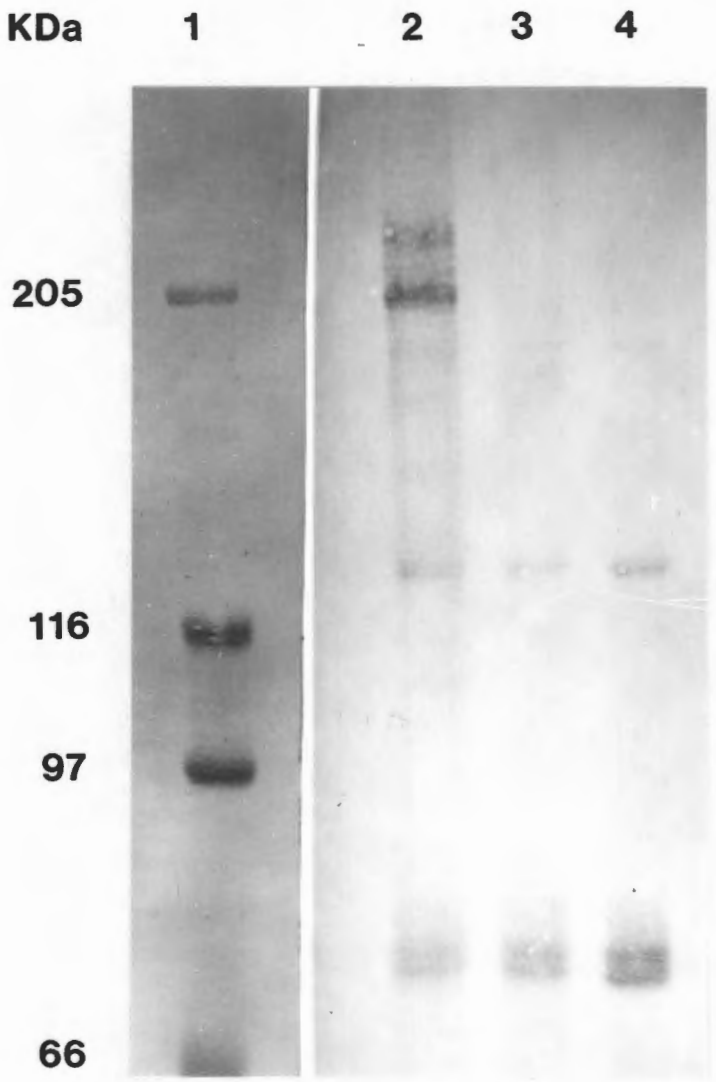


Figure 14. Western blot of nuclear RNA polymerase II photochemically biotinylated "in situ" with Bio-32-NAP-amanitin.

(See figure 11 legend and methods and materials for methodology.)

Lane 1. High molecular weight protein standard.
Lane 2. Nuclei photochemically biotinylated with Bio-32-NAP-amanitin.
Lane 3. Nuclei pre-incubated with α-amanitin, prior to the addition of Bio-32-NAP-amanitin and photoactivation.
Lane 4. Control nuclei (incubated in the absence of amanitins).

phosphatase positive proteins (Fig 14, lane 4). Initially it was assumed that the detection of these proteins was the result of non-specific binding by alkaline phosphatase, as no covalently biotinylated proteins have been previously detected in nuclei (Dakshinamurti and Chauhan, 1990). However, further analysis has shown that these proteins are naturally biotinylated, judged by their ability to bind to solid phase streptavidin. They appear to be cytoplasmic proteins that co-isolate with the nuclei and can be largely removed by increasing the sucrose concentration from 1.3 M to 1.5 M, during the nuclei isolation procedure.

The binding of ^{125}I-biotin-streptavidin complex to Bio-32-NAP-amanitin labelled native RNA polymerase II within permeabilized nuclei confirmed that the biotin moiety was accessible to streptavidin (Table 5). The specific labelling ratio observed was approximately 10:1 for the biotinylated nuclei versus the control nuclei or the α-amanitin pre-quenched nuclei. These results demonstrate that the spacer arm, hexaglycine, was of sufficient length to overcome the constraints imposed by the nitrophenyl azide group induced crosslink to protein (the azido group is converted to a nitrene radical which then reacts with any suitable group within the protein).

Demonstration RNA polymerase II within the nucleus.

Having shown that the transcription complexes can be specifically biotinylated, two questions arose: 1) Can the Bio-32-NAP-amanitin be applied for the isolation of the transcription complex and the associated transcriptionally active chromatin? (see chapter 2). 2) Can the Bio-32-NAP-amanitin be applied to determine the location of transcription complexes within the nuclei?

The nucleus is envisaged as a highly ordered structure in which chromatin regions are specifically positioned to facilitate transcription regulation and communication with the cytoplasm (Jackson, 1991; and Carter and Lawrence, 1991). Nick translation of the actively transcribing chromatin has suggested a peripheral location below the nuclear membrane (Hutchison and Weintraub, 1985; and Thiry, 1991a and 1991b). "In situ" hybridization and immuno-labelling of nascent RNA has demonstrated that transcription occurs at specific foci and the RNA is processed along tracks radiating away from the point of transcription (Xing *et al.*, 1993; Jackson *et al.*, 1993). However, as yet the spatial location of RNA polymerase II transcription complexes has not been directly demonstrated.

Table 5. "In situ" labelling of covalently biotinylated native nuclear RNA polymerase II with a ^{125}I-biotin-streptavidin complex.

	^{125}I-biotinyl-streptavidin[d]	
Nuclei preparation	(dpm)	(pmol)
Control[a]	84311	0.11
Control[b]	167025	0.22
Experiment[c]	1353302	1.78

a) Nuclei were incubated in the absence of α-amanitin or Bio-32-NAP-amanitin prior to labelling with ^{125}I-biotinyl-streptavidin.
b) Nuclei were pre-incubated with α-amanitin ($3x10^{-6}$ M) for 1 hour, followed by a second incubation with Bio-32-NAP-amanitin ($5x10^{-6}$ M) for 4 hours. Unbound amanitins were remove by washing, followed by photoactivation and labelling with ^{125}I-biotinyl-streptavidin.
c) Nuclei were incubated with Bio-32-NAP-amanitin ($5x10^{-6}$ M) for 4 hours, unbound amanitin was removed by washing, followed by photoactivation and labelling with ^{125}I-biotinyl-streptavidin.
d) Specific activity of ^{125}I-biotinyl-streptavidin was $7.59x10^5$ dpm/pmol and the molar ratio of biotin:streptavidin was 0.75:1.

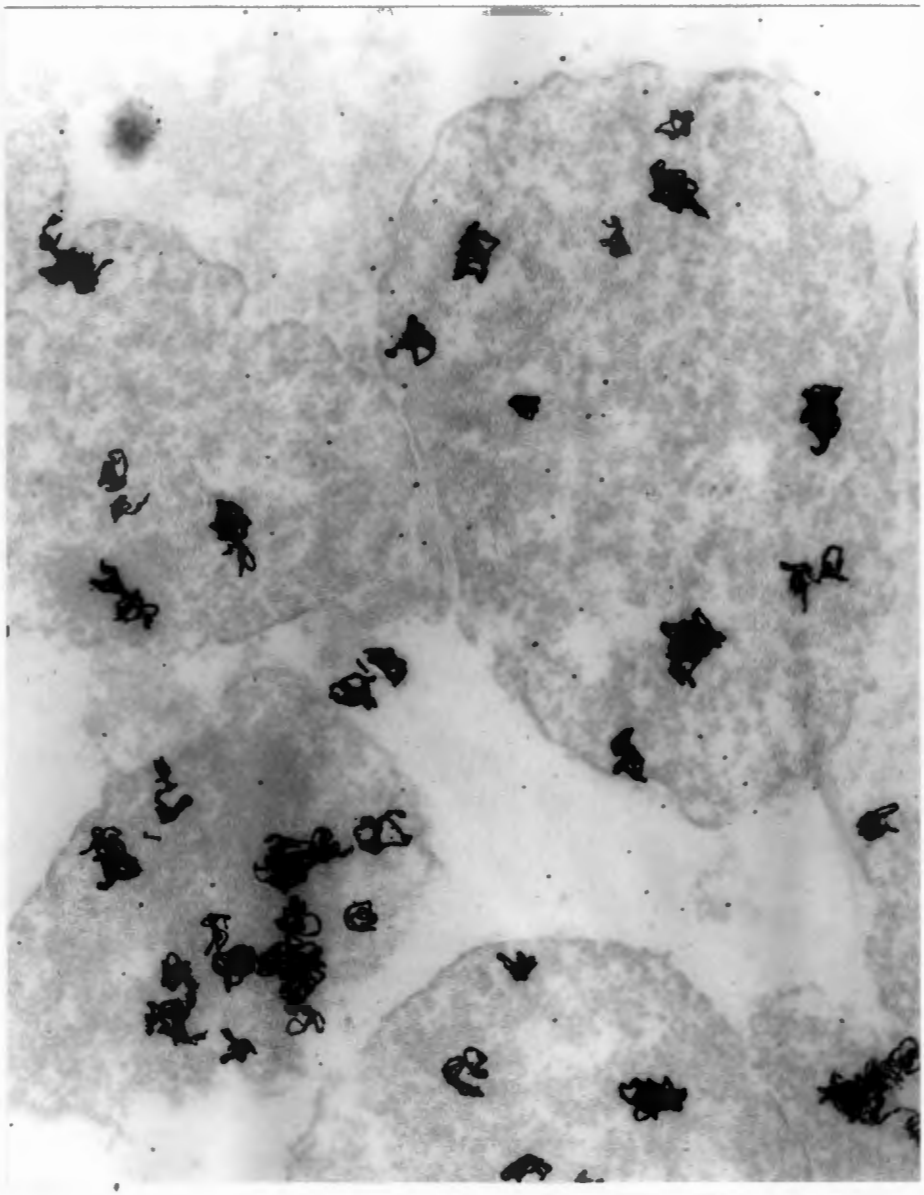
Visualization of the transcription complexes within the nucleus at the resolution of the electron microscope should further the understanding of the spatial location of the active chromatin.

The novel probe for the transcription complex, Bio-32-NAP-amanitin, opens up the possibility to label the transcription complexes within the nucleus for purposes of electron microscopy. Initially the technique of post embedding labelling was followed. Sea urchin embryo nuclear RNA polymerase II was labelled "in situ" with Bio-32-NAP-amanitin followed photolysis of the azido group and embedding of the nuclei in Epon 812 resin. Ultra thin nuclear sections were then treated with colloidal gold streptavidin (5 nm) to detect the biotinyl residues. The results (data not shown) showed a poor signal to noise ratio (2:1), this could have been due to two reasons. 1) The non porous nature of the resin restricts the accessibility of the biotinyl residues inside the sections as compared to those located on the surface of the section. Due to the low abundance of RNA polymerase II only a minor percentage of the latter molecule were detected. 2) The biotinyl residue within the native transcription complex is not accessible to the macromolecular streptavidin-gold complex, thus binding will only occurs if the transcription complex is in a partially denatured conformation (analogous to the denatured conformation visualized by Western blots). (The accessibility of the biotinyl residue in the native transcription complex is demonstrated in chapter 2.)

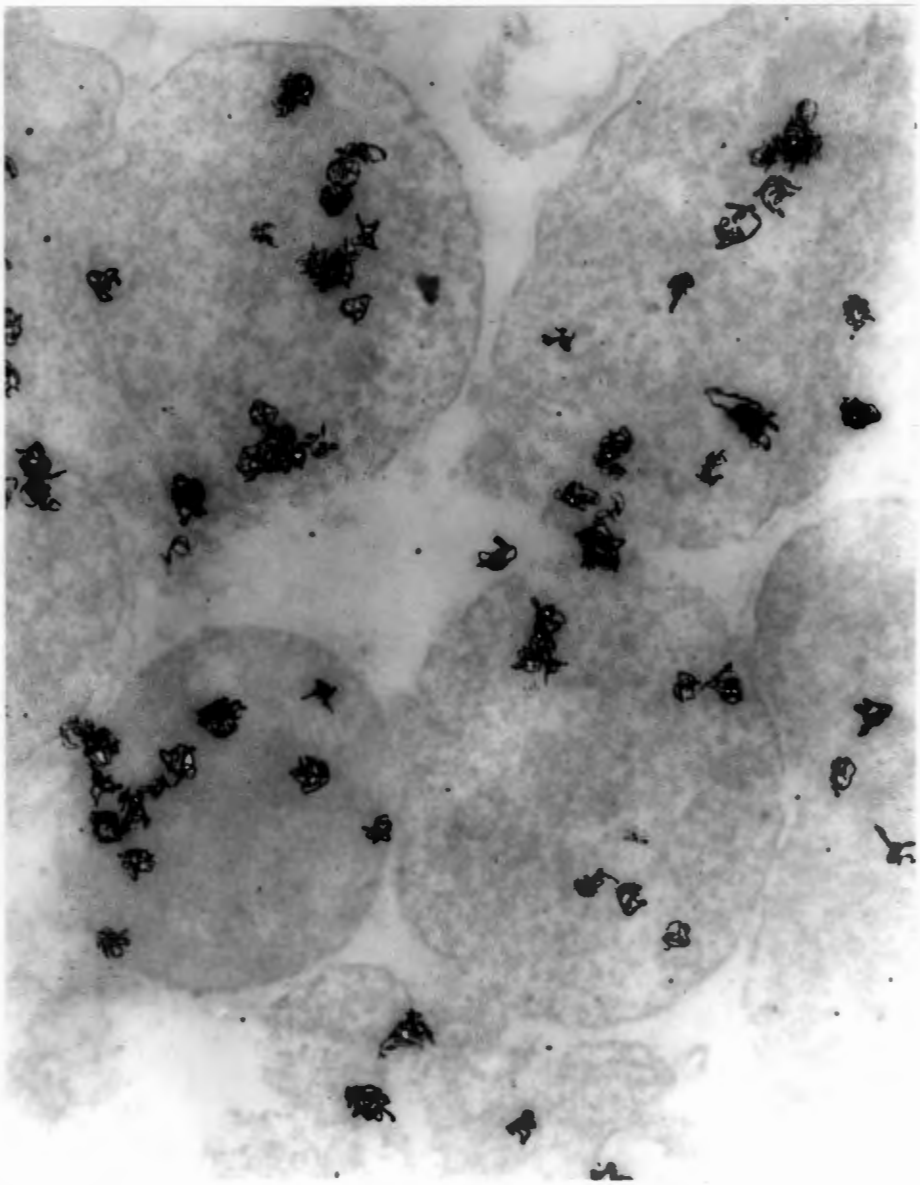
To circumvent the above problems, the technique of EM autoradiography was applied. Sea urchin embryo nuclei were labelled "in situ" with Bio-32-NAP-amanitin, followed by incubation with high specific activity ^{125}I-streptavidin (specific activity 3000 Ci/mmol), prior to embedding (this method of labelling has been shown to specifically label the transcription complex "in situ" (Table 4)). EM and LM autoradiography of ultra thin nuclear sections, 90-150 nm and 1 μm, respectively, (embedded in Epon 812), demonstrated that the Bio-32-NAP- amanitin is positioned in defined single loci, which by virtue of the amanitin residue within the probe, must be the transcription complexes (Fig. 15A and B; Fig 16A). These transcription complexes appear to be distributed throughout the nucleoplasm. This distribution may be the result of the cells being at a very early stage of differentiation, with a short cell cycle and a high degree of transcription.

EM and LM autoradiography of control nuclei (without Bio-32-NAP-amanitin) and α-amanitin quenched nuclei (with Bio-32-NAP-amanitin) show very low levels of labelling, demonstrating that the targeting of ^{125}I-streptavidin to

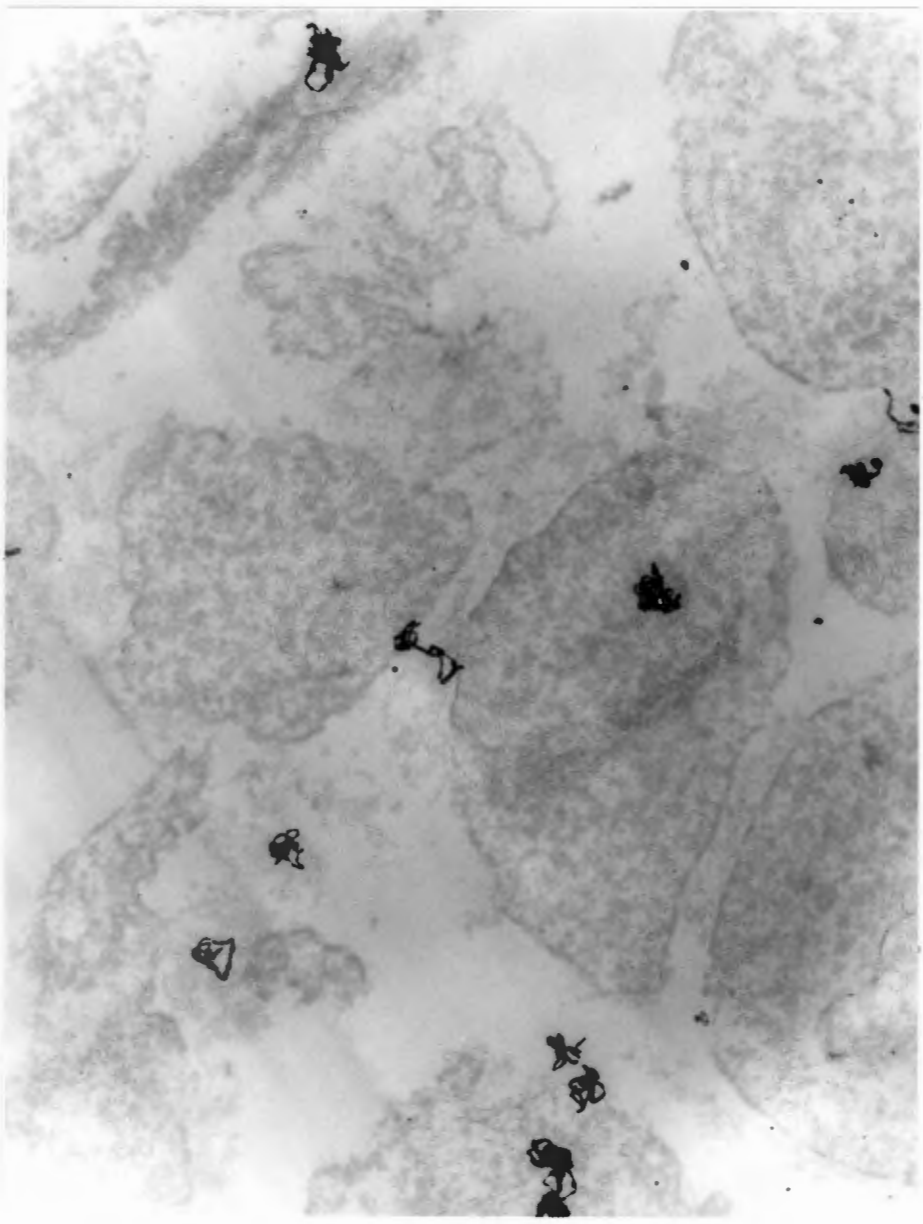
A



B



C



D

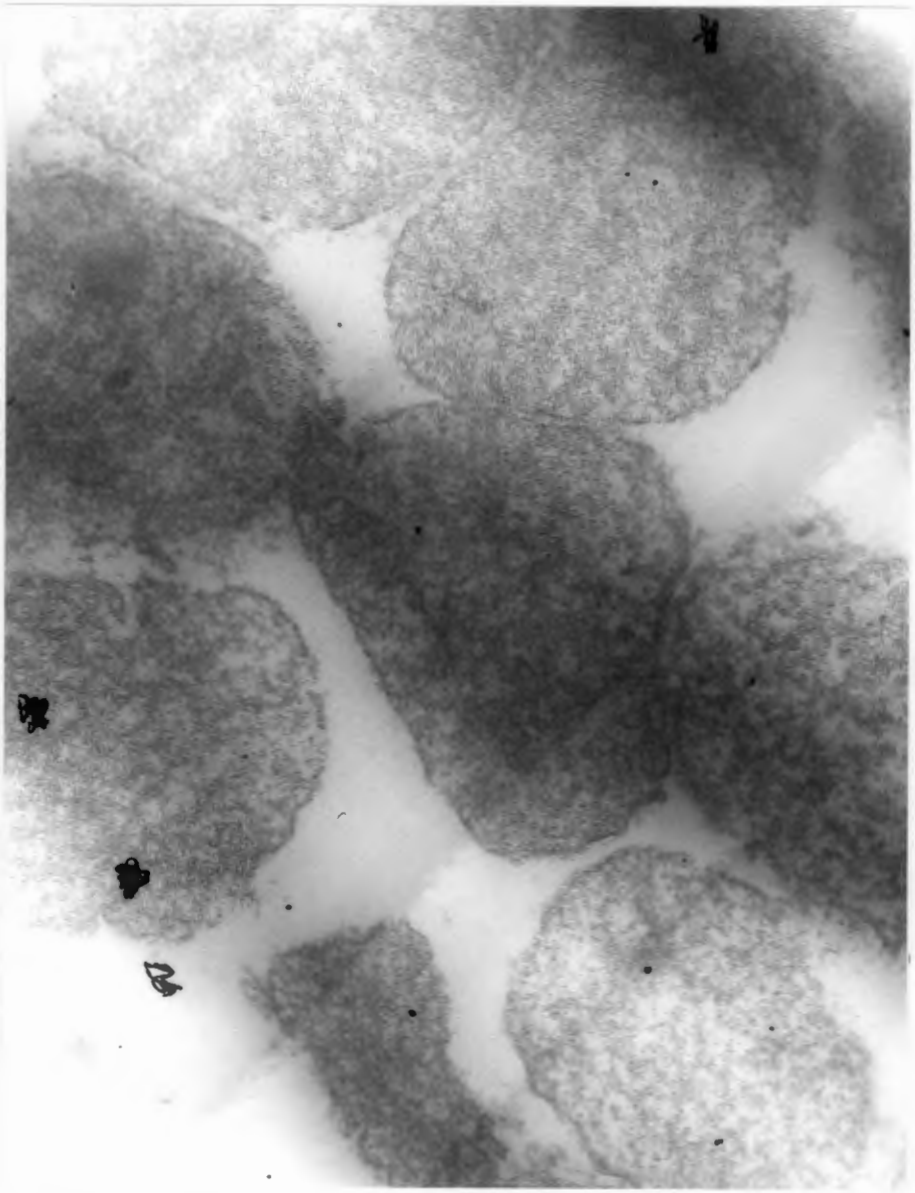


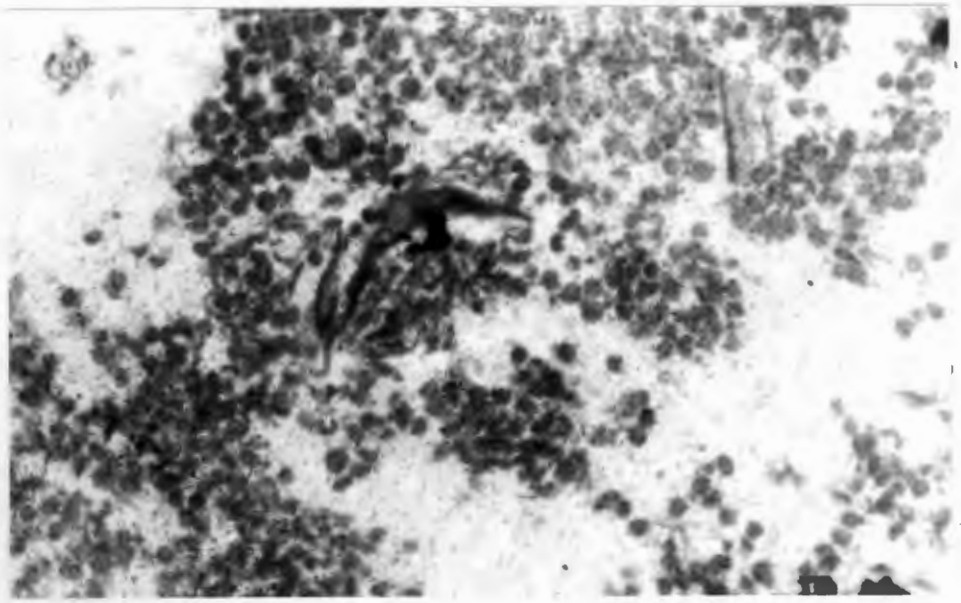
Figure 15. EM autoradiography of ultra thin sections of 14 hour sea urchin embryo nuclei.

A and B) Nuclear RNA polymerase II was covalently biotinylated "in situ" with Bio-32-NAP-amanitin and the targeted biotinyl residues were labelled with ^{125}I-streptavidin, as described in methods and materials. The nuclei were crosslinked with 1 % glutaraldehyde and 1 % formaldehyde and embedded into medium hard Epon 812. Ultra-thin sections (90-150 nm) were positioned onto formvar coated nickel grids, stained (Reynolds stain), coated with a carbon film followed by a mono layer of EM-1 emulsion (Amersham). Autoradiography was allowed to proceed for 9 weeks at 4° C and the emulsion was developed with D19 as described in methods and materials. The contrast was enhanced by straining with urinyl acetate and the nuclei and silver grains were visualized by electron microscopy.

C) Nuclei pre-incubated with α-amanitin to quench the amanitin binding sites on RNA polymerase II. Nuclei were then incubated with Bio-32-NAP-amanitin followed by photoactivation and labelling with ^{125}I-streptavidin as described in section A. This control was a measure of the non-specific biotinylation resulting from photoactivation of untargeted Bio-32-NAP-amanitin.

D) Nuclei incubated in the absence of α-amanitin or Bio-32-NAP-amanitin prior to the incubation with ^{125}I-streptavidin. This control was a measure of the non-specific binding of ^{125}I-streptavidin to nuclear proteins.

A



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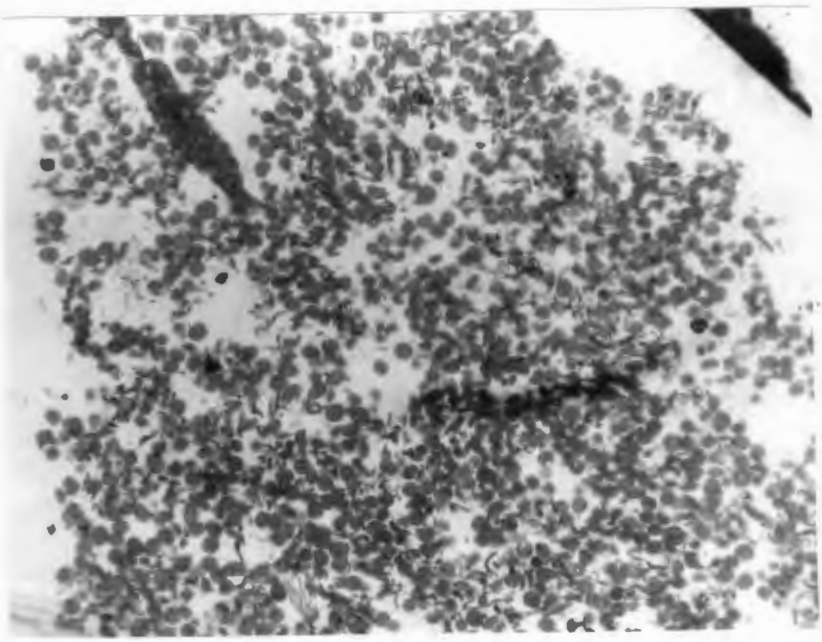

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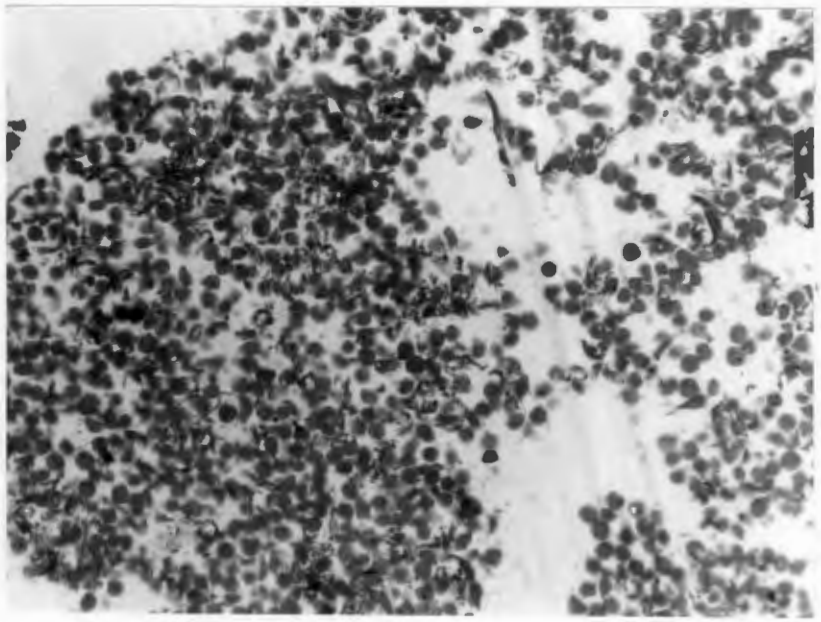

Figure 16. LM autoradiography of thin sections of 14 hour sea urchin embryo nuclei.

Nuclear sections (1 μm) were prepared as described in figure 15, positioned on formvar coated glass slides, stained (Reynolds stain), coated with a thin film of carbon followed by a thin film of EM-1 emulsion. Autoradiography was allowed to proceed for 2 weeks and the emulsion was then developed with D19. The sections were stained with methylene green prior to visualization by light microscopy.

A) Nuclear RNA polymerase II, photochemically biotinylated with Bio-32-NAP-amanitin.
B) Nuclei were pre-incubated with α-amanitin to saturate the RNA polymerase II α-amanitin binding sites prior to the addition of Bio-32-NAP-amanitin and photoactivation.
C) Control nuclei incubated in the absence of amanitins.

the transcription complex is dependent of the amanitin-RNA polymerase II interaction (Fig 15C and D; Fig 16B and C). Thus, it can be concluded that the observed labelling as seen in figure 15A and B, and figure 16A is not an artifact of non-specific binding.

These results demonstrate that the high affinity of α-amanitin for RNA polymerase II can be applied to specifically target an affinity ligand (biotin) onto the transcription complex. Thereby introducing a handle that can be subsequently labelled, via the binding of streptavidin, for the localization of the transcription complexes within the nucleus. This methodology is novel when compared to previously described methods for the covalent radioactive labelling of RNA polymerase and transcription complexes (Bartholomew *et al.*, 1990, Khanna *et al.*, 1991; and Sheng *et al.*, 1993). These methods as yet, have not been applied to localize the transcription complexes within the nucleus. Current methodologies depend on the non-covalent targeting of fluorescent markers via the binding of anti-RNA polymerase II or amanitin, which implies that the resolution is not at a molecular level.

Certain technical problems have limited the sensitivity of the described methodology. These can be summarized as follows. 1) The translocation of the ^{125}I ß-particle limits the resolution to 1-10 µm. 2) The random direction of ß-particle emission results in a loss of 50 % of the signal, as the emulsion is only applied to one side of the section. 3) The half-life of decay limits the level of detection due to the number of radioactive molecules deposited per biotinyl residue. It could be envisaged that the sensitivity would be enhanced by combining the following techniques. 1) Ultra-thin Cryo-sections would allow optimal penetration of streptavidin conjugates into the section, thereby maximizing the degree of labelling, however this technique was not available. 2) Colloidal gold streptavidin (1 nm) would eliminate the problems associated with a radioactive marker, but may have to be used in conjunction with long flexible spacer moieties.

A further advantage of introducing an affinity ligand (biotin) directly onto the native transcription complex, would be the affinity purification of the complex via streptavidin affinity chromatography. Because the RNA polymerase transcriptional complex is an integral component of transcriptionally active chromatin it could be envisaged that this methodology be applied to isolate transcriptionally active chromatin (see chapter 2).

CHAPTER 2.

AFFINITY CHROMATOGRAPHY OF PHOTOCHEMICALLY BIOTINYLATED TRANSCRIPTION COMPLEXES.

INTRODUCTION:

Methods to enrich transcriptionally active chromatin have been explored for the past 30 years. The techniques developed so far have not produced a procedure that specifically enriches transcribing genes. The reasons for this can be sought in the criteria on which these methods are based (ie. nuclease sensitivity, solubility, HMG binding, histone acetylation and nucleosome conformation), which are not unique to the transcribed regions. Thus, these methods are fundamentally unsuitable to distinguish between the transcribed regions and the flanking regions (van Holde *et al.*, 1992). Future methodologies will have to be based on known unique characteristics of the transcriptionally active chromatin that allow for the enrichment of only those genes involved in active transcription, at the time of isolation. The only unique characteristic that has been explored, was based on using the DNA sequence of specific transcribed genes (Workman and Langmore, 1985b; Vincenz *et al.*, 1991) (see chapter 3). Other possibilities not yet explored, to get a handle on transcribing chromatin, would consist in the utilization of other specific components of the transcription machinery .

I have investigated the potential usefulness of RNA polymerase II as a handle for the isolation of the transcription complex and it's attached chromatin, as well as the potential of mercurated nascent RNA for this purpose.

Nascent RNA.

The nascent RNA synthesized during transcription remains bound to the transcription machinery and thereby forms a component of the ternary transcription complex. Incorporation of derivatized nucleotide analogues into the nascent RNA is a means to specifically label the transcription complexes and could be proposed as a target for an affinity purification technique. However, the eucaryotic polymerases will not accept nucleotide derivatives with large side groups (ie. Bio-11-UTP, Langer *et al.*, 1981) as

a substrates for transcription. Nucleotide derivatives with small side chain groups (ie. Hg-UTP) have been shown to be accepted as a substrate for transcription in the presence of reducing agents (Beebee and Buterworth, 1976). Thus, it should be possible to purify the mercurated nascent RNA and associated transcription complexes (ternary transcription complex) by sulfhydryl affinity chromatography. The investigation of the suitability of this approach to target active chromatin is reported in chapter 4.

RNA polymerase.

RNA polymerase II forms the major component of the transcription apparatus and is present in two forms in the nucleus, template bound or unbound. The distribution on the various states of transcriptionally active chromatin is not conclusive. Ip *et al.* (1988) suggested that a major portion of the bound RNA polymerase II is associated with the transcribing chromatin, however, crosslinking shows that in some cases the RNA polymerase II may be bound to the promoter region of inducible genes (Gilmore and Lis, 1985). The transcription complex, by definition, is an integral component of actively transcribing chromatin and therefore can be proposed to be a suitable target for affinity chromatography purification of active chromatin.

The toxin α-amanitin binds specifically with a high affinity ($Kd=10^{-9}$ M) to the 140 kD subunit of RNA polymerase II within the transcription complex (Cochet-Meilhac and Chambon, 1974; Brodner and Wieland, 1976). This binding inhibits the enzymatic translocation of the RNA polymerase II along the DNA template without destabilizing the template interactions (Vaisius and Weiland, 1982). In comparison, monoclonal antibodies directed against RNA polymerase II have been shown to inhibit the initiation process, however, they are only very weak inhibitors of elongation and the mechanism of inhibition is not understood. (Carrol and Stollar, 1982; Laybourn and Dahmus, 1989). Thus, α-amanitin appears to be the affinity ligand of choice for the affinity purification of transcription complexes and associated transcriptionally active chromatin. Lutter (1984), suggested this in the context of using an amanitin-Sepharose 4B column to purify RNA polymerase II. However, this technique has not been further pursued in the context of transcription complexes. The synthesis of soluble biotinylated α-amanitin derivatives offers an added advantage over solid phase α-amanitin, due to the high degree of accessibility of the former to the macromolecular transcription complex. A soluble biotinylated derivative has been successfully used in the isolation of micro quantities of soluble RNA polymerase II

(Vaisius and Faulstich, 1986). However, no evidence has been published on the stability of a biotinyl-α-amanitin-transcription complex, once streptavidin was bound to the biotin moiety. It could be envisaged that steric hindrance could cause a increase in the inhibition constant. This is supported by the fact that the inhibition constants observed for protein-amanitin conjugates are less than 1 % of that seen for α-amanitin (Faulstich and Fiume, 1985). Extension of the spacer arm length between the amanitin and the biotinyl residue, has been shown to overcome steric hindrance and thereby facilitates the binding of streptavidin without dramatically lowering the inhibition constant (see chapter 1). Thus, I am suggesting that biotinylation of the transcription complexes with the amanitin derivative Bio-32-NAP-amanitin, would be a method of specifically introducing an affinity target, that would enable the purification of transcriptionally active chromatin via the interaction with streptavidin.

Streptavidin isolated from *Streptomyces avidinii* is a tetrameric biotin binding protein, that binds biotin with a dissociation constant of $4x10^{-14}M$ (see review Bayer and Wilchek, 1990b). This high affinity to biotin has been utilized in numerous molecular biology techniques, ie. affinity purification, non isotopic labelling of DNA probes and ELISA assays (for review see Wilchek and Bayer 1988). The molecular size of streptavidin is similar to that of avidin 55 Å x 55 Å x 41 Å with a 2 fold symmetry. Each of the two pairs of binding sites is located in a surface depressions, on opposite ends of the 40 Å axis (Green *et al.*, 1971; Bayer and Wilchek, 1990b). Polymerization of avidin with a bisbiotinyl derivative, showed that the binding sites on a given surface are positioned 15 Å apart with an overall depth of 15 Å (Green *et al.*, 1971). Therefore streptavidin will bind to a biotinylated macromolecule with optimum affinity and without any sterical clash if the spacer length is greater than 6 carbon bonds (7.5Å). The two fold symmetry of streptavidin and avidin offers an added advantage in that if one biotin binding surface is bound to a biotinylated molecule the second binding surface will be positioned to allow a further biotinylated molecule to bind. This feature has been used to amplify the biotin signal by depositing more than one streptavidin per biotin moiety (review Wilchek and Bayer, 1988).

The arrangement of the amino acids in the biotin binding pocket has been determined from the crystal structure of streptavidin (Weber *et al.*, 1989; Hendrickson *et al.*, 1989), establishing that lysine is not involved in biotin binding (different from avidin. Glitin *et al.*, 1987), confirming previous suggestions (Glitin *et al.*, 1988). The exteriorly positioned lysine residues allows for the covalent coupling of streptavidin to either a solid support or enzymes, without any loss of the binding affinity for the biotinylated target molecule.

Thus, by combining the specific biotin binding characteristics of immobilized streptavidin with Bio-32-NAP-amanitin directed transcription complex biotinylation, it can be envisaged that these complexes and associated transcriptionally active chromatin could be specifically enriched.

METHODS AND MATERIALS:

Synthesis of streptavidin or bis-biotinyl-dextran microspheres.

Coupling of hexaglycine to oxirane acrylic microspheres.

Hexaglycine (25 mg; 66 µmol) was dissolved in 100 µl H_2O by titration with 1 M NaOH and added to oxirane acrylic microspheres (1 g; 600 µmol epoxide, Sigma) suspended in 2 ml 200 mM $NaHCO_3$ pH 8.5. The suspension was vigorously stirred at 37° C for 72 hours to allow coupling to proceed. The non-coupled hexaglycine was removed by extensive dialysis against H_2O, followed by washing twice with 10 mM HCl to protonate the carboxyl group. The hexaglycine microspheres were dried in vacuo and then further dried in vacuo against phosphorous pentoxide at 37° C for 72 hours.

Coupling of streptavidin or bis-biotinyl-dextran to hexaglycine microspheres.

The hexaglycine carboxyl groups on the microspheres were converted to active N-hydroxysuccinimide esters by incubating the hexaglycyl-microspheres (1 g) in 5 ml dry dimethylformamide containing dicyclohexylcarbodiimide (103 mg; 100 mM) and N-hydroxysuccinimide (57.5 mg; 100 mM) at 37° C for 6 hours. The activating solvent was then removed by washing the N-hydroxysuccinimide-hexaglycyl-microspheres 4 times with dry dimethylformamide and once with H_2O.

Streptavidin (1 mg; 16.67 nmol) or bis-biotinyl-dextran lysine fixable (Sigma) (1 mg; 100 nmol) dissolved in 2 ml 100 mM $NaHCO_3$ pH 8.0 was added to the N-hydroxysuccinimide-hexaglycyl-micorspheres (1 g) and coupling was allowed to proceed for 24 hours at 24° C. The streptavidin or bis-biotinyl-dextran-hexaglycyl-microspheres were then extensively washed with STE (100mM NaCl, 10mM Tris-HCl pH 8.0 and 1 mM EDTA) and resuspended to a final volume of 8 ml in STE pH 8.0 and stored at 4° C.

The accessibility of the immobilized streptavidin was determined by incubating a volume range of streptavidin-hexaglycyl-microspheres with a constant amount of 5'-32P labelled biotinylated probe DNA (0.8 µg) for 1 hour with rolling. The matrix was

then pelleted by centrifugation 10000 g for 1 min and the DNA content of the supernatant assayed by agarose gel electrophoresis.

Conversion of streptavidin microspheres to biotinyl-dextran microspheres (BIO-DEX-SA-hexaglycyl-microspheres) or bis-biotinyl-dextran microspheres to streptavidin microspheres (SA-BIO-DEX-hexaglycyl-microspheres).

Streptavidin-hexaglycyl-microspheres or bis-biotinyl-dextran-hexaglycyl-microspheres (100 µl) were incubated with 50 µg bis-biotinyl-dextran or 10 µg streptavidin, respectively at 4° C for 16 hours. The matrices were washed 5 time with 1 ml aliquots of TE pH 8.0 containing 0.05 % Tween 20 to remove unbound material. Non-specific binding was quenched by a further incubation in TE pH 8.0 containing 100 µg/ml BSA and 0.05 % Tween 20 for 1 hour. The efficiency of binding for these matrices was determined as described above.

Preparation of streptavidin polymers.

Crosslinking streptavidin with glutaraldehyde.
(According to Bayer and Wilchek, 1990a).

Poly-streptavidin was prepared by dissolving 200 µg streptavidin in 200 µl 100 mM $NaHPO_4$ pH 7.0, 1 % glutaraldehyde (5 µl) was added and the reaction was incubated at 24° C for 2.5 hours with vigorous shaking. The excess glutaraldehyde was quenched with the addition of 50 µl 1 M ethanolamine pH 8.0 and incubated for 1 hour. The polymeric streptavidin was fractionated from the monomer on a AcA 34 column equilibrated in TE pH 8.0 containing 2 µg/ml BSA. The outer volume peak was collected and concentrated by ultrafiltration on a PM 10 Amicon membrane, followed by storage at 4° C.

Crosslinking of streptavidin with DSP.

DSP (16.8 µg; 41.156 nmol) was dissolved in dry dimethylformamide (100 µl) and added to streptavidin (500 µg; 8.33 nmol) dissolved in 200 mM $NaHCO_3$ pH 7.5 (500 µl) and incubated at 24° C for 16 hours. Excess crosslinking reagent was removed by dialysis against H_2O and the polymeric streptavidin was then fractionated by gel

exclusion chromatography on AcA 34 (equilibrated in 50 mM KCl, Tris-HCl pH 8.0 and 1 µg/ml BSA). The outer volume peak was pooled and stored in 50 % glycerol at -20° C.

Crosslinking streptavidin by sulfhydryl exchange.
(according to New, 1990).

The crosslinking reagent bis-maleimido-diaminedipropylamin was prepared by incubating maleimido N-hydroxysuccinimide (5.3 mg; 14.9 µmol) dissolved in dimethylformamide together with diaminedipropylamin (0.55 µmol) at 24° C for 2 hours. The product was then purified by reverse phase chromatography on a µBonda Pak C18 column (eluted with a linear acetonitrile gradient ranging from 0 to 70 % containing 0.05 % TFA). The major peak was collected and the solvent was removed in vacuo.

PdP-streptavidin was prepared as described in chapter 5. The ratio of derivatization was spectrophotometrically shown to be two moles PdP per mole streptavidin (the molar extinction coefficient of 2 thiopyridine was 8330 at 343 nm). The pyridylthiol groups were reduced by incubating the streptavidin in 500 mM DTT and 100 mM sodium acetate pH 5.5 at 24° C for 30 min, followed by extensive dialysis against 100 mM NaH_2PO_4 pH 6.6 (continuously flushed with N_2).

Bis-maleimido-diaminedipropylamin (9.3 nmol) dissolved in dry dimethylformamide (50 µl) was added to freshly prepared SH-streptavidin (1.54 nmol, dissolved in 50 µl NaH_2PO_4 buffer). The reactants were incubated at 24° C for 16 hours and the efficiency of dimerisation was shown to be complete, as assayed by electrophoresis of the ^{125}I-biotin labelled complexes on pH 9.5 non-denaturing polyacrylamide. The putative di-streptavidin was store in 50 % glycerol at -20° C.

BSA biotinylation.

BSA (4.66 mg; 68.5 nmol) dissolved in 1.4 ml 100 mM $NaHCO_3$ pH 9.0 was added to biotin N-hydroxysuccinimide (0.24 mg; 685 nmol) dissolved in 0.4 ml dry dimethylformamide. Biotinylation was allowed to proceed at 4° C for 16 hours, followed by extensive dialysis against H_2O. The biotinylated BSA was the freeze

dried and stored at -20° C. The ratio of biotinylation could be varied by increasing or deceasing the input biotin N-hydroxysuccinimide.

Assaying the accessibility of the biotin moiety within the RNA polymerase II transcription complex.

Solubilization of biotinylated transcription complexes.

The transcription complexes were biotinylated "in situ" as previously described (chapter 1). The chromosomal DNA was then extensively digested by incubating the nuclei with 150 units micrococcal, 20 µg DNAse I (per mg DNA) in NSB containing 1 mM $CaCl_2$, 1 mM $MgCl_2$. The digestion was stopped with the addition of EDTA to a final concentration of 3 mM and the transcription complexes were solubilized by dialysis against TE ph 8.0 containing 0.05 % Nonidet LE and 0.2 mM PMSF at 4° C for 16 hours. Insoluble nuclear debri was pelleted by centrifugation 10000 xg for 1-2 min.

Assay for the binding of streptavidin to the biotin moiety.

The soluble biotinylated transcription complexes were radioactively labelled by incubation with an excess of either ^{125}I-streptavidin or ^{125}I-biotin streptavidin at 4° C for 1-2 hours. These labelled transcription complexes were then fractionated by electrophoresis in 0.8 % agarose in 0.25x TAE (10 mM Tris-HCl ph 8.3; 0.5 mM EDTA) at 1.5 volts per cm, with recirculation. The agarose gel was dried and the transcription complexes were visualized by autoradiography.

Accessibility of the biotin moiety within the transcription complex was determined by two methods. 1) Pre-incubation of the soluble biotinylated transcription complexes with an excess of the streptavidin conjugate or matrix for 1-6 hours. The unquenched biotin residues were then labelled by a second incubation with ^{125}I-streptavidin or ^{125}I-biotin-streptavidin for 1 hour and detected by gel electrophoresis. 2) The soluble biotinylated transcription complexes were pre-incubated with ^{125}I-streptavidin (as above), followed by incubation with poly-biotinylated molecules or matrices. Accessibility of the positioned streptavidin was indicated by either a change in electrophoretic mobility or loss (when retained on matrix) of the positive signal when fractionated by electrophoresis.

RESULTS AND DISCUSSION:

Most methodologies published, which have resulted in some enrichment of actively transcribing chromatin, are based on features postulated to be characteristic of these regions, ie. HMG proteins, acetylated and ubiquitinated histones, altered nucleosome structure and nuclease susceptibility (see overview). However, these methodologies have shown only a limited degree of enrichment, which is a direct function of the uniqueness of the target within the transcribed region.

The transcription complex can be defined as a unique characteristic of the transcriptionally active chromatin. Thus, by targeting the transcription complex, the characterizing of the associated chromatin should reveal specific features of active chromatin.

The proposed purification procedure can be summarized as follows:

Step 1: Amanitin directed photochemical biotinylation of the RNA polymerase II transcription complexes.

Step 2: Solubilization of the transcription complexes by nuclease digestion.

Step 3: Affinity purification by solid phase streptavidin.

Solubilization of biotinylated transcription complexes.

In order to assay for the presence of biotinylated transcription complexes within the soluble chromatin fraction, sea urchin embryo nuclei RNA polymerase II transcription complexes were biotinylated "in situ" with Bio-32-NAP-amanitin as described in chapter 1. These biotinylated transcription complexes were solubilized by extensive nuclease digestion, followed by extraction at low ionic strength. The soluble extract was radioactively labelled by incubation with an excess of either [^{125}I-biotin-streptavidin] complex or ^{125}I-streptavidin and the resulting ^{125}I labelled transcription complexes were subsequently fractionated by electrophoresis in 0.8 % agarose (0.25x TAE pH 8.2; 1.5 V/cm). Autoradiography of the dried gel demonstrated specific labelling within a broad band, which was presumed to contain transcription complexes associated with varying lengths of chromatin

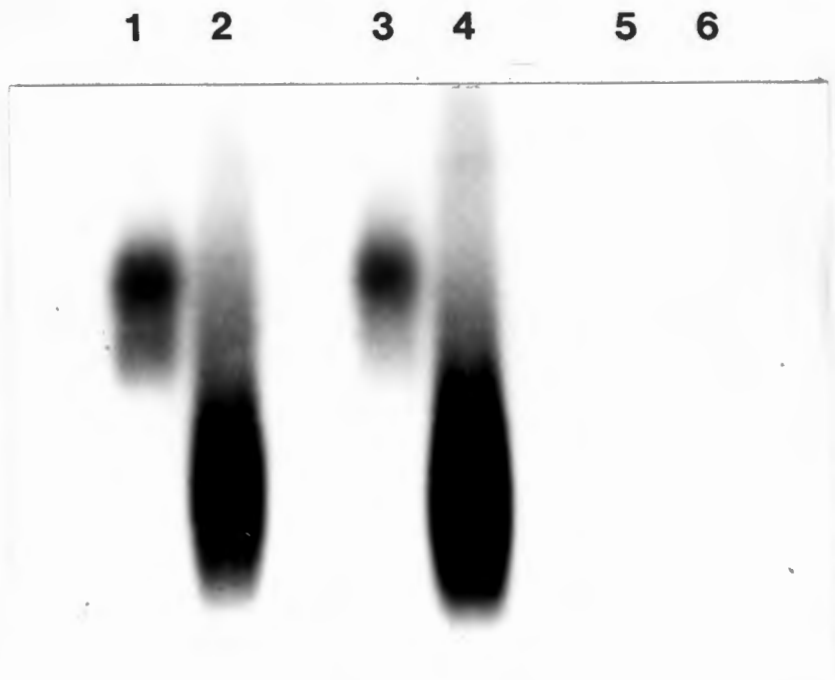

Figure 1. Electrophoretic fractionation of covalently biotinylated RNA polymerase II transcription complexes targeted with 1) ^{125}I-biotinyl-streptavidin or 2) streptavidin, followed by ^{125}I-biotin.

RNA polymerase II transcription complexes were biotinylated "in situ", as described in methods and materials (chapter 1). The biotinylated transcription complexes were solubilized by incubating the nuclei with DNAse I (5 µg/mg DNA) and micrococcal nuclease (150 U/mg DNA) in the presence of 5 mM $MgCl_2$ and 1 mM $CaCl_2$, on ice for 1 hour. The soluble chromatin was extracted at low ionic strength (TE pH 8.0) and the insoluble debri was pelleted by centrifugation 8000 xg for 2 min. Aliquots of the soluble chromatin (biotinylated and non-biotinylated) were incubated at 4°C for 2 hours with either ^{125}I-biotinyl-streptavidin (molar ratio 0.75:1; 0.5 pmol) or streptavidin (0.5 pmol) followed by a second incubation with ^{125}I-biotin (1 hour). The targeted chromatin was then electrophoretically fractionated on 0.8 % agarose (0.25x TAE pH 8.2) at 1 volt/cm for 16 hours with recirculation of the buffer. The gel was dried and the labelled transcription complexes were visualized by autoradiography.

Lane 1. Non-biotinylated chromatin incubated with ^{125}I-biotinyl-streptavidin.
Lane 2. Biotinylated chromatin incubated with ^{125}I-biotinyl-streptavidin.
Lane 3. Non-biotinylated chromatin incubated with streptavidin followed by a second incubation with ^{125}I-biotin.
Lane 4. Biotinylated chromatin incubated with streptavidin followed by a further second with ^{125}I-biotin.
Lane 5. Non-biotinylated chromatin pre-incubated with an excess of streptavidin (40 pmol) followed by a second incubation with ^{125}I-biotinyl-streptavidin.
Lane 6. Biotinylated chromatin pre-incubated with an excess of streptavidin (40 pmol) followed by a second incubation with ^{125}I-biotinyl-streptavidin.

(Fig 1, lane 2 and 4). (The unbound streptavidin remained near origin, as was shown by a diffuse haze at the top of the gel). This labelling was dependent on the [Bio-32-NAP-amanitin-RNA polymerase II] interaction, as demonstrated by the absence of a signal in non-biotinylated chromatin (Fig 1, lane 1 and 3). (Similarly, pre-incubation of the nuclei with α-amanitin quenched the specific biotinylation of the transcription complexes). However, some chromatin preparations did show non-specific targeting of streptavidin, which was as a function of contaminating naturally biotinylated cytoplasmic proteins (Fig 1, lane 1; see also chapter 1).

The binding of ^{125}I-biotinyl-streptavidin to the biotinylated transcription complexes was biotin dependent, as this binding was totally quenched by pre-incubation with excess streptavidin (Fig 1, lane 6). Thus demonstrating specific biotin directed targeting of the streptavidin versus a non-specific interactions.

Incubation of the streptavidin targeted transcription complexes with ^{125}I-biotin resulted in the specific labelling of the electrophoretically fractionated transcription complexes (Fig 1, lane 4). Demonstrating that the unoccupied biotin binding sites of the targeted streptavidin were accessible (low molecular weight biotin), and retained their binding capacity. This would suggest that vacant biotin binding sites would have the potential to link the biotinylated transcription complex to other biotinylated molecules (ie. biotinylated solid phase).

Synthesis of solid phase streptavidin.

Having demonstrated the solubilization and specific targeting of streptavidin towards the transcription complexes, the following question arose: could these transcription complexes be affinity purified?

The molecular complexity of the transcription complex and it's associated chromatin, as well as the unknown location and topology of the α-amanitin binding site, complicated the design of a solid phase affinity technique. Optimal binding and thus purification could only be achieved if two factors were taken into account. 1) Maximum accessibility of the immobilized streptavidin to macromolecular weight biotin (biotinylated transcription complex). 2) Low non-specific binding of chromatin.

Various solid phase matrices were tested for their ability to efficiently bind macromolecular weight biotin (see chapter 5). Non-porous microspheres (oxirane-acrylic microspheres; 1μm diameter) should be the solid phase of choice. The high surface area and density of the functional groups allows for an optimal coupling efficiency and the accessibility of the immobilized ligand should not be restricted by the microsphere surface topology. Streptavidin was covalently coupled to the microspheres via a hexaglycine spacer arm (see methods and materials for synthesis). The accessibility of the matrix bound streptavidin to biotin attached to macromolecules was demonstrated using 5'-biotinylated DNA as a model system (Fig 2, 300 fmol biotinylated DNA was specifically retained by a 10 μl aliquot of streptavidin-hexaglycyl-microspheres (1 pmol immobilized streptavidin)). Specific binding was totally quenched when the streptavidin-hexaglycyl-microspheres were pre-incubated with an excess of biotin (Fig 2, lane 6), thus demonstrating that non-specific binding was not responsible for the retention of the DNA.

These streptavidin-hexaglycyl-microspheres were then tested to determine their biotin binding capacity in the context of biotinylated transcription complexes. Pre-incubation of soluble biotinylated transcription complexes with streptavidin-hexaglycyl-microspheres followed by a subsequent incubation with ^{125}I-biotinyl-streptavidin, demonstrated that the immobilized streptavidin was unable to quench the binding of the soluble ^{125}I labelled streptavidin (Fig 3, lanes 2,3 and 4). This result suggests that the immobilized streptavidin could not reach biotinyl residue within the RNA polymerase II, due to a sterical clash between the transcription complex and the solid phase matrix.

Two approaches have been followed to overcome this steric hindrance effect. 1) Translocation of the biotin residue or the streptavidin from within the transcription complex to a position more accessible. 2) Extension of the matrix spacer arm length.

1. Translocation of the biotin residue or targeted streptavidin within the transcription complex.

Poly-streptavidin.

The binding of a poly-streptavidin to the biotinylated transcription complex would be a means to translocate the biotin binding activity, from within the transcription complex, to a more accessible surface position or directly into the solvent.

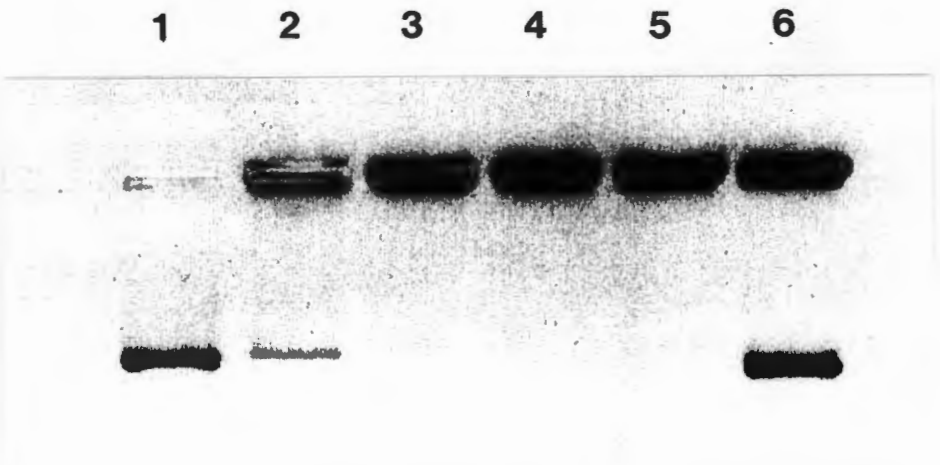

Figure 2. Binding of macromolecular weight biotin to streptavidin-hexaglycyl-microspheres.

Streptavidin-hexaglycyl-microspheres (prepared as described in method and materials) were pre-incubated in STE pH 8.0 containing BSA (250 µg/ml) and tRNA (100 µg/ml) at 24° C for 1 hour to block non-specific binding. Aliquots of the streptavidin-hexaglycyl-microspheres ranging form 5 to 20 µl (packed microsphere volume approximately 20 % of total aliquot volume) were incubated with 5'-biotinylated DNA (0.8 µg; 300 fmol) at 24° C for 1 hour. The total binding mixture was then fractionated by electrophoresis in 1 % agarose (1x TBE pH 8.3) and the DNA was visualized by staining with ethidium bromide.

Lane 1. Biotinylated DNA in the absence of microspheres.
Lane 2. Biotinylated DNA incubated with 5 µl microspheres.
Lane 3. Biotinylated DNA incubated with 10 µl microspheres.
Lane 4. Biotinylated DNA incubated with 15 µl microspheres.
Lane 5. Biotinylated DNA incubated with 20 µl microspheres.
Lane 6. Biotinylated DNA incubated with 20 µl microspheres pre-quenched with excess biotin.

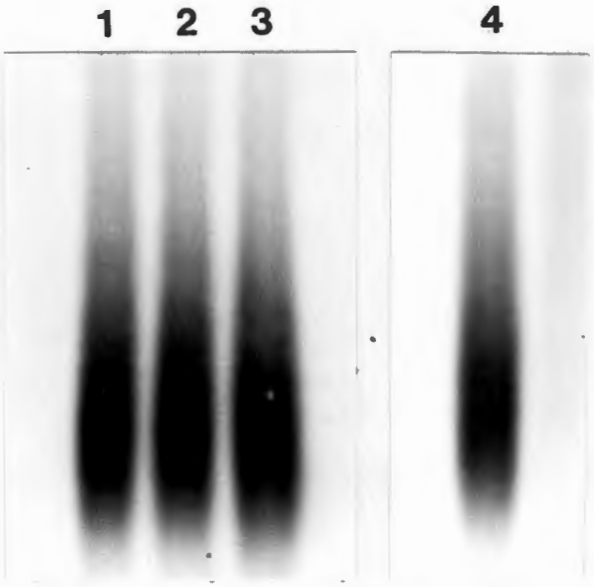

Figure 3. Accessibility of the biotinyl residue within the transcription complex to immobilized streptavidin.

RNA polymerase II transcription complexes were biotinylated and solubilized as described previously. Aliquots of the soluble chromatin were incubated with streptavidin microspheres at 4° C for 1 hour to allow binding of the biotinylated transcription complexes. The microsphere were then pelleted by centrifugation at 8000xg for 1 min and the supernatant was retained. Excess ^{125}I-biotin-streptavidin (0.75 pmol) was added to the supernatant, followed by a further incubation for 1 hour. The chromatin was then fractionated by electrophoresis in 0.8 % agarose (0.25x TAE pH 8.2) as described above.

Accessibility of the biotinyl residue was indicated by quenching of the binding of ^{125}I-biotin-streptavidin.

Lane 1. Pre-incubation with 5 µl streptavidin-hexaglycyl-microspheres.
Lane 2. Pre-incubation with 10 µl streptavidin-hexaglycyl-microspheres.
Lane 3. Pre-incubation with 20 µl streptavidin-hexaglycyl-microspheres.
Lane 4. Biotinylated transcription complexes targeted with ^{125}I-biotinyl-streptavidin.

Thus, the targeted poly-streptavidin molecules would be more accessible for interaction with solid phase biotin. Streptavidin was crosslinked with glutaraldehyde or DSP (dithiobis[succinimidylpropionate]) and the polymers were purified by gel exclusion chromatography on AcA 34 (see methods and materials). Incubation of the biotinylated transcription complexes with poly-streptavidin showed that these polymers were unable to quench the binding of ^{125}I-streptavidin (Fig 4). Thereby demonstrating that the α-amanitin binding pocket was not located on or near to surface of transcription complex and suggested that the pocket may accommodate only a single molecule of the tetrameric streptavidin. Similar results were obtained when the biotinylated transcription complexes were incubated with di-streptavidin (Fig 5, lane 3) or with streptavidin gold (5 nm) (Fig 5, lane 4). This result confirmed the observation that the biotinylated transcription complexes could not be efficiently labelled via streptavidin gold, as assayed by a post embedding procedure (see chapter 1).

Poly-biotin.

The molecular symmetry and tetrameric biotin binding characteristics of streptavidin facilitates the binding of more than one biotinylated protein per streptavidin molecule (Wilchek *et al.*, 1988). Thus, the biotinyl residue within the transcription complex could selectively position further biotin residues (poly-biotinylated complex) via the binding of streptavidin. These targeted biotinyl residues in turn could react with the solid phase streptavidin.

Initially poly-biotinylated BSA was synthesized for this purpose (ratios biotin:BSA ranging from 5:1 to 15:1) and was shown to bind to streptavidin. However, the high localized concentration of biotin on the surface of albumin resulted in the displacement of ^{125}I-biotin from the binding pocket of streptavidin (Fig 6). Therefore it could be postulated that the poly-biotinylated BSA may displace the streptavidin from the biotinylated transcription complex if sterically accessible. However, conclusive evidence for displacement could not be demonstrated, because it appeared, in the electrophoretic test that streptavidin targeted transcription complexes incubated with $(Bio)_5$-BSA, failed to quench the binding of ^{125}I-biotin. Initially suggesting: the BSA biotinyl residues were unable to interact with the targeted streptavidin, and the vacant biotin binding sites bound the ^{125}I-biotin (Fig 5, lane 6). However, further analysis showed that the [biotinylated-BSA-^{125}I-streptavidin] complex and [^{125}I-streptavidin-Bio-32-NAP-NAP-amanitin-transcription complex] had similar

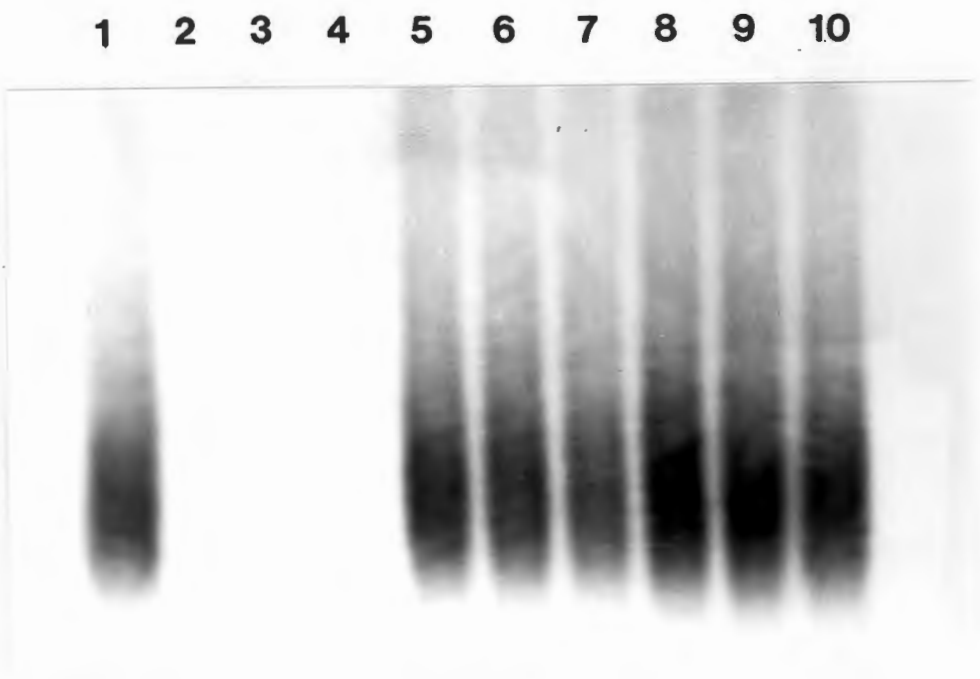

Figure 4. Accessibility of the transcription complex biotinyl residue to streptavidin polymers, measure by their ability to quench the binding of ^{125}I-streptavidin.

Soluble biotinylated RNA transcription complexes were prepared as previously described. Aliquots of the soluble chromatin were pre-incubated with increasing concentrations of 1) streptavidin or 2) poly-streptavidin (glutaraldehyde crosslink) or 3) poly-streptavidin (DSP crosslink), at 4° C for 1 hour. ^{125}I-streptavidin was added to each aliquot and incubated a further 1 hour. The chromatin was then electrophoretically fractionated in 0.8 % agarose (0.25x TAE pH 8.2 with recirculation of the buffer. The gel was dried and the labelled transcription complexes were visualized by autoradiography.

Lane 1. Without pre-incubation with streptavidin.
Lane 2. Pre-incubation with 0.10 μg streptavidin.
Lane 3. Pre-incubation with 0.25 μg streptavidin.
lane 4. Pre-incubation with 0.50 μg streptavidin.
Lane 5. Pre-incubation with 0.10 μg poly-streptavidin (crosslinked with glutaraldehyde).
Lane 6. Pre-incubation with 0.25 μg poly-streptavidin (crosslinked with glutaraldehyde).
Lane 7. Pre-incubation with 0.50 μg poly-streptavidin (crosslinked with glutaraldehyde).
Lane 8. Pre-incubation with 0.10 μg Poly streptavidin (crosslinked with DSP).
Lane 9. Pre-incubation with 0.25 μg Poly streptavidin (crosslinked with DSP).
Lane 10. Pre-incubation with 0.50 μg Poly streptavidin (crosslinked with DSP).

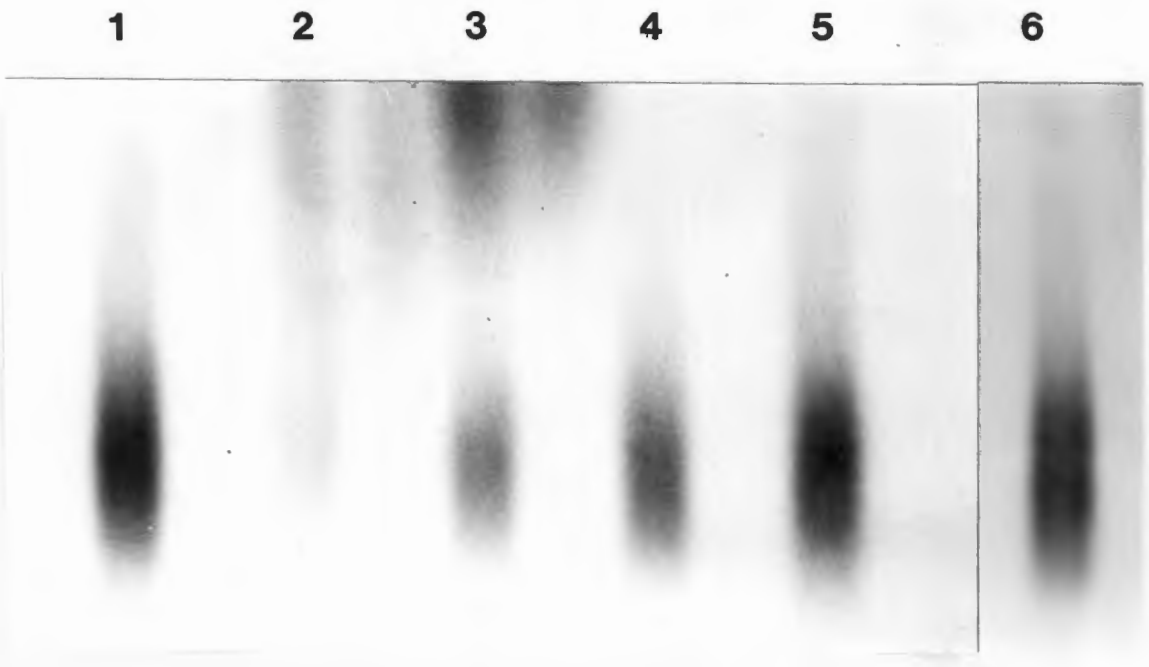

Figure 5. Accessibility of the transcription complex biotinyl residue to streptavidin conjugates, measured by their ability the quench the binding of ^{125}I-biotinyl-streptavidin.

Biotinylated RNA polymerase II transcription complexes were prepared as previously described. Aliquots of the soluble chromatin were pre-incubated at 24° C for 1 hour with various streptavidin derivatives. ^{125}I-biotinyl-streptavidin (0.5 pmol) was then added to each fraction followed by a further incubation for 1 hour. The targeted chromatin was fractionated by electrophoresis in 0.8 % agarose and the labelled complexes were visualized by autoradiography of the dried gel. (The hazy region at the top of the gel in certain lanes is as a result of the poor electrophoretic mobility of the streptavidin and also demonstrates that the di-streptavidin is able to displace the ^{125}I-biotin residue from the monomeric streptavidin, lane 3).

Lane 1. No competitor.
Lane 2. Streptavidin (1.0 pmol).
Lane 3. Di-streptavidin (1.0 pmol).
Lane 4. Streptavidin 5 nm colloidal gold (2 µl).
Lane 5. Streptavidin-hexaglycyl-microspheres (2.5 µl).
Lane 6. Streptavidin (0.5 pmol), followed by a second incubation with $(bio)_5$-BSA (4 pmol) and the complex was labelled by incubation with 0.25 pmol ^{125}I-biotin.

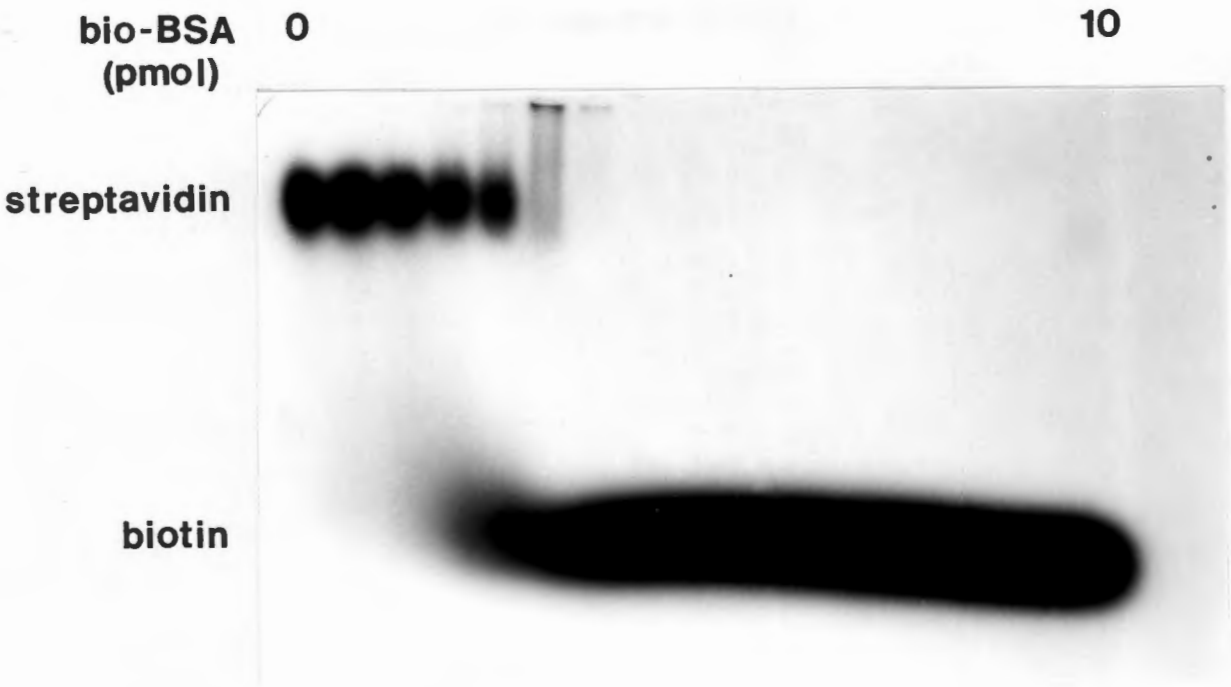

Figure 6. Titration of ^{125}I-biotin-streptavidin complex with poly-biotinylated BSA.

^{125}I-biotinyl-streptavidin (molar ratio 0.5:1) was prepared by slowly adding 10 pmol ^{125}I-biotin to 21 pmol streptavidin. 10 µl aliquots of the ^{125}I-biotinyl-streptavidin (0.8 pmol) were incubated with a dilution series of $(bio)_{15}$-BSA (ranging from 20 fmol to 10 pmol) at 24° C for 1 hour. The total mixture was then fractionated by electrophoresis in 5 % non-denaturing polyacrylamide (1x TAE pH 8.2) and the ^{125}I labelled complexes were visualized by autoradiography of the wet gel.

Figure 7. Accessibility of the transcription complex targeted ^{125}I-streptavidin to poly-biotinylated macromolecules, measured by their ability to change electrophoretic mobility.

RNA polymerase II transcription complexes were biotinylated as described in chapter 1. ^{125}I-streptavidin was positioned within the transcription complexes by incubating aliquots (50 µl) of the solubilized chromatin with ^{125}I-streptavidin (0.5 pmol), on ice for 1 hours. Excess bis-biotinyl-dextran (lysine fixable) or $(bio)_{15}$-BSA was added and incubated for a further 1 hour. The chromatin was then fractionated by electrophoresis in 0.8 % agarose (0.25x TAE pH 8.2). Accessibility was demonstrated by a electrophoretic mobility shift.

Lane 1. Excess $(bio)_{15}$-BSA.
Lane 2. Without addition of poly-biotinylated macromolecules.
Lane 3. Excess bis-biotinyl-dextran (lysine fixable).

electrophoretic mobilities (Fig 7, compare the intensity of the signal between lane 1 and 2). This implies that the excess ^{125}I-streptavidin becomes bound to the electrophoretic mobilities (Fig 7, compare the intensity of the signal between lane 1 and 2). This implies that the excess ^{125}I-streptavidin becomes bound to the biotinyl-BSA and co-migrates with the transcription complexes. Thus making it impossible to determine whether the triple complex [poly-biotinyl-BSA-streptavidin-biotinyl-transcription complex] had formed. For these reasons biotinyl-BSA was not further investigated.

The accessibility of the bound streptavidin within the transcription complex to high molecular weight biotin was shown by incubation with bis-biotinyl-dextran (lysine fixable). The binding of this biotin derivative resulted in a total loss of electrophoretic mobility of the transcription complex (Fig 7, lane 3). This was not a function of streptavidin displacement, as the biotinyl residues were spatially separated and at a low concentration. This result demonstrated that the targeted streptavidin was accessible to a biotinylated macromolecule.

2. Extension of the matrix spacer arm.

In it's entirety the results, at this stage point at the chemical nature of the spacer and it's length between the solid phase and streptavidin, as the limiting factor controlling penetration of the streptavidin into the α-amanitin binding pocket. Initial attempts to lengthen the spacer arm on the microsphere surface, included coupling of 1) two hexaglycine peptides, or 2) insulin A-chain, or 3) poly-(DL)-alanine, or 4) poly-proline, followed by activation and streptavidin coupling. However, due to low coupling efficiencies and/or activation efficiencies of the respective linker arms, none of the matrices prepared with these spacers yielded sufficient biotin binding (data not shown).

The accessibility of targeted streptavidin, within the transcription complex, to bis-biotinyl-dextran dextran as demonstrated in the previous section, suggests that this molecule may be attached as a spacer arm to the solid phase. Two matrices were prepared. 1) Bis-biotinyl-dextran dextran (lysine fixable) was covalently coupled to N-hydroxysuccinamide hexaglycine-microspheres. Which in turn was incubated with an excess of streptavidin producing a long arm flexible streptavidin microsphere (SA-BIO-DEX-hexaglycyl-microspheres) (Fig 8). 2) Streptavidin-hexaglycyl-microspheres were incubated with excess Bis-biotinyl-dextran dextran producing a

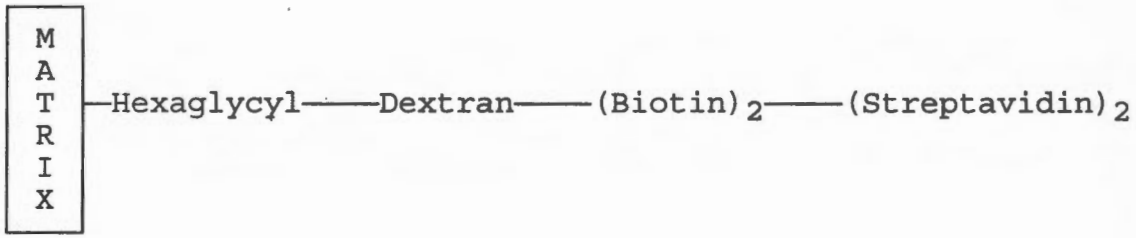

Figure 8. Schematic diagram of streptavidin-biotinyl-dextran-hexaglycyl-microspheres (SA-BIO-DEX-hexaglycyl-microspheres).

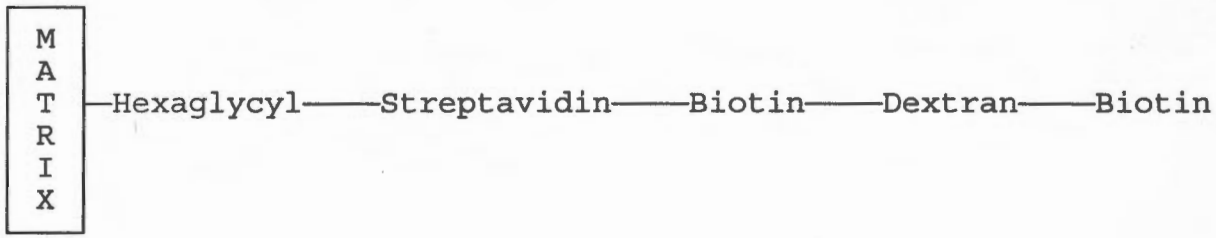

Figure 9. Schematic diagram of biotinyl-dextran-streptavidin-hexaglycyl-microspheres (BIO-DEX-SA-hexaglycyl-microspheres).

long arm flexible biotinyl microsphere without the constraints of a covalent link within the dextran (BIO-DEX-SA-hexaglycyl-microspheres) (Fig 9).

SA-BIO-DEX-hexaglycyl-microspheres.

Soluble biotinylated transcription complexes were incubated with increasing volumes of SA-BIO-DEX-hexaglycyl-microspheres, followed by centrifugation. The unbound chromatin fraction was then incubated with ^{125}I-streptavidin and fractionated by agarose gel electrophoresis. The results demonstrated that the transcription complexes were retained (Fig 10). Specificity of binding could not be shown as the excess biotin used to quench the matrix also saturated the binding sites of the ^{125}I-streptavidin (Fig 10, lane 8).

BIO-DEX-SA-hexaglycyl-microspheres.

Pre-targeting of the soluble biotinylated transcription complexes with ^{125}I-streptavidin followed by incubation with increasing volumes of BIO-DEX-SA-hexaglycyl-microspheres demonstrated that the transcription complexes were retained (Fig 11). However, the efficiency of retention was lower than that observed for SA-BIO-DEX-hexaglycyl-micropsheres, possibly due to the fact that untargeted ^{125}I-streptavidin partially quenched the matrix binding activity. The specificity of retention could be shown as the addition of excess biotin quenched the binding of the transcription complex to the solid phase (Fig 11, lane 8). Similar results were demonstrated for biotinylated transcription complexes isolated from rat liver nuclei. Thus, confirming that this methodology could be applied to all tissues that are actively transcribing mRNA genes.

Having demonstrated the specific retention of transcription complexes on a solid phase matrix, the aim was to determine if these complexes could be specifically released. This would enable further analysis of the transcription complex associated proteins and DNA.

The dextran arm was proposed as the target for a release mechanism, as dextran is rapidly digested with dextranase (Janson and Porath, 1968). Control digestions of the complex [5'-32P-$(Bio)_4$-oligonucleotide-streptavidin-BIO-DEX-hexaglycyl-microsphere], with dextranase, however, demonstrated that the [5'-32P-$(Bio)_4$-oligonucleotide-streptavidin] complex was only partially released (Fig 12). The activity of the dextranase was demonstrated by the complete solubilization of

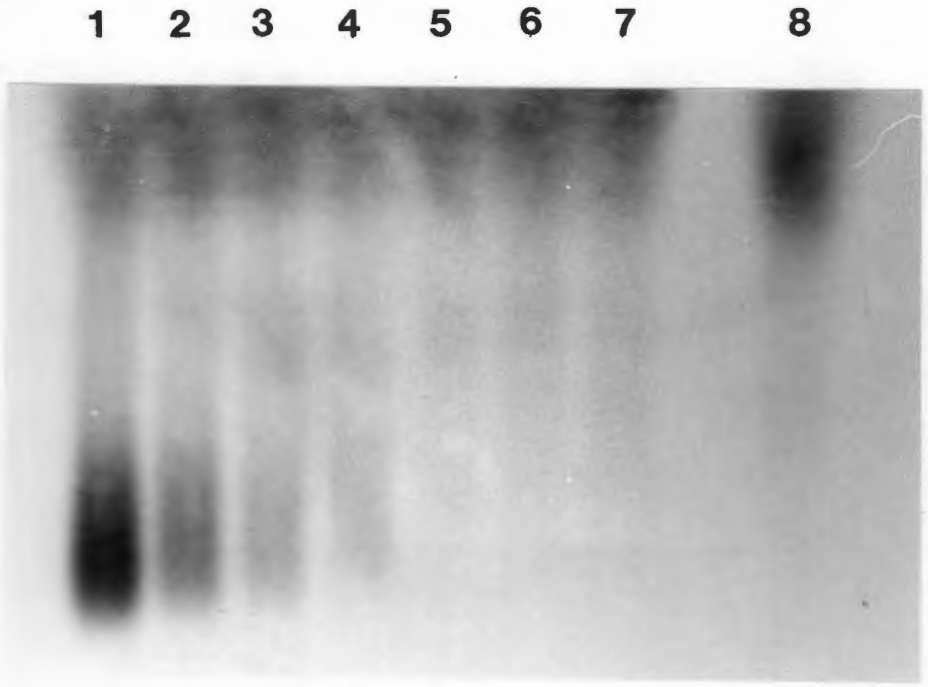

Figure 10. Retention of biotinylated transcription complexes on SA-BIO-DEX-microspheres.

RNA polymerase II transcription complexes were biotinylated and solubilized as described previously. Aliquots (200 µl) of the soluble chromatin were incubated with increasing volumes of SA-BIO-DEX-hexaglycyl-microspheres (ranging from 0 to 160 µl) at 4° C for 4 hours. The microspheres were pelleted by gentle centrifugation (8000 xg, 1 min.) and the supernatants were retained. ^{125}I-streptavidin (2 pmol) was added to the supernatants and incubated for 1 hours at 4° C. The supernatants were then fractionated by electrophoresis in 0.8 % agarose (0.25x TBE pH 8.2).

Lane 1. Without matrix.
Lane 2. 10 µl matrix.
Lane 3. 20 µl matrix.
Lane 4. 40 µl matrix.
Lane 5. 80 µl matrix.
Lane 6. 120 µl matrix.
Lane 7. 160 µl matrix.

The degree of non-specific binding could not be shown as the excess biotin added to quench the binding activity of the SA-BIO-DEX-microspheres (160 µl) also quenched the binding activity of the ^{125}I-streptavidin (Lane 8).

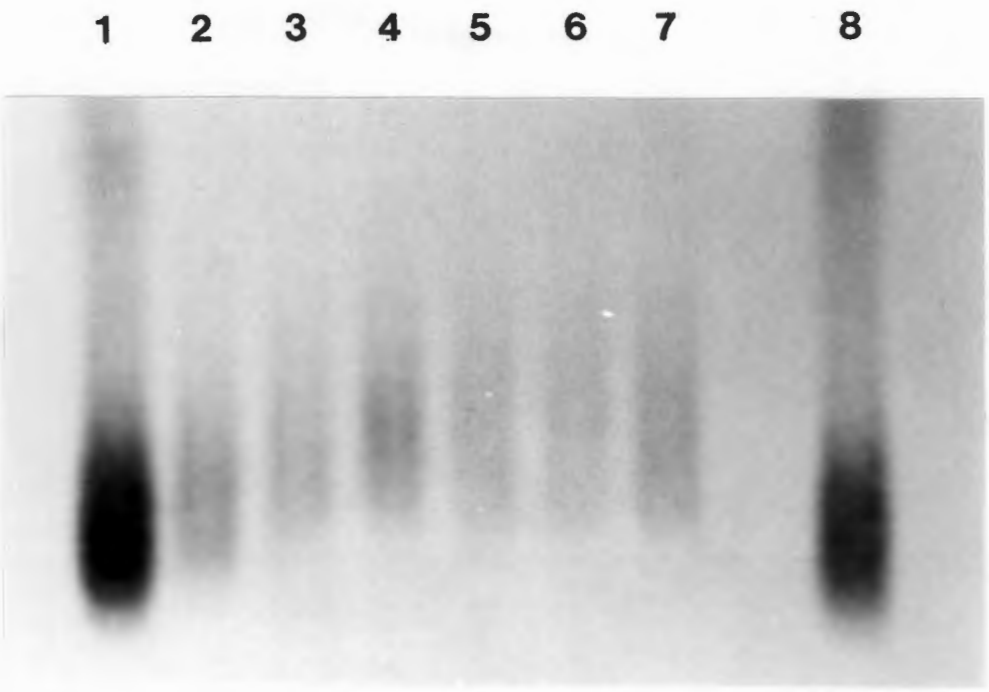

Figure 11. Retention of biotinylated transcription complexes on BIO-DEX-SA-microspheres.

RNA polymerase II transcription complexes were biotinylated and solubilized as described previously. ^{125}I-streptavidin (9 pmol/ml soluble chromatin) was added and incubated at 4° C for 2 hours. Aliquots (200 µl) of the labelled soluble chromatin then incubated with increasing volumes of BIO-DEX-SA microspheres (ranging from 0 to 160 µl) at 4° for 4 hours. The microspheres were pelleted by gentle centrifugation (8000 xg, 1 min.), and the supernatants were fractionated by electrophoresis in 0.8 % agarose (0.25x TAE pH 8.2).

Lane 1. Without matrix.
Lane 2. 10 µl matrix.
Lane 3. 20 µl matrix.
Lane 4. 40 µl matrix.
Lane 5. 80 µl matrix.
Lane 6. 120 µl matrix.
Lane 7. 160 µl matrix.

Non-specific binding to the matrix was determined by addition of excess biotin to the streptavidin labelled transcription complexes prior to the addition of 160 µl matrix (Lane 8).

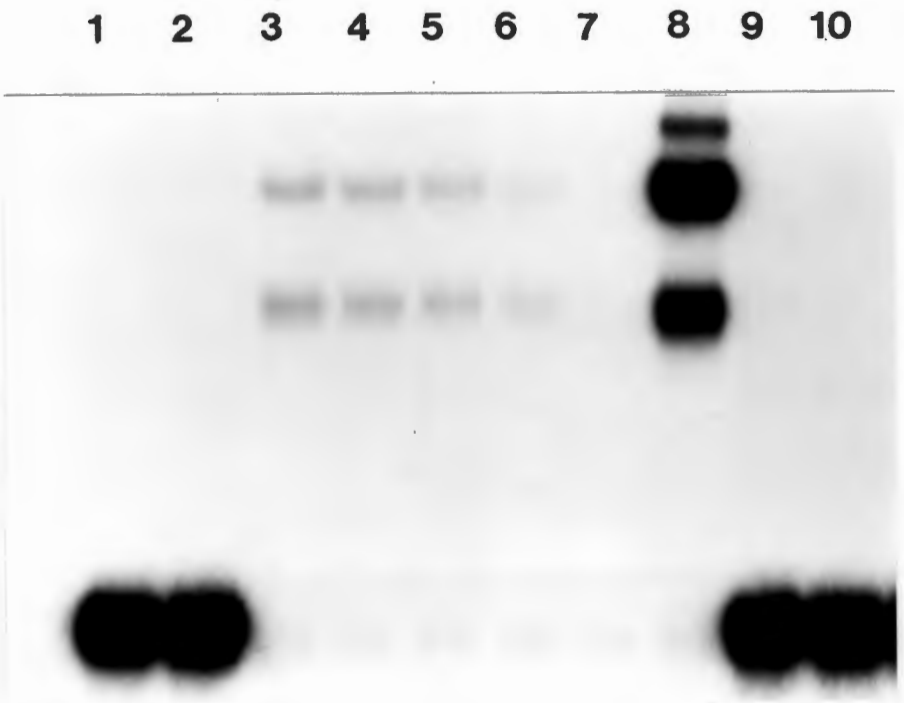

Figure 12. Digestion of the [^{32}P-biotinyl-primer-SA-BIO-DEX-hexaglycyl-microsphere] complex with dextranase.

SA-Bio-DEX-hexaglycyl-microspheres were prepared as described in methods and materials and equilibrated in 100 mM KH_2PO_4 pH 7.3 containing 0.1 % Tween 20 and 100 µg/ml BSA. 0.5 pmol 5'-32P-$(Bio)_4$-primer (probe 5, chapter 3) was added to 10 µl aliquots of the microspheres and incubated at 24° C for 1 hour to allow for the formation of the [^{32}P-biotinyl-primer-streptavidin-BIO-DEX-hexaglycyl-microsphere] complex.

Increasing amounts of dextranase (dissolved in 100 mM KH_2PO_4 pH 7.2), ranging from 0 to 2 units, was then added to the respective microsphere complexes and incubated at 37° for 1 hour. The microsphere were then pelleted by gentle centrifugation (8000 xg for 1 min) and the supernatants were fractionated by electrophoresis in 9 % non-denaturing polyacrylamide (1x TBE pH 8.3).

Lane 1 and 2. 5'-32P-$(Bio)_4$-oligonucleotide (0.5 pmol)
Lane 3. Microsphere complex incubated with 2.0 units dextranase.
Lane 4. Microsphere complex incubated with 1.0 units dextranase.
Lane 5. Microsphere complex incubated with 0.5 units dextranase.
Lane 6. Microsphere complex incubated with 0.25 units dextranase.
Lane 7. Microsphere complex incubated without dextranase.
Lane 8 and 9. 5'-32P-$(Bio)_4$-oligonucleotide-streptavidin complex (0.5 pmol).

Sephadex G50 beads, under the same reaction conditions resulted. The lack of digestion was interpreted as a result of a structural change of the dextran in the cause of it's derivatization, which inhibits recognition by dextranase and thus results in only a partial release. direct function of the structure of the dextran molecule, which is a chemically altered derivative that facilitates biotinylation and therefore was not efficiently recognized by the dextranase. Because an enzyme concentration dependent release was observed it could be envisaged that the addition of high concentrations of dextranase may increase the efficiency of digestion. However, the addition of such excess of enzyme would not only mask further biochemical analysis of the released complexes, but would also introduce proteolytic and nuclease activity due to the purity of the enzyme preparation. Thus, it was concluded that a dextranase directed release mechanism could not be applied in the context of the above matrices. However, this technology is only limited by the structure of the dextran molecule and therefore if a bis-biotinyl-dextran dextran derivative was synthesized without complete modification of the glucose residues, specific release would be expected.

Alternatively, it was tested whether poly-biotinylated BSA could compete with the immobilized biotinyl dextran for the binding of the streptavidin, as was previously demonstrated for ^{125}I-biotin (Fig 6) and through this mechanism release the biotinylated oligonucleotide. However, displacement could not be shown, which may be a function of the matrix being poly-biotinylated and therefore the localized concentration of biotin would be similar to that of the biotinylated BSA.

Non-specific release was also attempted, whereby the immobilized transcription complexes were released by denaturing with SDS (boiled for 5 min). Western blot analysis of the released RNA polymerase was not possible due to partial release of the biotinyl dextran, which saturated the signal. This emphasizes that not only a specific capture mechanism must be available to bind the desired macromolecule to the matrix surface, but that of equal importance a specific release mechanism must be incorporated.

The above results suggest that specific of the biotinylated transcription complexes and associated transcriptionally active chromatin is possible. However, the release of these complexes depends on the development of new streptavidin matrices. These matrices must be constructed to fulfill the following criteria. 1) The spacer arm must be of sufficient length to penetrate the α-amanitin binding pocket to overcome steric hindrance. 2) A specific release mechanism must be incorporated. 3) Non-specific of both DNA and proteins binding must be low.

The permutations that I have investigated were unable to fulfill all of the above criteria. The development of new affinity matrices exhibiting the above properties, in combination with Bio-32-NAP-amanitin, should make it possible to purify transcription complexes and the associated transcriptionally active chromatin utilizing the high affinity between biotin and avidin.

CHAPTER 3.

NUCLEOPROTEIN HYBRIDIZATION: DEVELOPMENT OF A METHODOLOGY TO ENRICH SPECIFIC GENES.

INTRODUCTION:

The characterization of proteins associated *in vivo* with transcriptionally active genes is essential for the understanding of the chromatin structure and those proteins that facilitate the transcription process. However, the methodologies developed to enrich active chromatin up to the present, are unable to conclusively determine these specific characteristics. This may be explained by the fact that criteria on which these methods are based are not unique to the transcriptionally active chromatin domains, resulting in the co-isolation of bulk chromatin.

The DNA nucleotide base sequence is a unique characteristic of an individual gene, which in turn translates into a specific protein product. Therefore, the DNA sequences can be proposed as a means to enrich a specific target gene via hybridization. This was initially demonstrated by Yenikolopov *et al.* (1976) and Georgiev *et al.* (1977), who showed that DNA treated with an exonuclease (exonuclease III or λ exonuclease) to expose single stranded regions of the target genes, could be enriched after hybridization to complementary mRNA or Hg labelled mRNA by affinity chromatography on poly-U or sulfhydryl Sepharose, respectively. The enrichment factor that was obtained varied between 100-1000 fold.

Restriction endonuclease digestion of chromatin has been used to determine the accessibility of specific DNA sequences and sensitivity suggests the absences of a protein-DNA complex (Varshavsky *et al.*, 1979). As a result of these findings Reynolds *et al.* (1983) showed that the 5S RNA chromatin from *Xenopus* could be efficiently solubilized. Workman and Langmore (1985a) optimized the digestion conditions for a single step solubilization of the histone gene chromatin from *Strongylocentrotus purpuratus*. This method of chromatin fragmentation generates chromatin lengths as a function of DNA sequence. The physicochemical properties of this chromatin allowed for the partial purification of the histone genes (16 fold) by differential density ultracentrifugation (Workman and Langmore, 1985a).

Amendment.

The development of methodologies for the enrichment of transcriptionally active chromatin domains, has over the past three decades, focused on chromosomal features proposed to be associated with these domains. Thus, the extent of enrichment achieved, was a direct gauge of the uniqueness of structural characteristic associated with the active domains.

The primary step in all the procedures described thus far involved the solubilization of the chromatin by nuclease digestion (Micrococcal nuclease) (Bloom and Anderson (1978). However, this enzyme is non-specific and only a 5 fold enrichment of actively transcribed sequences was observed, besides the simultaneous release of non-transcribed domains. It was on the other hand not possible to solubilize the complete set of transcriptionally active domains, which might be due to an association with the insoluble nuclear matrix and/or precipitation due to nuclear collapse. Further enrichment of the solubilized active domains required secondary purification steps. These methods require chromosomal structural features shown to be enriched in the nuclease digestion. Consecutive gel exclusion chromatography was shown to enhance the enrichment factor to 50 fold, as extended the active chromatin moves differently from the compact non-transcribed chromatin (reduced H1 content) (Ridsdale and Davie (1987). High mobility group protein (HMG) affinity chromatography has once been shown to enriched transcribing domains (no enrichment factor was given) (Wiesbrod and Wientraub 1981 and Weisbrod,1982), but these results were not repeated successfully, possibly due to the preferential binding of HMG's to nucleosomes with intact linker DNA (Swedlow and Varshavsky, 1983). To circumvent this obstacle Dorbic and Wittig (1986) developed an immunoprecipitation protocol to selectively precipitate nucleosomes containing HMG 17. An enrichment of 60-100 fold was observed. These authors showed that HMG 14 and 17 are also associated with non-transcribed nucleosomes. This finding cast some doubt on the integrity of the enriched material. A further matter of concern arises with this methodology, that non-specific binding was observed between the antibody and histone H1. To minimize this effect nucleosomes with a reduced H1 content were produced by salt precipitation. Similar non-specific binding was observed by Hebbs *et el.* (1988), who demonstrated the selective immunoprecipitation of hyperacetylated nucleosomes depleted of H1. This method showed an enrichment factor of 30 fold, hinting at a link between acetylation and transcriptional activity. Allegra *et el.* (1987)

suggested that acetylation of the core histones unfolds the nucleosome core, which exposes the H3 sulfhydryl group. Consecutive Hg affinity chromatography enriched active domains 3-4 fold. However, subsequent reports suggested that additional sulfhydryl containing proteins were immobilized (Walker *et el.*, 1990 and Chen *et el.*, 1991). As early as 1983 Baer and Rhodes demonstrated that the transcriptionally active chromatin had an altered conformation, lacking one H2A-H2B dimer per core. These altered nucleosomes could be purified by density gradient ultracentrifugation when associated with RNA polymerase II. Transcribing sequences are enriched 10 fold.

All of the methods listed above failed to demonstrate a definite enrichment of pure transcriptionally active chromatin, because the chromosomal features identified as targets were not unique to these domains. Furthermore, no attempts were made to differentiate between potentially active and active domains. What can be said with certainty is the following: 1) the transcriptionally active chromatin has an extended conformation with a reduced H1 content, 2) the histone are highly modified and 3) non-histone proteins are associated preferentially with these domains.

In a new approach to discriminate between active and non-active chromatin, Workman and Langmore (1985a) developed a restriction endonuclease solubilization method to solubilize defined units of transcriptionally active chromatin. These domains were subsequently enriched 16 fold by density ultracentrifugation. Furthermore, Workman and Langmore (1985b) demonstrated that these domains could be enriched a further 115 fold by nucleoprotein hybridization in combination with SH affinity chromatography . However, this methodology required acetylation of all the histone to minimize non-specific interactions between the Hg labelled probe and the histones. The level of acetylation required will most probably irreversibly alter the conformation of the transcriptionally active chromatin structure. Other complicating factors were demonstrated by the authors. These included a high degree of non-specific binding (up to 3 %) between matrix and chromatin and the size of the probe. To improve this methodology Vincenz *et el.* (1991) labelled an oligonucleotide probe with biotin and captured the nucleoprotein hybrids on an avidin-D matrix. This method also required the acetylation of the nuclear proteins to prevent disulfhyde exchange between the probe and sulfhydryl groups on the nuclear proteins. The authors claim that they can achieve an overall enrichment of >700 fold for the

particular target genes (histone gene quintet), which is the highest purification reported to date. However, the yield of target chromatin was only 2-15 %. Analysis of the data reported by Vincenz *et el.* (1991) can shed some light on the low yield, which can be summarized as follows.

Step 1: Restriction endonuclease digestion (Hind III) only released 15-30 % of the target gene. The remaining target genes must either be associated with the insoluble nuclear matrix or the size of the fragments released promoted entanglement in the nuclear debri.

Step 2: 5' exonuclease (lamda exonuclease) digestion of the solubilized chromatin only exposed 80 % of the 3' ends, and no data were given when the chromatin was digested with T7 gene 6 exonuclease instead, the enzyme used by the authors in preparative scale experiments. Furthermore, the conditions given for T7 gene 6 exonuclease digestion failed to list a reducing agent, an absolute requirement for enzyme activity (Kerr and Sadowski. 1972).

Step 3: The nucleoprotein hybridization efficiency was shown to be approximately 70 %, which corresponds well with the efficiency of exonuclease digestion, suggesting that 90 % of the exposed 3' ends formed hybrids. However, the authors failed to elude to the observed non-specific binding between the probe and chromatin (personal communication with Vincenz).

Step 4: Avidin-D matrix retains approximately 70 % of the target genes (100% of the hybrids). However, only 16 % of these hybrids were specifically released with the addition of DTT, suggesting a high degree of non-specific binding. The authors do not discuss this further, but they demonstrate that non-specifically retained chromatin is not released with the addition of DTT.

Based on the reviewed data the overall yield was recalculated to be maximally 4 %. Two main factors contribute to the low yield; 1) the inefficiency of solubilization, and 2) the lack of hybrid release.

But these are shortcomings which can be overcome.

Because the nucleoprotein hybridization method showed the greatest promise in the ability to enrich transcriptionally active chromatin, it would be the obvious method to improve in the future. This thesis has focused on characterizing each step of the method with the view to improve the technique and increase yields.

Restriction endonuclease digestion has an added advantage to that of nonspecific nuclease digestion, in that a specific target sequence will be positioned at the 5' end of the released fragment. This implies that the target sequence could be exposed by subsequent 5' exonuclease digestion. Workman and Langmore (1985b) developed a nucleoprotein hybridization technique, whereby a complementary mercurated DNA probe was hybridized under physiological conditions to the exposed target sequence of SV40 chromatin. The resulting hybrids were specifically enriched (115 fold) by sulfhydryl-Sepharose affinity chromatography.

The histone gene quintet contains the genes coding for the four nucleosome core histone H2A, H2B, H3 and H4, and the linker binding histone H1 (Gross *et al.*, 1976). This gene family is highly reiterated and comprises 0.5-0.8% of the total genome in the sea urchin (Birnstiel *et al.*, 1974). These genes are maximally transcribed during the early blastula stage of development and transcription ceases at gastrulation (see review Kedes, 1979; Maxson and Wilt, 1981). Enrichment of this chromatin region would allow for the characterization of the proteins associated with active chromatin (Workman and Langmore, 1985b).

This section describes the adaption of the methods reported by Workman and Langmore (1985b) to a streptavidin/biotin affinity purification technique. This method of affinity chromatography was selected due to the low dissociation constant (10^{-15} M) and the low non-specific binding properties of streptavidin (Wilchek and Bayer, 1990). Two main aspects were focused on: 1) The preparation of a biotinylated DNA or RNA probe that could be released from the hybrid by nuclease digestion. 2) The construction of a streptavidin matrix that efficiently binds the macromolecular biotinylated hybrid (see chapter 5).

METHODS AND MATERIALS:

Construction of the pPA(H2B-H3S) plasmid probe (probe 1).

Cloning of pPA(H2B-H3S).

The plasmid pPA(HQ) (Bluescribe SK containing the histone gene quintet, cloned by J. Rees) was digested with the restriction endonucleases PstI (cleaves in the poly-linker region) and AccI (cleaves in the H3 spacer region) in a medium salt buffer at 37° C for 3 hours. The total digest was electrophoretically fractionated on 0.7 % agarose (1x TBE pH 8.3; 1.5 V/cm) and the 3.8 Kb fragment (2.8 Kb Bluescribe vector plus the 1 Kb region of the H2B gene and H3 spacer) was excised and collected by electro-elution. The PstI end was converted to a blunt end by incubation with T4 DNA polymerase and blunt end ligated to a BamHI linker (Beckman). Excess BamHI linker was removed by gel exclusion chromatography on Sepharose CL-6B (equilibrated in TE pH 8.0). The AccI end was then converted to a blunt end by incubation with Klenow, and the linear plasmid (pPA(H2B-H3S)) was circularized by blunt end ligation and transformed into the E.coli (strain JM109). The plasmid pPA(H2B-H3S) was amplified in JM109, isolated by the lysozyme/Triton x100 method and purified by CsCl equilibrium density ultracentrifugation (above methods according to Sambrook *et al.*, 1989).

Biotinylation of linearized pPA(H2B-H3S).

pPA(H2B-H3S) was linearized by digestion with SacI and the 3' protruding ends were poly-biotinylated by incubation with terminal tranferase (according to Current Protocols, 1987). Briefly, SacI linearized pPA(H2B-H3S) was incubated in tailing buffer (100 mM sodium cacodylate pH 7.0, 1 mM $CoCl_2$, 0.1 mM DTT, 20 µM Bio-11-dUTP and 50 µg/ml BSA) containing 0.2 units terminal transferase per µg DNA and incubated at 37° C for 60 min. The tailing reacting was stopped with the addition of EDTA to a final concentration of 5 mM, followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Biotinylation was demonstrated by a gel retardation assay. Poly-biotinylated pPA(H2B-H3S) was digested with Hind III (generating two biotinylated DNA fragments, approximately 1100 and 2900 base pair respectively) and incubated with an excess of streptavidin.

The streptavidin-DNA complexes were then electrophoretically fractionated in 1 % agarose (in 1x TBE pH 8.3, 1.5 V/cm) and biotinylation was demonstrated by a retardation in the electrophoretic mobility when compared to non complexed poly-biotinylated DNA.

The poly-biotinylated DNA was then digested with SmaI to remove the biotinyl residues on the 5' end of the H2B gene, which in turn generated a substrate end for exonuclease III digestion. Partial exonuclease III digestion (0.5 units exonuclease III per µg DNA incubated in exonuclease buffer (66 mM Tris-HCl pH 8.0 and 6.6 mM $MgCl_2$) at 37° C for 5 min followed by incubation at 70° C for 10 min), exposed the non-coding nucleotide sequence of the H2B gene (approximately 500 bases). The exonuclease activity was monitored electrophoretically. This construct was referred to as probe 1.

Oligonucleotide 5' end labelling.

Briefly, 50 pmol oligonucleotide was incubated at 37° for 1 hour in 50 µl end labelling buffer (according to enzyme manufacturer) containing 200 µCi γ-^{32}P-ATP and 20 units T4 polynucleotide kinase. The labelling reaction was stopped with the addition of EDTA to a final concentration of 15 mM and desalted by centrifugation through a Sephadex G25 or G50 spin column equilibrated in STE pH 8.0. For post 5' end labelling biotinylation the spin column was equilibrated in terminal transferase tailing buffer excluding Bio-11-dUTP, BSA and enzyme.

Oligonucleotide 3'-poly-biotinylation.

The oligonucleotide 5'TCCAAGAAAGCAGTGAAG 3' was poly-biotinylated using a modification of the conditions described above. 15 pmol 5'-32P labelled oligonucleotide was incubated in tailing buffer containing 100 µM Bio-11-dUTP and 25 units terminal transferase, and incubated at 37° C for 1.5 min. The tailing reaction was stopped with the addition of EDTA (5 mM) and desalted by centrifugation through a Sephadex G25 spin column equilibrated in STE pH 8.0. Biotinylation was demonstrated by electrophoretic fractionation on a 15 % non-denaturing polyacrylamide gel (1x TBE pH 8.3). These conditions allowed for the addition of a average of 4 biotinyl residues per 3' end.

The above protocol could also be applied for 3' end labelling of the oligonucleotide, where Bio-11-dUTP was substituted with α-^{32}P-dCTP.

Linear PCR amplification of the 5' end of the H2B gene.

Cloning of pPA(H2BS-H2B-H3S).

The plasmid pPA(H2B-H3S) was digested with the restriction endonuclease BamHI and the excised insert containing the H2B gene and H3 spacer was purified from low melting agarose using a Clean Gene Kit (Bio 101).

The plasmid pPA(H2BS-H2B) (Bluescript containing the H2B spacer and the 5' end of the H2B gene (EcoRI-BamHI fragment), cloned by J. Rees) was linearized by digestion with BamHI, dephosphorylated by incubation with phosphatase (CIP) and purified from low melting agarose. The BamHI fragment (H2B-H3S) was then ligated into the BamHI site of pPA(H2BS-H2B) and transformed into competent XL1-Blue cells. Recombinants plasmids were screened by restriction endonuclease digestion to determine the orientation of insertion. The plasmid p(H2BS-H2B-H3S) containing the intact H2B gene was then purified by cesium chloride equilibrium density ultracentrifugation.

PCR amplification.

The plasmid pPA(H2B2-H2B-H3S) was digested with AluI which cleaves the DNA 70 bases down stream of the H2B BamHI site. The 5' end of the H2B gene was then linearly amplified by the PCR reaction using the oligonucleotide primer 5'ATCATGGCTCCAACAGGTC3'. The conditions used for amplification were as follows, 50 mM KCl, 10 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.05 mM Bio-11-dUTP, 0.01 % BSA and 0.05 % Tween 20. 1 µg AluI digested template was amplified with the addition of 200 pmol primer and 5 units of Taq polymerase. The thermocycles used were, 92° C for 30 seconds, 58° C for 30 seconds and 72° C for 90 seconds and repeated 35 times. On completion of amplification formamide (final concentration 50 %) was added to the reaction mixture, which was heated to 100° C for 10 min followed by electrophoretic fractionation on 12 % denaturing polyacrylamide gel. The amplicons were visualized

by florescent quenching over a thin layer silica F_{254} plate. These amplicons (103 bases) were excised and extracted by incubation in 0.3 M sodium acetate pH 7.0, 0.1 % SDS and 10 mM EDTA at 37° for 16 hours. The acrylamide was pelleted by centrifugation (8000 xg for 5 min.) and the amplicons were then ethanol precipitated. This biotinylated probe (probe 4) was then 5' end labelled by incubation with T4 polynucleotide kinase and γ-^{32}P-ATP as described above.

Solubilization of the histone gene quintet chromatin.

BamHI digestion of 14 hour sea urchin embryo nuclear chromatin.

(adapted with modifications from Workman and Langmore 1985a).

14 hour sea urchin embryo nuclei (stored at -70° C) were thawed on ice and then permeabilized by washing 4 times with NSB containing 0.1% Nonidet LE. The detergent was removed by a further 2 washes with NSB. The permeabilized nuclei were then diluted to a concentration of 125 µg DNA/ml in 50 mM NaCl, 50 mM Tris/HCl pH 8.0, 0.5 mM $MgCl_2$ and 1 mM ß-mercaptoethanol. The chromatin was then digested with the addition of BamHI (5 units/µg chromatin) followed by incubation at 37°C for 3 hours, without shaking. Insoluble nuclear debri was pelleted by centrifugation at 1000xg for 30 min and the soluble chromatin (supernatant) was collected and dialysed against T7 gene 6 exonuclease digestion buffer (20 mM KCl, 20 mM Tris/Cl pH 8.0, 1 mM $MnCl_2$ and 5 mM ß-mercaptoethanol) at 4°C for 16 hours.

T7 Gene 6 Exonuclease Digestion of BamHI Soluble Chromatin.

The BamHI soluble chromatin (approximately 50µg/ml, measured by OD_{260}) was digested with T7 gene 6 exonuclease (1 unit/µg chromatin) at 37° C for 3 hours. Following digestion the chromatin was dialysed into hybridization buffer (100 mM NaCl, 10 mM Tris/Cl pH 8.0 and 1 mM EDTA) at 4°C for 16 hours.

The degree of exonuclease digestion was determined by electrophoretic fractionation of the deproteinized chromatin, pre-digested with P1 nuclease and Sal I endonuclease (1 % agarose (1x TBE pH 8.3) (1.5 V/cm)). The H2B gene fragment (the site of

exonuclease digestion) was visualized by autoradiography after being Southern transferred to Hybond-N (Amersham) and hybridized with ^{32}P labelled pPA(H2B-H3S) (nick translation).

Nuclear Protein Hybridization.
(according to Workman and Langmore 1985b).

5'-32P labelled biotinylated probe complementary to the 5' sequence of the H2B gene was added in a ratio of approximately 1000:1 to [BamHI-T7 gene 6 exonuclease] digested chromatin or deproteinized chromatin (approximately 50 µg/ml). Hybridization was allowed to proceed at 37° C for 24 hours. The degree of hybridization was monitored by autoradiography of the hybrids (deproteinized by treatment with Proteinase K or 0.25 % Sarkosyl or 0.25 % SDS) fractionated by electrophoresis in 0.7 % agarose (1x TBE pH 8.3, 1.5 V/cm).

Immobilization of Hybrid onto Solid Phase Streptavidin.

Streptavidin-hexaglycyl-microspheres (see chapter 2 for synthesis) or streptavidin magnetic beads (Advanced Magnetics and Dynal) were pre-incubated in STE pH 8.0 containing 250 µg/ml BSA 0.1-0.25 % Tween 20 and 5 % glycerol to quench non-specific binding. The hybridized chromatin or DNA (Tween 20, BSA and glycerol were added to a final concentration of 0.1-0.25 %, 250 µg/ml and 5 %, respectively) was then added to an aliquot of solid phase streptavidin (total biotin binding capacity 30 fold higher than that required to bind all the probe) and binding was allowed to proceed for 2 hours with gentle agitation. The solid phase streptavidin was then pelleted by brief centrifugation (8000xg for 1 min) or by being positioned against a magnet and the pelleted matrix was then washed with P1 nuclease digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0 and STE, 0.25 mM $MgCl_2$, 0.1-0.25 % Tween 20, 250 µg/ml BSA and 5 % glycerol). The washed matrix was resuspended in 200 µl P1 nuclease buffer and the bound hybrids were digested with 3 units P1 nuclease at 37° C for 15-40 min, followed by the addition of EDTA to 1 mM. The matrices were then pelleted as above and the supernatant, putatively containing the histone gene quintet chromatin or DNA was retained.

To determine the degree of non-specific binding of the hybrids to the solid phase streptavidin, the matrices were resuspended in Proteinase K buffer containing 100

μg/ml Proteinase K and incubated at 37° C for 2 hours. The matrices were then pelleted and the supernatants retained.

All chromatin or DNA fractions were deproteinized by incubation with Proteinase K (100 μg/ml) followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation in the presence of 10 μg tRNA. The DNA was then fractionated by electrophoresis in 0.7 % agarose (1x TBE pH 8.3, 1.5 V/cm) and southern transferred to Hybond-N and the histone gene quintets were visualized by hybridization with nick translated pPA(HQ). Controls included were: 1) Total hybridization mixture before incubation with the microspheres and 2) Unbound chromatin or DNA in the presence or absence of excess biotin.

Dot Blot Hybridization.

P1 nuclease released hybrids were deproteinized as described previously and redissolved in TE pH 8.0. The DNA was denatured by heating to 100° C for 10 min followed by rapid cooling. An equal volume of 20x SSC pH 7.0 was added and the denatured DNA was spotted onto Hybond-N+ (Amersham) using a commercial vacuum dot blotter. Sea urchin embryo genomic DNA (ranging from 5000-2 ng) was also spotted as a control.

The DNA was then fixed to the membrane by alkaline treatment (according to manufacturer) and the histone gene quintet content of each dot was determined by hybridization with ^{32}P labelled pPA(HQ). The DNA content was determined by hybridization with ^{32}P labelled genomic DNA.

Southern transfer and hybridization.

Southern transfer.
(according to Sambrook *et al.*, 1989).

Agarose gels were stained with Ethidium Bromide (0.5 mg/ml) for 30 min and the DNA was visualized by UV light. Exposure to UV light (approximately 5 min) resulted in DNA nicking to facilitate transfer. The DNA was then denatured by soaking the gel in 0.5 M NaOH and 1.5 M NaCl for 30 min, followed by neutralizing for 30 min in 1 M Tris-HCl pH 8.0 and 1.5 M NaCl. The DNA was transferred to Hybond N (Amersham) by capillary action with 10x SSC pH 7.0 and fixed onto the membrane by UV radiation.

Alternatively the DNA was denatured by soaking the agarose gel in 0.4 M NaOH for 20 min followed by capillary transfer in the same buffer to Hybond N+ (Amersham). No fixing was required.

Hybridization.

The membranes were incubated at 42° C for 6 hours in prehybridization solution (50 % formamide, 5x SSPE pH 7.4, 5x Denhardts, 0.2 % SDS and 100 µg/ml denatured sonicated Herring sperm DNA) (0.1 ml per cm^2). Following this step the prehybridization solution was discarded and fresh prehybridization solution was added (0.05 ml per cm^2) containing denatured ^{32}P labelled probe (5 ng per ml). Hybridization was allowed to proceed for 16 hours at 42° C. The membranes were then washed twice with 2x SSC pH 7.0 and 0.1 % SDS at room temperature for 30 min. This step was repeated at 45° C followed by a moderate stringency wash at 45° C (0.2x SSC and 0.1 % SDS). The target DNA was visualized by autoradiography.

RESULTS AND DISCUSSION:

The nucleotide sequence is a unique characteristic of an individual gene and if the respective gene is in a state of being actively transcribed, it could be said that the nucleotide sequence is a specific characteristic of transcriptionally active chromatin. This definition suggests that the nucleotide sequences of transcriptionally active genes could be proposed as targets for the affinity isolation of transcriptionally active chromatin via nucleoprotein hybridization, in order to enrich protein components (Workman and Langmore 1985b). However, due to the limited sensitivity of current protein detection methods, this methodology could only be applied to highly reiterated genes or gene families, thereby increasing the yield to facilitate the characterization of the associated chromatin proteins. The sea urchin embryo histone gene quintet which codes for the five histones H1, H2A, H2B, H3 and H4 is one such gene family that is tandemly repeated approximately 300 times per genome (see review Kedes, 1979). These genes are actively expressed during early embryonic development (Maxson and Wilt, 1981) and therefore would be the ideal target for the isolation of active chromatin via the nucleoprotein hybridization technique.

Solubilization of the histone gene quintet chromatin by restriction endonuclease digestion.

14 hour sea urchin embryo nuclei (*Parachinus Angulosus*) were digested with the restriction endonuclease BamHI, using the digestion conditions previously described by Workman and Langmore (1985a). The BamHI recognition sequence (GGATCC) occurs once per quintet and is located 31 bases down stream from the A of the ATG start codon of the H2B gene (Fig 1). Thus, by cleaving the chromatin at this site, the intact histone gene quintet repeat or multiples thereof would be solubilized. Furthermore, this would enable the coding region of the H2B gene to be exposed by subsequent exonuclease digestion (see below).

The ionic conditions employed during digestion allowed for the immediate solubilization of approximately 40-50 % of the chromatin as measured by OD_{260}. Further extraction of the insoluble chromatin at low ionic strength (10 mM Tris-HCL pH 8.0 and 1 mM EDTA) failed to improve the yield, presumably due to irreversible aggregation or association with the nuclear debri. Hybridization analysis of the solubilized DNA, fractionated by agarose

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                                                                  B
                                                                  a
                                                                  m
                                                                  H
                                                                  I
TACACCAAAAAACTTCATCTC**ATCATGGCTCCAACAGGTC**AAGTCGCTAAGAAAGGATCC
---------+---------+---------+---------+---------+---------+---------+
ATGTGGTTTTTTGAAGTAGAGTAGTACCGAGGTTGTCCAGTTCAGCGATTCTTTCCTAGG

AAGAAAGCAGTGAAGGCACCTCGCCCTAGCGGTGGCAAGAAGAGGAACAGGAAAAGGAAG
---------+---------+---------+---------+---------+---------+---------+
TTCTTTCGTCACTTCCGTGGAGCGGGATCGCCACCGTTCTTCTCCTTGTCCTTTTCCTTC

    A
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    I
GAGAGCTACGGCATCTACATCTACAAGGTCCTCAAGCAGGTTCACCCTGACACTGGTATC
---------+---------+---------+---------+---------+---------+---------+
CTCTCGATGCCGTAGATGTAGATGTTCCAGGAGTTCGTCCAAGTGGGACTGTGACCATAG

TCCAGCAAGGCCATGACCATCATGAACAGCTTTGTCAACGACATCTTCGAGCGAATCGCC
---------+---------+---------+---------+---------+---------+---------+
AGGTCGTTCCGGTACTGGTAGTACTTGTCGAAACAGTTGCTGTAGAAGCTCGCTTAGCGG

GGTGAAGCCTCTCGTCTCTCCCAGTACAACAAGAAGTCAACCATCAGCAGTCGCGAGATT
---------+---------+---------+---------+---------+---------+---------+
CCACTTCGGAGAGCAGAGAGGGTCATGTTGTTCTTCAGTTGGTAGTCGTCAGCGCTCTAA

CAGACCGCTGTGGCCCTCCTTCTCCCAGGAGAGCTGGCAAAGCACGCCGTGAGGCAGGGG
---------+---------+---------+---------+---------+---------+---------+
GTCTGGCGACACCGGGAGGAAGAGGGTCCTCTCGACCGTTTCGTGCGGCACTCCGTCCCC

ACCAAAGCCGTGACAAAGTACACCACCGCCAAATAAATGGTACACAACTGGTCCT
---------+---------+---------+---------+---------+-----
TGGTTTCGGCACTGTTTCATGTGGTGGCGGTTTATTTACCATGTGTTGACCAGGA
```

Figure 1. Partial nucleotide sequence of the histone H2B gene derived from *Parachinus Angulosis* as according to J. Rees (unpublished).

1) The BamHI restriction site denotes the site at which the histone gene quintet was cleaved during solubilization.

2) The oligonucleotide depicted in bold was used to linearly amplify the 5' end of the H2B gene sequence up to AluI restriction site with the simultaneous incorporation of Bio-11-dUMP.

3) The ATG transcription start codon of the H2B gene is located 31 bases upstream of the BamHI site.

gel electrophoresis, demonstrated that the histone gene quintet chromatin was solubilized in the forms monomer, dimer, trimer and tetramer (Fig 2). The relative intensities of the quintet bands was not related to their respective yields, as the hybridization signal was a function of the fragment length when probed with total histone quintet DNA (pPA(HQ)). In solution hybridization with a primer complementary only to the 5' end of each quintet fragment showed that the monomeric quintet was preferentially solubilized (Fig 11). The reason for incomplete digestion was not investigated, but it was presumed that either sequence heterogeneity or a protein-DNA complexes prevented BamHI recognition.

Exonuclease digestion.

The nucleoprotein hybridization technique was designed to enrich intact chromatin, thus standard DNA hybridization conditions could not be applied. Hybridization under non-denaturing conditions would only proceed if both the probe DNA and the target DNA were in a single stranded conformation. In the context of chromatin this could only be achieved by controlled exonuclease digestion to specifically expose one of the DNA strands. Two 5' exonucleases were investigated, λ exonuclease and T7 gene 6 exonuclease. Control digestions showed that λ exonuclease was unfavorable due to the high processitivity which resulted in the incomplete digestion on all the 5' ends, furthermore the high affinity of this enzyme for the 5' ends may influence hybridization. In contrast the non-processive T7 gene 6 exonuclease showed complete digestion of the 5' ends and therefore was the exonuclease of choice.

The BamHI solubilized chromatin was dialysed against T7 gene 6 exonuclease buffer (optimized digestion conditions as described by Kerr and Sadowski, 1972) and digested with 1 unit T7 gene 6 exonuclease per μg DNA at 37° C for 3 hours to expose the coding strand. The extent of exonuclease digestion was shown to be approximately 60 bases from the 5' end of the histone gene quintet (Fig 3). The uniformity of digestion suggests the presence of a positioned protein complex approximately 60 bases from the 5' end, which prevented further exonuclease digestion.

Probe design.

Workman and Langmore (1985b), who described the nucleoprotein hybridization technique, used a single stranded mercurated DNA probe complementary to the exposed

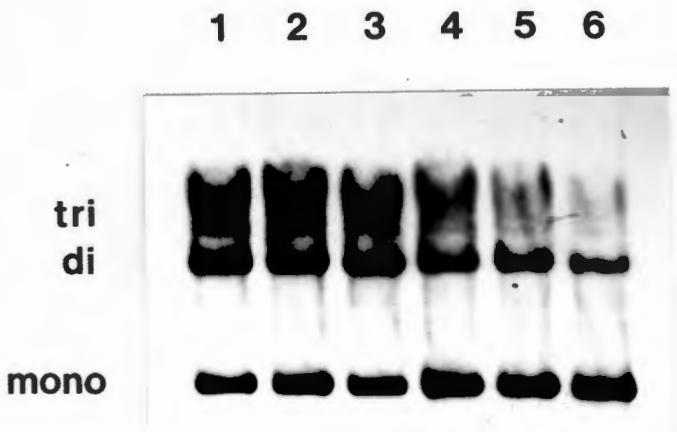


Figure 2. Solubilization of the histone gene quintet by digestion with the restriction endonuclease BamHI.

Detergent permeabilized 14 hour sea urchin embryo nuclei (early gastrula) were digested with the restriction endonuclease BamHI as a function of time (see methods and materials). The solubilized chromatin was deproteinized and ethanol precipitated and redissolved in TE pH 8.0. The purified DNA was then electrophoretically fractionated in 0.7 % agarose (1x TBE pH 8.3) and Southern transferred to Hybond N (Amersham). The histone gene quintet fragments were visualized by hybridization with ^{32}P labelled pPA(HQ) (nick translation), followed by autoradiography.

Lane 1. Digestion at 37° C for 1 hour.
Lane 2. Digestion at 37° C for 2 hours.
Lane 3. Digestion at 37° C for 3 hours.
Lane 4. Digestion at 37° C for 4 hours.
Lane 5. Digestion at 37° C for 5 hours.
Lane 6. Digestion at 37° C for 6 hours.

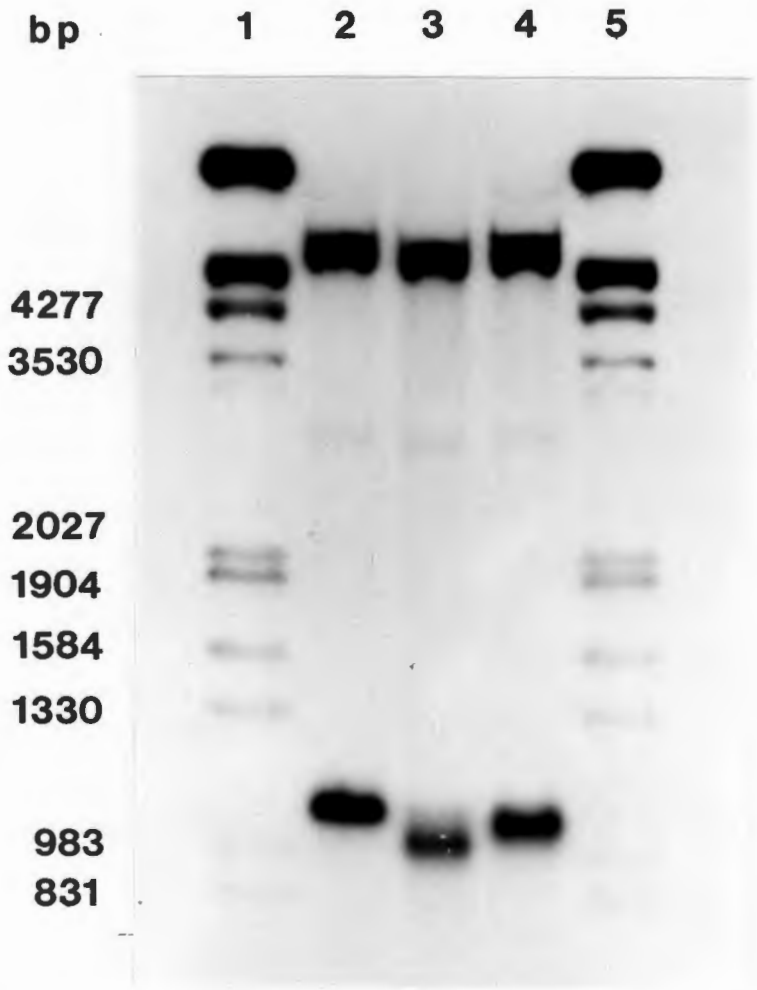


Figure 3. T7 gene 6 exonuclease digestion of the 5' end of the histone H2B gene.

BamHI solubilized chromatin was prepared as described in methods and materials. The solubilized chromatin was dialysed against T7 gene 6 exonuclease buffer (10 mM Tris-HCl pH 8.0, 20 mM KCl, 5 mM ß-mercaptoethanol and 1 mM $MnCl_2$) at 4° C for 16 hours. T7 gene 6 exonuclease (1 unit/μg) was added and digestion was allowed to proceed at 37° C for 3 hours. The chromatin was then deproteinized and ethanol precipitation according to standard protocols. The purified DNA (15 μg) was redissolved in high salt buffer (Buffer H, Boehringer Mannheim) and digested with the restriction endonuclease SalI (15 units) at 37° C for 3 hours. P1 nuclease (3.3 units) was then added and the incubation was continued for a further 1 hour. Digestion was stopped with the addition of Proteinase K digestion buffer and Proteinase K and incubated at 42° C for 1 hour followed by phenol/ chloroform extraction and ethanol precipitation. The DNA was then redissolved in TE pH 8.0, fractionated by electrophoresis in 1.2 % agarose and Souther transferred to Hybond N (Amersham). The histone H2B gene was visualized by hybridization with ^{32}P labelled pPA(H2B-H3S) (nick translation) followed by autoradiography.

Lane 1. EcoRI/HindIII digest of λ DNA.
Lane 2. BamHI solubilized genomic DNA digested with SalI and P1 nuclease.
Lane 3. BamHI-T7 gene 6 exonuclease digested genomic DNA, digested with SalI and P1 nuclease.
Lane 4. BamHI-T7 gene 6 exonuclease digested genomic DNA, digested with Sal I.
Lane 5. EcoRI/HindIII digest of λ DNA.

5' region. This method of probe labelling requires that the sulfhydryl groups of the chromatin proteins must be acetylated prior to hybridization in order to prevent non-specific binding to the probe. To circumvent this problem, biotinylation was chosen as a means of introducing an affinity ligand into the probe. The advantages of a biotinyl residue are: 1) The low non-specific binding characteristics. 2) The avid binding by streptavidin and avidin. 3) The incorporated biotinyl residue has only a minimal effect on the stability of the hybrids formed (Langer *et al.*, 1981). The disadvantage was that the streptavidin-biotin interaction was not easily reversed and therefore structural prerequisites for the release of the hybridized complex would have to be incorporated into the probe.

Restriction endonuclease digestion as initial mechanism for release has been investigated. The aim was to achieve sequence specific digestion to cleave the probe between the biotinylated region and the hybridizing region, thereby releasing the hybrid from the solid phase without further fragmenting the histone quintet. To achieve this, the plasmid pPA(H2B-H3S) was constructed, containing an additional BamHI recognition sequence inserted downstream of the H2B gene (Fig 4). SacI linearized pPA(H2B-H3S) (cleaved at 5' end of H2B gene) was 3'-poly-biotinylated by incubation with terminal transferase and Bio-11-dUTP, followed by digestion with SmaI (removes biotinylation on 5' end of the H2B gene and generates a recognition end for exonuclease III). The non-coding strand of the H2B gene was the exposed by partial exonuclease III digestion, as described in methods and materials. This probe was referred to as probe 1.

Increasing amounts of probe 1 were hybridized in solution to a constant amount of [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S) using similar conditions to those described by Workman and Langmore (1985b). Agarose gel electrophoresis of the hybridization mixtures showed the formation of specific hybrid, which were demonstrated by a mobility shift (retardation) (Fig 5). Incubation of these hybrids with streptavidin and biotinylated liposomes followed by centrifugation through a 5 % Ficoll gradient demonstrated the specific retention of the hybrids (Fig 6, lane 5). Digestion of the [hybrib-liposome] complexes with BamHI demonstrated that the hybrid were selectively released (Fig 6, lane 6). Together these results indicated that probe 1 would be a candidate as a handle for the enrichment of histone gene quintet chromatin. However, when probe 1 was hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin or deproteinized chromatin, efficient hybridization could not be demonstrated (Fig 7). This was thought to be a direct function of the hybrid stability, which would be related to the

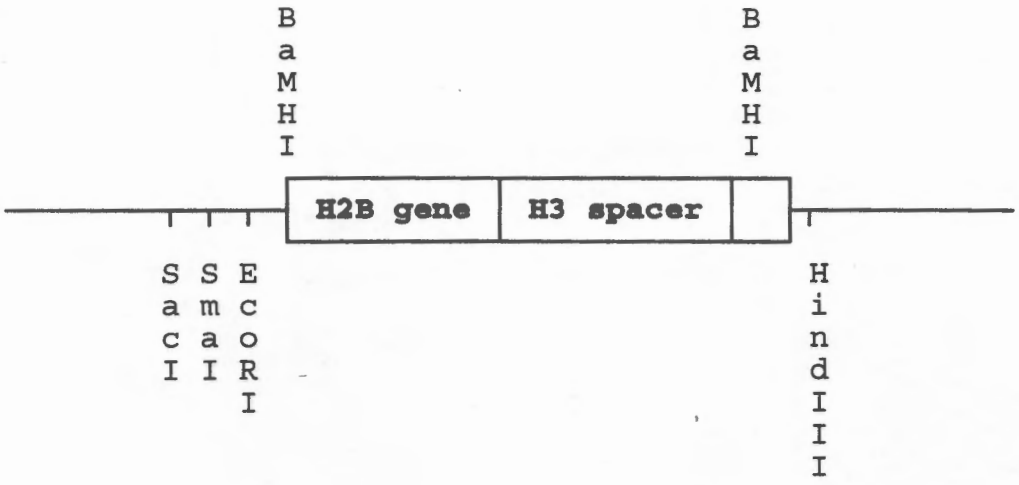


Figure 4A. Plasmid map of pPA(H2B-H3S).

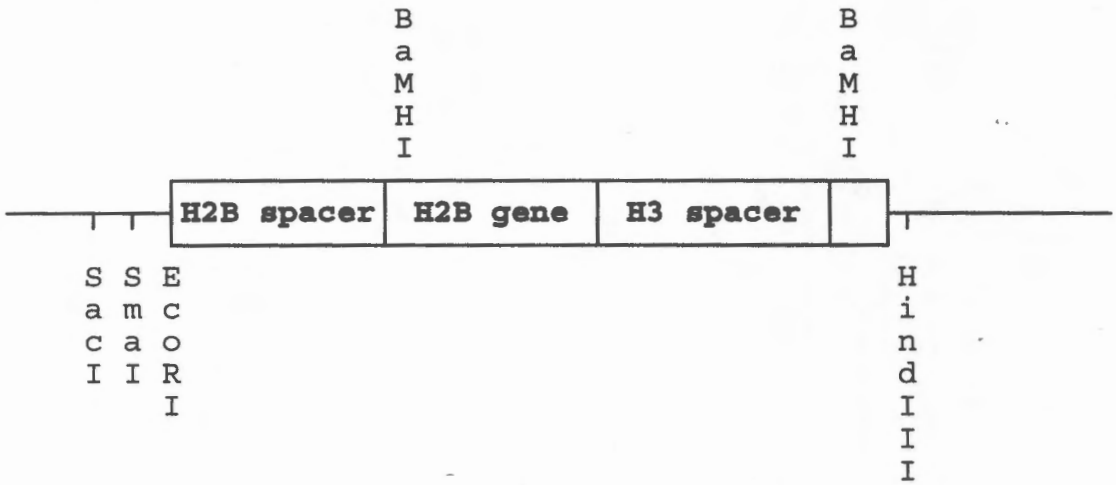


Figure 4B. Plasmid map of pPA(H2BS-H2B-H3S).

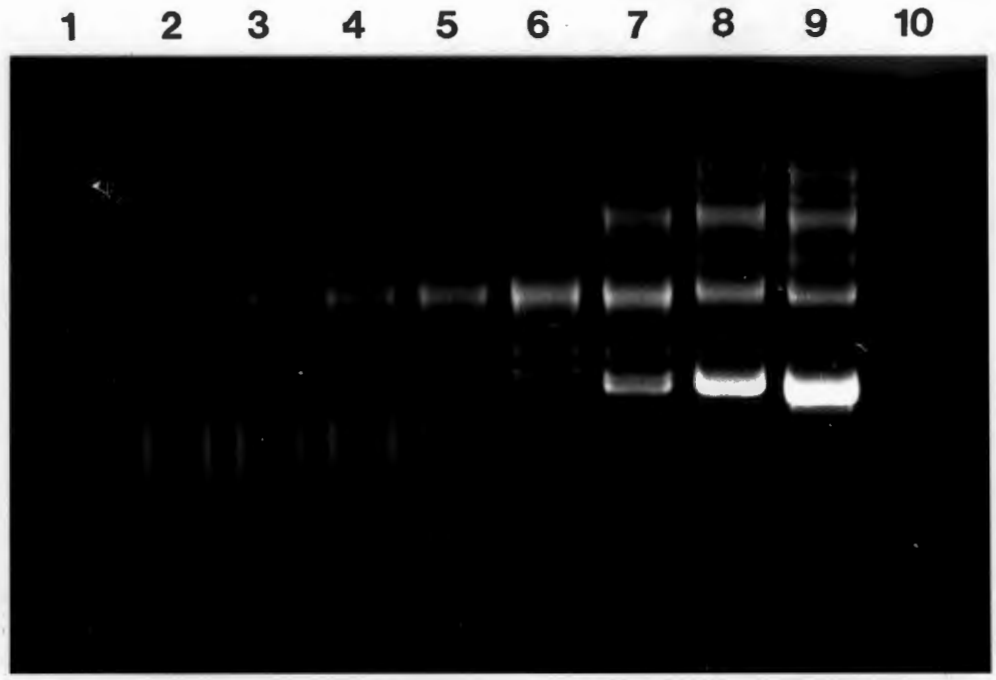


Figure 5. Hybridization of probe 1 to [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S).

pPA(H2B-H3S) was linearized by digestion with EcoRI and the 5' coding stand of the H2B gene was exposed by partial digestion with T7 gene 6 exonuclease (0.8 units T7 gene 6 exonuclease per μg DNA, incubated at 20° C for 3.5 min). Digestion was stopped by heating to 70° C for 10 min followed by phenol/chloroform extraction, ethanol precipitation and the pellet was redissolved in STE pH 8.0. Aliquots of [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S) (approximately 0.5 μg) were added to various dilutions of probe 1 (ranging from approximately 0-5 μg, dissolved in STE ph 8.0) and the final volume was adjusted to 12 μl with buffer. The DNA fragments were allowed to hybridize at 37° C for 20 hours and the resulting hybrids were fractionated by electrophoresis in 0.7 % agarose (1x TBE pH 8.3). Hybridization was demonstrated by the retardation of the [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S] plasmid. The formation of multiple hybrids at high concentrations of probe 1 suggests that more that one probe 1 was hybridized.

Lane 1. AccI digest of pPA(HQ).
Lane 2. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S).(0.5 μg).
Lane 3. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (0.05 μg)
Lane 4. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (0.125 μg)
Lane 5. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (0.25 μg)
Lane 6. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (0.5 μg)
Lane 7. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (1.0 μg)
Lane 8. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (2.5 μg)
Lane 9. [EcoRI-T7 gene 6 exonuclease] pPA(H2B-H3S) (0.5 μg) hybridized to probe 1 (5.0 μg)
Lane 10. AccI digest of pPA(HQ).

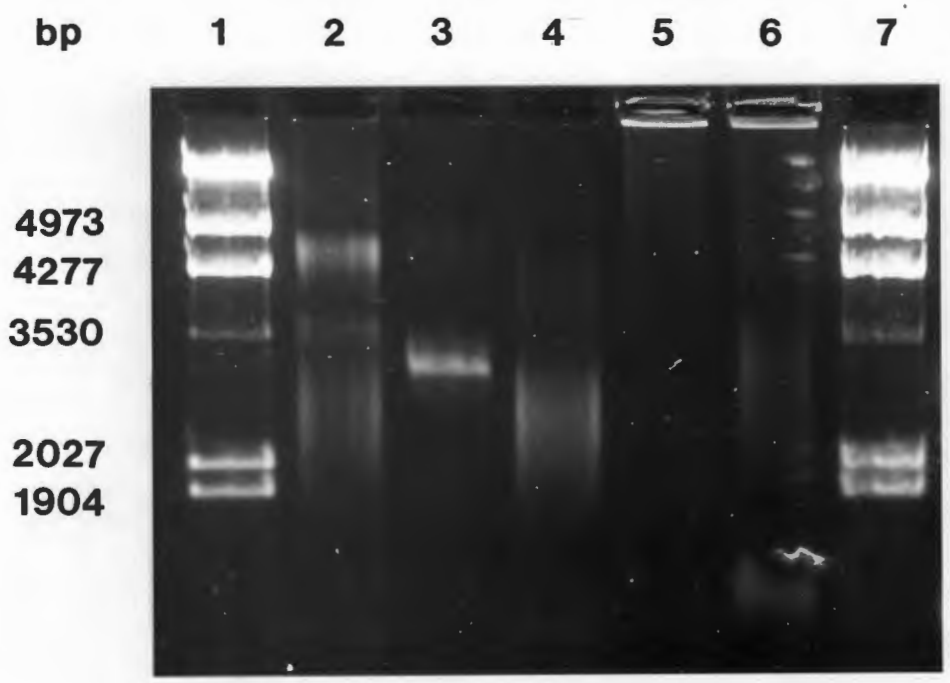


Figure 6. Binding of the hybrids formed between probe 1 and [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S), to biotinylated liposomes via streptavidin.

Probe 1 was hybridized to [EcoRI-T7 gene 6 exonuclease] digested pPA(H2B-H3S) at a molar ratio of 1:1 as described in figure 5. Streptavidin was then added to the hybridization reaction (ratio; 0.05 µg streptavidin: 0.5 µg probe 1) and incubated at 24° C for 1 hour. Biotinylated liposomes (optimal streptavidin binding ratio; 20 µl liposomes per 0.5 µg streptavidin) were then added and binding was allowed to proceed for 1 hour at 24° C. The liposome-hybrid complexes were purified by buoyant density centrifugation through 5 % Ficoll 400 in STE pH 8.0. Following purification, $MgCl_2$ and ß-mercaptoethanol were added to give final concentrations of 10 mM and 1 mM, respectively. The hybrids were released from the liposome by the addition of BamHI (20 units) and incubation at 37° C for 2 hours.
Binding and release of the hybrids was demonstrated by electrophoretically fractionating the respective fractions in 0.7 % agarose (1x TBE pH 8.3).

Lane 1. EcoRI-HindIII digest of λ.
Lane 2. Partial hybridization of probe 1 to [EcoRI-T7 gen 6 exonuclease] digested pPA(H2B-H3S).
Lane 3. Probe 1.
Lane 4. [EcoRI-T7 gen 6 exonuclease] digested pPA(H2B-H3S).
Lane 5. Purified liposome-hybrid complexes (hybrids remain associated with the liposomes in the wells).
Lane 6. Liposome-hybrid complexes digested with BamHI.
Lane 7. EcoRI-HindIII digest of λ.

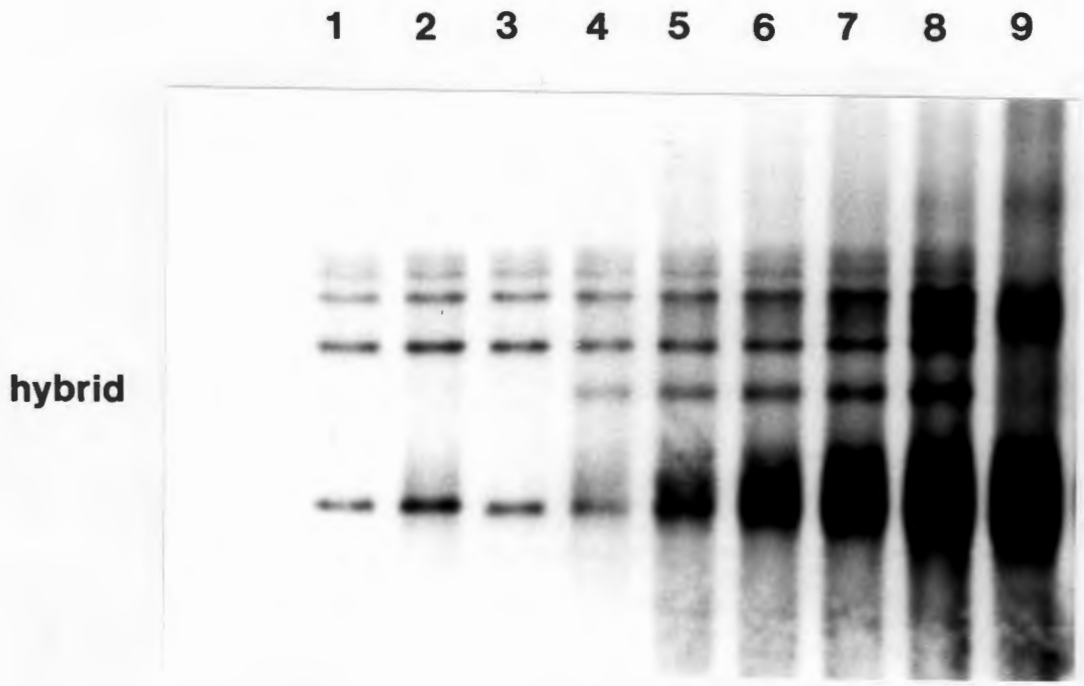


Figure 7. Hybridization of probe 1 to [BamHI-T7 gene 6 exonuclease] digested sea urchin embryo chromatin.

[BamHI-T7 gene 6 exonuclease] digested chromatin was prepared as described in methods and materials. KCl was added to the solubilized chromatin (2.5 μg) to give a final concentration of 100 mM, followed by the addition of increasing amounts of probe 1 (ranging from 0 to 2.5 μg). The reactants were allowed to hybridize at 37° C for 16 hours. The chromatin was then deproteinized, ethanol precipitated (according to standard protocols), redissolved in STE pH 8.0 and fractionated by electrophoresis in 0.6 % agarose (1x TBE pH 8.3). The histone gene quintet fragments and hybrids were visualized after Southern transfer to Hybond N and hybridization with ^{32}P labelled pPA(HQ).

Lane 1. BamHI solubilized chromatin.
Lane 2. BamHI solubilized chromatin hybridized with 0.5 μg probe 1.
Lane 3. [BamHI-T7 gene 6 exonuclease] digested chromatin.
Lane 4-8. [BamHI-T7 gene 6 exonuclease] digested chromatin hybridized with 0.5, 1.0, 1.5, 2.0 or 2.5 μg probe 1.
Lane 9. 2.5 μg probe 1.

length of the single stranded region exposed by T7 gene 6 exonuclease digestion (approximately 60 bases). Dissociation, thus may have occurred when the hybrids were placed in an electric field as a result of the differential in electromotive forces applied to the probe 1 and the histone quintet DNA, in conjunction with the shearing effect of the agarose barrier. Because efficient hybridization could not be demonstrated, it was felt that the probe design should to be changed.

The observed shearing effect should be minimized by decreasing the probe size. Synthetic oligonucleotides were therefore substituted for the linearized plasmid as the probe, with an alternative method of release. It was envisaged that the oligonucleotide probe would hybridize only to a defined number of bases at the 5' end of the single stranded region, thus a single stranded region would remain downstream of the hybrid. This single stranded region would be a substrate for single strand specific nuclease digestion (P1 nuclease), thereby releasing the histone quintet from the solid phase streptavidin or avidin.

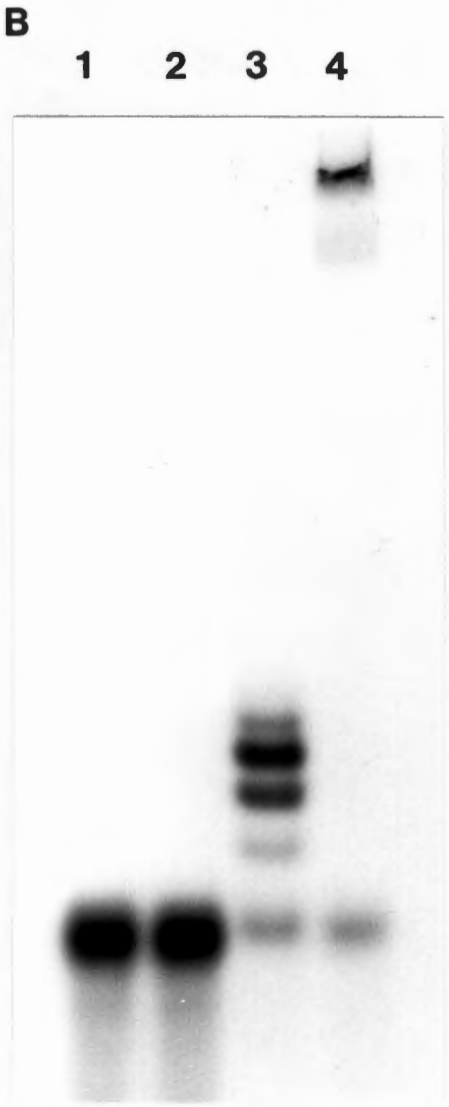
The oligonucleotide 5' TCCAAGAAAGCAGTGAAG 3' complementary to the 5' terminal sequence of the H2B gene was synthesized with or without the inclusion of a 5' C6 thiomodifier (Amersham). The sulfhydryl group was biotinylated by incubation with biotin maleimide and the biotinylated probe was purified by reverse phase HPLC [probe 2]. 3'-^{32}P labelled probe 2 was hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin using standard conditions. Electrophoretic fractionation of the resulting hybrids showed specific hybridization to the histone quintets, however, the level of non-specific binding to bulk chromatin was very high (data not shown). The reason for this was not known, but it was thought that the spacer arm causes this phenomenon, as probes lacking the spacer arm do not show this non-specific binding.

Alternatively, the oligonucleotide probe could be biotinylated on the 3' end. The 18 mer oligonucleotide probe was biotinylated by brief incubation with terminal transferase and Bio-11-dUTP, resulting in the addition of an average of 4 biotinyl residues per 3' end [probe 3] (see methods and materials) (Fig 8A). Streptavidin binding was demonstrated by incubating probe 3 with an excess of streptavidin and biotinylated liposomes (Fig 8B, lane 4). Hybridization of probe 3 to [BamHI-T7 gene 6 exonuclease] digested chromatin was demonstrated by the formation of specific hybrids with the histone gene quintets (no non-specific hybridization was observed) (Fig 9, lane 1). Incubation of the deproteinized chromatin hybrids with a variety of streptavidin or avidin matrices, however, failed to

Figure 8. 3'-Biotinylation of the oligonucleotide

A) 50 pmol oligonucleotide was 5' end labelled by incubating with γ^{32}P-ATP and T4 polynucleotide kinase and desalted by centrifugation through a G25 Sephadex spin column equilibrated in 1x terminal transferase buffer (Current Protocols). Bio-11-dUTP (final concentration 0.1 mM) together with terminal transferase (1 unit per 1 pmol oligonucleotide) were added and tailing was allowed to proceed at 37° C for 1.5 min. The tailing reaction was stopped with EDTA and the poly-biotinylated oligonucleotide was desalted through G25 sephadex equilibrated in STE pH 8.0 containing 0.1 % Sarkosyl and precipitated with the addition of LiCl (1 M) and 6 volumes ethanol/acetone (1:3) The degree of tailing was determined by electrophoretically fractionating aliquots of both the biotinylated and non biotinylated oligonucleotide in 15 % non-denatureing polyacrylamide (1x TBE pH 8.3) followed by autoradiography.

Lane 1. 5'-^{32}P labelled oligonucleotide.
Lane 2. 3'-poly-biotinylated oligonucleotide (5'-^{32}P labelled) (probe 3).



B) Accessibility of the biotinyl residues was determined by incubating the 3'-poly-biotinylated oligonucleotide (5'-^{32}P labelled) (probe 3) with biotinyl liposomes or streptavidin and biotinyl liposomes. The resulting complexes were fractionated as described in part A.

Lane 1 and 2. 5'-^{32}P labelled oligonucleotide.
Lane 3. 3'-poly-biotinylated oligonucleotide (probe 3; 1 pmol) was incubated with an excess of biotinylated liposomes.
Lane 4. 3'-poly-biotinylated oligonucleotide (probe 3) was pre-incubated with streptavidin (5 μg) for 1 hour at 24° C followed by the addition of biotinylated liposomes.

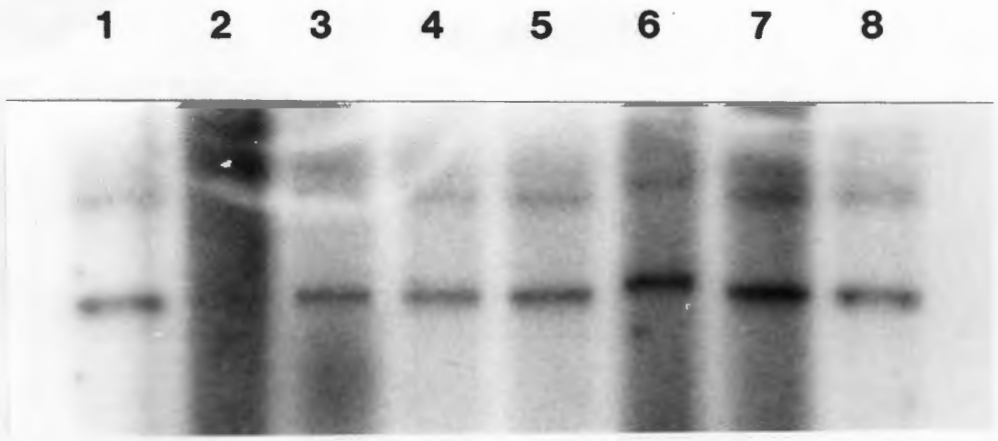


Figure 9. Binding of the hybrids formed between probe 3 and [BamHI-T7 gene 6 exonuclease] digested sea urchin embryo chromatin to streptavidin or avidin matrices.

[BamHI-T7 gene 6 exonuclease] digested chromatin was prepared as previously described and dialysed into STE pH 8.0. 5'-^{32}P labelled probe 3 (4 pmol) was added to 25 µg solubilized chromatin and incubated at 37° C for 24 hours. The hybrids were then deproteinized, ethanol precipitated and redissolved in STE pH 8.0 at a concentration of 0.125 µg DNA/µl. 25 µl aliquots were incubated with a variety of avidin or streptavidin matrices at 4° C for 2 hours. The total binding reactions were then electrophoretically fractionated in 0.7 % agarose and hybrids were visualized by autoradiography.

Lane 1. Probe 3 hybridized to the histone quintet fragments.
Lane 2. Hybrids incubated with avidin-fragmented Sephadex G15.
Lane 3. Hybrids incubated with streptavidin-Sepharose 4B.
Lane 4. Hybrids incubated with avidin-AcA 54.
Lane 5. Hybrids incubated with avidin-Avicell cellulose.
Lane 6. Hybrids incubated with avidin-aminocaproyl-microsphers.
Lane 7. Hybrids incubated with avidin-microspheres.
Lane 8. As per lane 1.

show any specific retention suggesting that the biotinyl residues were inaccessible to the solid phase streptavidin or avidin (Fig 9, lanes 2 to 7).

In an attempt to increase the accessibility of the biotinyl residues the 5' end of the H2B gene was linearly amplified with the simultaneous incorporation of Bio-11 dUMP [probe 4] (see methods and materials; see also Fig 1 for amplified region). The aim being that 50 % of the biotinyl residues would be positioned within the 5' domain of the probe and thereby would be more accessible for binding by immobilized streptavidin. Simultaneously the matrix arm was also extended with the incorporation of a hexaglycine spacer.

Probe 4 biotinylation was demonstrated by specific electrophoretic retardation in the presence of streptavidin and specific retention on streptavidin-hexaglycyl-microspheres (Fig 10). Hybridization of probe 4 to [BamHI-T7 gene 6 exonuclease] digested chromatin and deproteinized chromatin, showed the formation of hybrids (Fig 11A and 11B, lanes 1). The observed strength of the hybridization signal suggested that the efficiency of hybridization was higher than that observed for the oligonucleotide probe (probe 3). This may be directly related to the length of probe 4, thereby increasing the number of possible nucleation events.

Incubation of these hybrids with streptavidin-hexaglycyl-microspheres demonstrated their complete and specific retention (Fig 11A and 11B, lanes 2 and 3). Thus, suggesting that the biotinyl residues were accessible when positioned towards the 5' end of the probe. Digestion of the retained hybrids with P1 nuclease, however, failed to show any significant release (Fig 11C). This could be attributed to the absence of an accessible single stranded domain as a function of the probe length, if the streptavidins were bound to the biotinyl residues within the hybridizing domain. Because 50 % of the biotinyl residues are positioned upstream of the hybridizing domain, which was in a single stranded conformation, partial release should have been observed. Due to the absence of release, other inhibiting factors may have been involved. Non-specific binding was not suspected at the time because the addition of excess biotin fully quenched the retention of the chromatin hybrids.

To circumvent the above observations it was felt that the probe design should again be changed. 5' Biotinylation had been shown to facilitate optimal streptavidin, as the biotinyl residues were positioned away from the chromatin protein complexes, thereby

Figure 10. Assay to determine biotinylation of probe 4.

Probe 4 was synthesized and 5'-^{32}P labelled as described in methods and material. Aliquots (approximately 1 pmol) were incubated with streptavidin (5 μg) or streptavidin-hexaglycyl-microspheres (30 μl) with or without pre-quenching with excess biotin, at 24° C for 2 hours. The total binding mixtures were then electrophoretically fractionated in 9 % non-denaturing polyacrylamide (1x TBE pH 8.3) and probe 4 was visualized by autoradiography.

Lane 1. Probe 4.
Lane 2. Probe 4 incubated with streptavidin.
Lane 3. Probe 4 incubated with streptavidin pre-quenched with excess biotin.
Lane 4. Probe 4 incubated with streptavidin-hexaglycyl-microspheres.
Lane 5. Probe 4 incubated with streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.

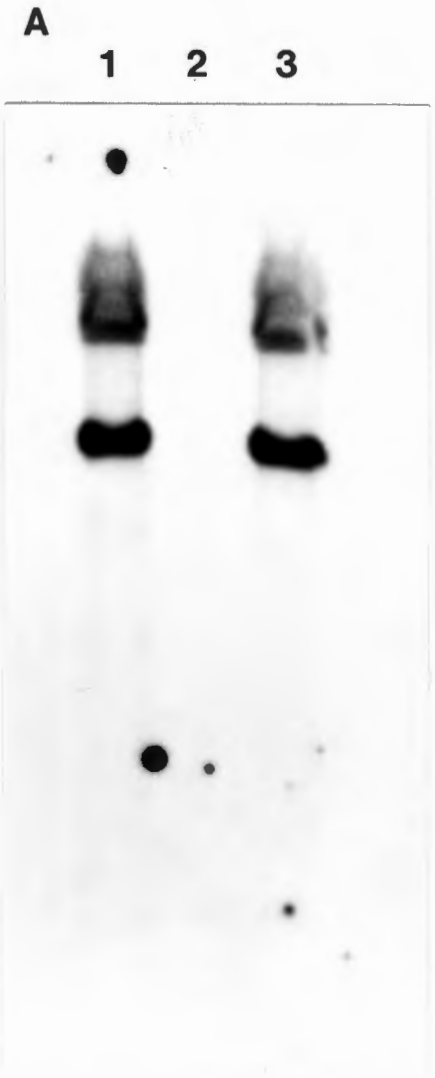
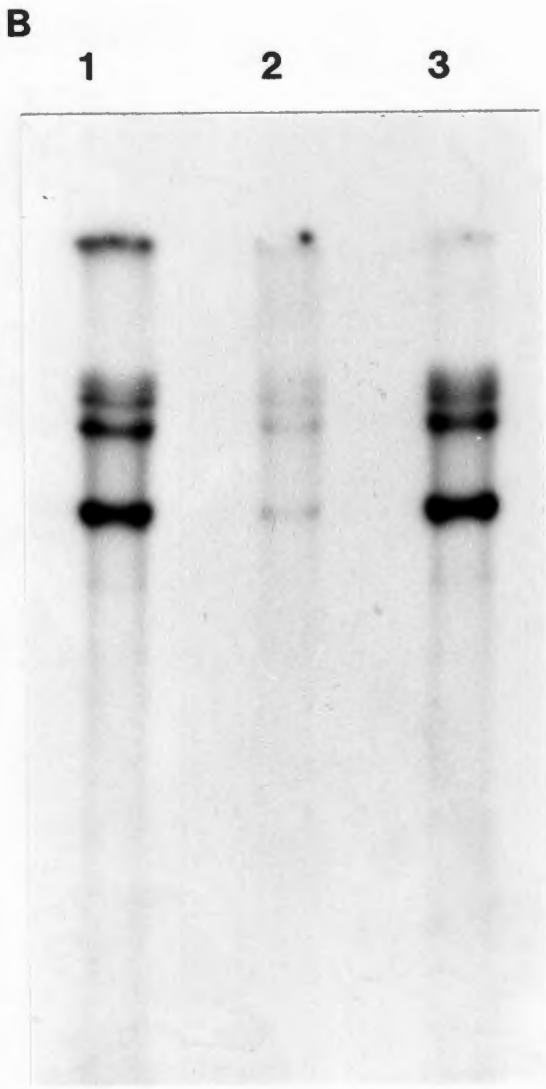


Figure 11. Hybridization of probe 4 to [BamHI-T7 gene 6 exonuclease] digested sea urchin chromatin or DNA and retention of hybrids on streptavidin or avidin matrices.

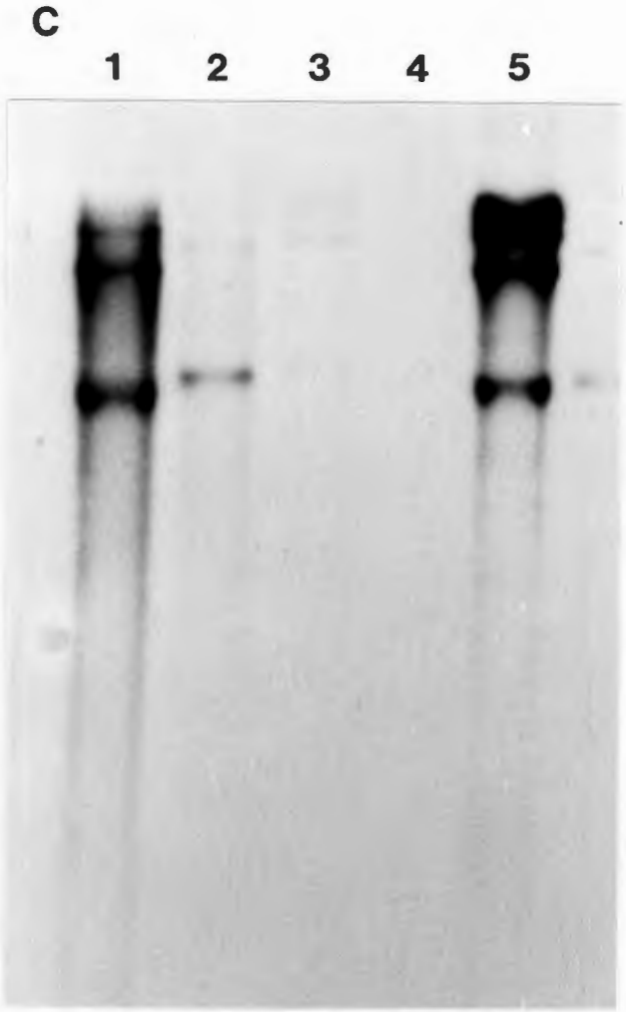
A) [BamHI-T7 gene 6 exonuclease] digested DNA was prepared as described previously. ^{32}P labelled Probe 4 (approximately 0.3 pmol) was hybridized to the [BamHI-T7 gene exonuclease] digested DNA (approximately 5 μg) by incubating the mixture at 37° C for 24 hours. The resulting hybrids were then incubated with avidin-hexaglycyl-microspheres (with or without pre-quenching of the biotin binding), for 2 hours at 24° C. The matrices were pelleted by gentle centrifugation and the supernatants were fractionated by electrophoresis in 0.7 % agarose. The gel was dried and the hybrids were visualized by autoradiography.

Lane 1. The hybrids formed between probe 4 and [BamHI-T7 gene 6 exonuclease] digested DNA.
Lane 2. Retention of the hybrids on avidin-hexaglycyl-microspheres.
Lane 3. Retention of the hybrids on avidin-hexaglycyl-microspheres pre-quenched with excess biotin.



B) [BamHI-T7 gene 6 exonuclease] digested chromatin was prepared as described previously and hybridized to ^{32}P labelled probe 4 (ratio 0.3 pmol probe 4 per 5 µg chromatin) by incubating at 37° C for 24 hours. The hybrid were the incubated with streptavidin-hexaglycyl-microspheres (with or without pre-quenching with excess biotin). The matrices were pelleted by gentle centrifugation and the supernatants were retained. The chromatin was then deproteinized by phenol extraction followed by ethanol precipitation. The deproteinized hybrids were then fractionated and visualized as in figure 10A.

Lane 1. The hybrids formed between probe 4 and [BamHI-T7 gene 6 exonuclease] digested chromatin.
Lane 2. Retention of the chromatin hybrids on streptavidin-hexaglycyl-microspheres.
Lane 3. Retention of the chromatin hybrids on streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.



C) 5'-^{32}P labelled probe 4 was hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin as in part B. Aliquots of the hybridization mixtures were incubated with streptavidin-hexaglycyl-microspheres (with or without pre-incubation with excess biotin) at 24° C for 2 hours. The microsphere were then pelleted by centrifugation, the supernatants were collected (unbound fraction) and the pelleted matrices were then resuspended in 100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 5 mM $MgCl_2$ and incubated with 3 units P1 nuclease at 37° C for 30 min. Digestion was stopped with the addition of EDTA (final concentration of 7.5 mM), the microspheres were then pelleted and supernatants were again retained (P1 released fraction). Both supernatants were deproteinized, ethanol precipitated and redissolved in STE pH 8.0 and fractionated by electrophoresis in 0.7 % agarose. The DNA was Southern transferred to Hybond N and the histone quintets were visualized by hybridization with ^{32}P labelled pPA(HQ) followed by autoradiography.

Lane 1. [BamHI-T7 gene 6 exonuclease] digested chromatin.
Lane 2. Hybrids released by P1 nuclease digestion from the streptavidin-hexaglycyl-microspheres.
Lane 3. Hybrids released without P1 nuclease digestion from the streptavidin-hexaglycyl-microspheres.
Lane 4. Hybrids released by P1 nuclease digestion from the streptavidin-hexaglycyl-microspheres (pre-quenched with excess biotin).
Lane 5. Chromatin not bound to streptavidin-hexaglycyl-microspheres.

increasing the accessibility. By increasing the length of the 5' overhang and thereby the P1 nuclease substrate, it was proposed that specific release would be obtained. To test this hypothesis a biotinylated oligonucleotide was designed and purchased (computer analysis showed that no stable secondary structure would be formed (GCG squiggles analysis)). This oligonucleotide [probe 5] (Fig 12) contained three functional regions. 1) Hybridizing region of 39 bases complementary to the 5' end of the H2B gene. 2) Non-hybridizing region positioned on the 5' side of the hybridizing region that would remain single stranded and thus be a substrate for P1 nuclease digestion. 3) Biotinylated region positioned 5' to the non-hybridizing region containing 4 biotinylated amino modified thymidine nucleotides. Biotinylation was demonstrated by specific retention on streptavidin-hexagylcyl-microspheres (Fig 13).

Hybridization of 5'-32P labelled probe 5 to deproteinized [BamHI-T7 gene 6 exonuclease] digested chromatin showed the formation of specific hybrids with the histone quintets (Fig 14, lane 1). These hybrids were specifically retained on streptavidin-hexaglycyl-microspheres (Fig 14, lane 2) and the binding was biotin dependent as pre-incubation with excess biotin totally quenched the retention (Fig 14, lane 3). Hybridization analysis of the unbound DNA, fractionated by agarose gel electrophoresis, showed that approximately 50 % of the histone quintet DNA was retained (Fig 15, compare lanes 1 and 2). Digestion of the matrix bound hybrids with P1 nuclease demonstrated that the hybridized histone quintet DNA was specifically released (Fig 15, lane 4). Comparison of the ethidium bromide stained DNA (Fig 15B) and the hybridization signals clearly demonstrated that the histone gene quintet DNA was specifically enriched. Furthermore, no non-specific matrix retention of the histone quintet DNA could be shown after digestion with P1 nuclease by incubation with SDS and Proteinase K (data not shown).

Having demonstrated the application of this methodology to enrich the histone quintet DNA it was felt that the histone quintet chromatin could be enriched using the same technique.

Hybridization of probe 5 to [BamHI-T7 gene 6 exonuclease] digested chromatin showed that specific hybrids were formed (Fig 16, lane 1). Incubation of the biotinylated chromatin hybrids with streptavidin-hexaglycyl-microspheres demonstrated their near complete retention (Fig 16, lane 2), and the specificity of retention was demonstrated be pre-quenching the streptavidin binding activity with an excess of biotin (Fig 16, lane 3).

```
                10              20              30
5' CXC CXA CXG GXC AAG TCG CTA AGA AAG GAT

                40              50              60
   CCA AGA AAG CAG TGA AGG CAC CTC GCC CTA

                70
   GCG GTG GCA AG 3'
```

Figure 12. Nucleotide sequence of probe 5.

1) Biotinylated domain (1-10), X indicates the sites of biotinylation (Bio-16-dT).

2) Single stranded domain (11-31).

3) Hybridizing domain (32-71), complimentary to the 5' end of the H2B gene.

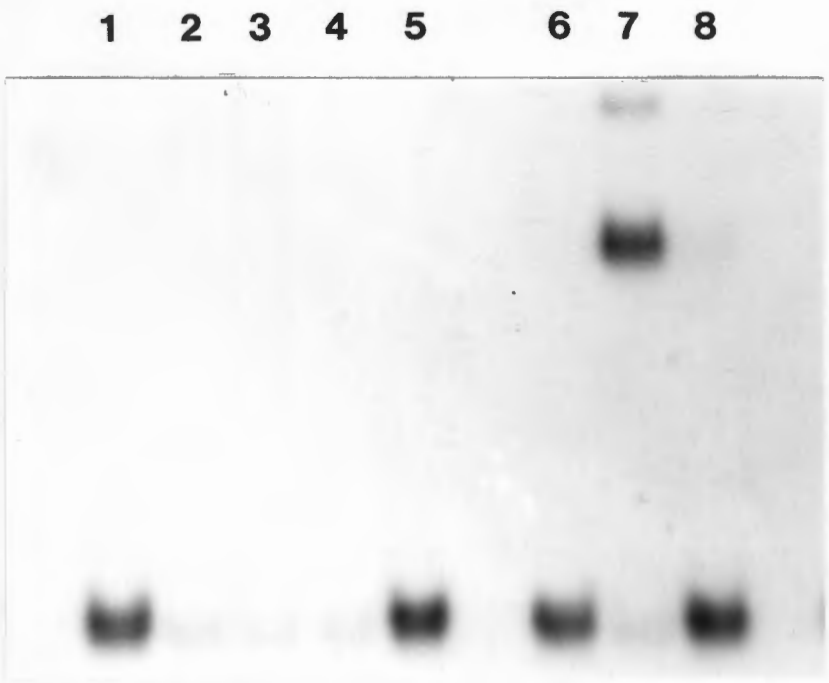


Figure 13. Titration of probe 5 with increasing volumes of streptavidin-hexaglycyl-microspheres.

5'-^{32}P labelled probe 5 (1 pmol) was incubated with increasing volumes of streptavidin-hexaglycyl-microspheres (ranging from 0 to 20 µl) at 24° C for 2 hours. The matrices were pelleted by centrifugation (8000 xg for 1 min) and the supernatants were fractionated by electrophoresis in 10 % non-denaturing polyacrylamide.

Lane 1. Probe 5 (0.5 pmol).
Lane 2. Probe 5 (0.5 pmol) incubated with 5 µl streptavidin-hexaglycyl-microspheres.
Lane 3. Probe 5 (0.5 pmol) incubated with 10 µl streptavidin-hexaglycyl-microspheres.
Lane 4. Probe 5 (0.5 pmol) incubated with 20 µl streptavidin-hexaglycyl-microspheres.
Lane 5. Probe 5 (0.5 pmol) incubated with 20 µl streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.
Lane 6. Probe 5 (0.5 pmol)
Lane 7. Probe 5 (0.5 pmol) incubated with excess streptavidin.
Lane 8. Probe 5 (0.5 pmol) incubated with excess streptavidin pre-quenched with excess biotin.

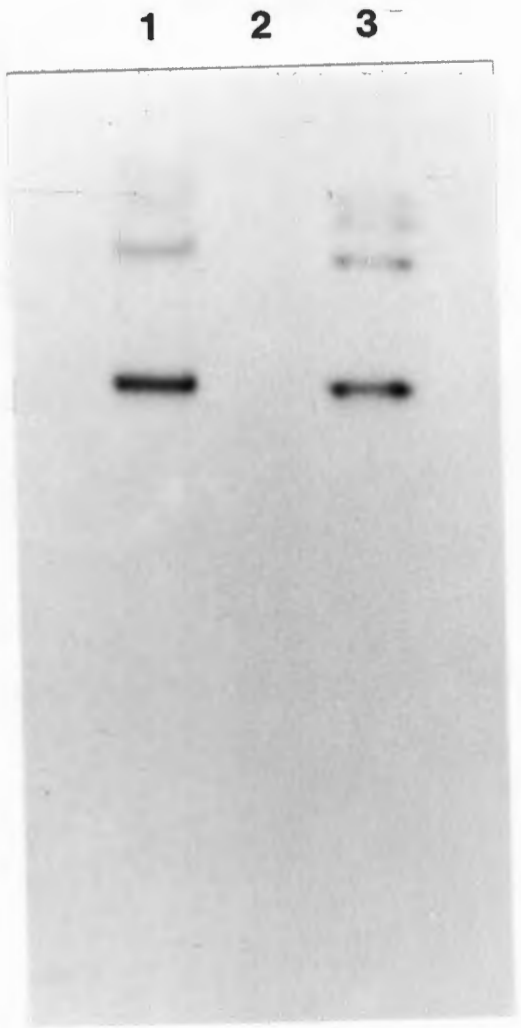


Figure 14. Hybridization of probe 5 to [BamHI-T7 gene 6 exonuclease] digested sea urchin embryo DNA and the binding of the resulting hybrids to streptavidin-hexaglycyl-microspheres.

[BamHI-T7 gene 6 exonuclease] digested sea urchin embryo DNA was prepared as described previously and redissolved in STE pH 8.0. 5'-^{32}P labelled probe 5 was added at a ratio of 0.06 pmol per µg DNA and hybridization was allowed to proceed at 37° C for 24 hours. Aliquots of the hybridization mixture (10 µg DNA) were then incubated with 60 µl aliquots of streptavidin-hexaglycyl-microspheres (with and without pre-quenching with excess biotin) at 24° C for 2 hours. The matrices were pelleted by centrifugation and the supernatants were fractionated by electrophoresis in 0.7 % agarose. The hybrids were visualized by autoradiography.

Lane 1. Probe 5 hybridized to [BamHI-T7 gene 6 exonuclease] digested DNA.
Lane 2. Retention of hybrids on 60 µl streptavidin-hexaglycyl-microspheres.
Lane 3. Retention of hybrids on 60 µl streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.

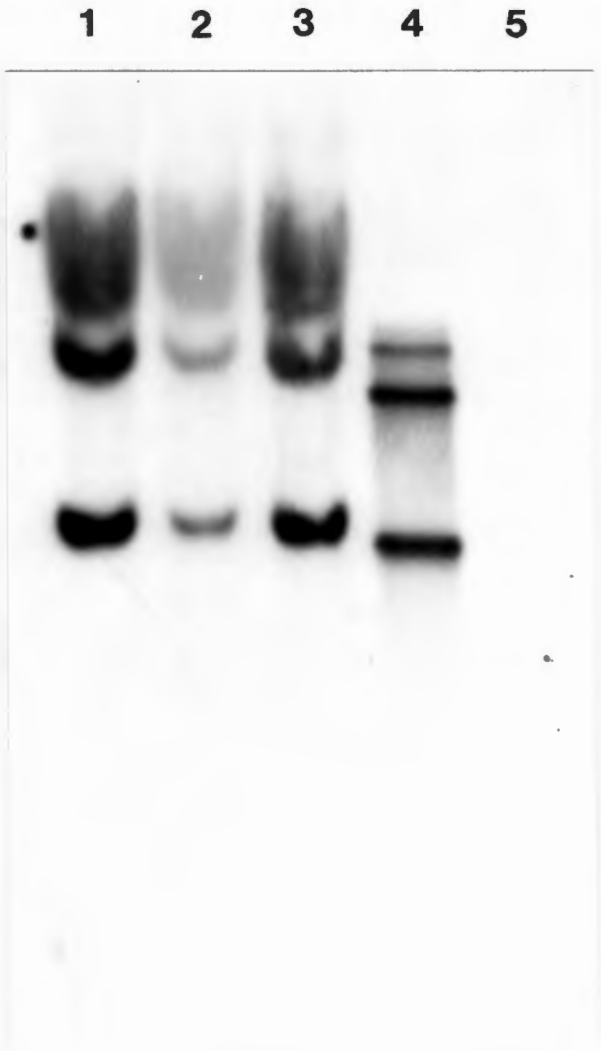
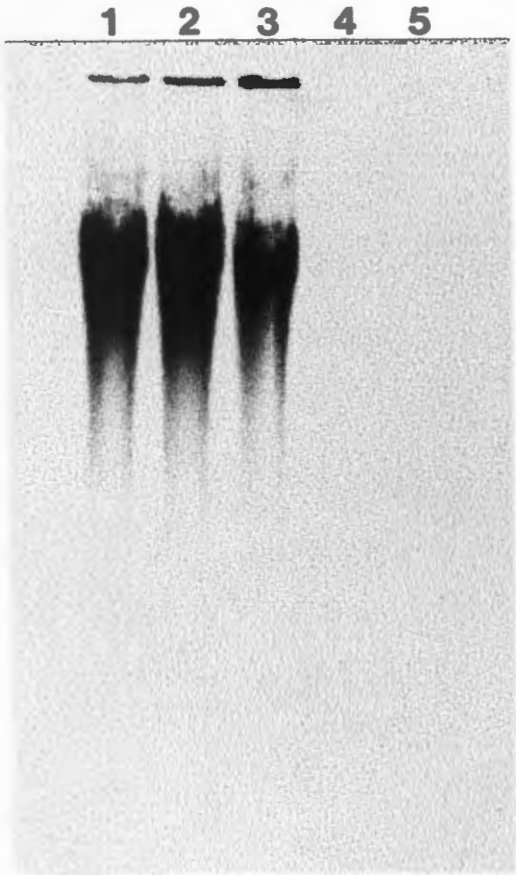


Figure 15. P1 nuclease directed release of the hybrid [probe 5-BamHI-T7 gene 6 exonuclease digested sea urchin embryo DNA] from streptavidin-hexaglycyl-microspheres.

[BamHI-T7 gene 6 exonuclease] digested sea urchin embryo DNA was prepared as described previously and redissolved in STE pH 8.0 at a concentration of 0.075 µg/µl. 5'-^{32}P labelled probe 5 (0.06 pmol per µg DNA) was added and hybridization was allowed to proceed at 37° C for 24 hours. Tween 20 and BSA were then added to the hybridization mixture to give a final concentration of 0.05 % and 250 µg/ml, respectively. Streptavidin-hexaglycyl-microspheres (60 µl per 15 µg DNA), pre-incubated in the above blocking buffer (with or without excess biotin, were then added followed by a further incubation at 24° C for 2 hours. The matrices were pelleted by gentle centrifugation and the supernatants were retained (unbound fraction). The matrices were resuspended in 200 µl P1 nuclease buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 5 mM $MgCl_2$), 3 units of P1 nuclease was added followed by incubation at 37° for 30 min. The matrices were again pelleted and the supernatants were retained (P1

released). All the fractions were treated with Proteinase K, phenol/chloroform/isoamyl alcohol extracted and ethanol precipitated. The DNA from the respective fractions were fractionated by electrophoresis in 0.7 % agarose, Southern transferred to Hybond N and the histone gene quintets were visualized by autoradiography after hybridization with ^{32}P labelled pPA(HQ).

Lane 1. [BamHI-T7 gene 6 exonuclease] digested DNA.
Lane 2. Unbound [BamHI-T7 gene 6 exonuclease] digested DNA released from streptavidin-hexaglycyl-microspheres.
Lane 3. Unbound [BamHI-T7 gene 6 exonuclease] digested DNA released from streptavidin-hexaglycyl-microspheres (pre-quenched with excess biotin).
Lane 4. Hybrids released by P1 nuclease digestion from the streptavidin-hexaglycyl-microspheres.
Lane 5. Hybrids released by P1 nuclease digestion from streptavidin-hexaglycyl-microspheres (pre-quenched with excess biotin).



B) DNA (as in part A), visualized by ethidium bromide straining prior to southern transfer.

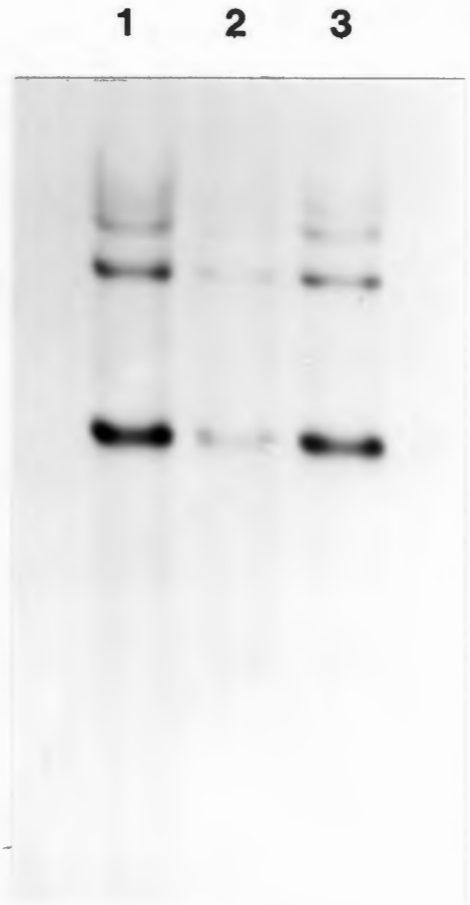


Figure 16. Hybridization of probe 5 to [BamHI-T7 gene 6 exonuclease] digested sea urchin embryo chromatin and the binding of the resulting hybrids to streptavidin-hexaglycyl-microspheres.

BamHI-T7 gene 6 exonuclease digested chromatin was prepared as described previously. 5'-^{32}P labelled probe 5 (0.06 pmol per μg chromatin) was added and hybridization was allowed to proceed at 37° C for 24 hours. Tween 20 and BSA were then added to the hybridization mixture to give a final concentration of 0.1 % and 250 μg/ml, respectively. Aliquots (18 μg chromatin) were then incubated for 2 hours at 24° C with streptavidin-hexaglycyl-microspheres (80 μl) equilibrated in detergent/BSA blocking buffer (with or without pre-quenching with excess biotin). The matrices were pelleted by gentle centrifugation and the supernatants were retained. SDS was added to each supernatant to a final concentration of 0.25 % to displace chromosomal proteins and the hybrids with fractionated by electrophoresis in 0.7 % agarose containing 0.25 % SDS. The hybrids were visualized by autoradiography of the dried gel.

Lane 1. Probe 5 hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin.
Lane 2. Retention of hybrids on streptavidin-hexaglycyl-microspheres.
Lane 3. Retention of hybrids on streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.

Hybridization analysis of the unbound chromatin fraction demonstrated that approximately 50 % of the histone gene quintet chromatin was retained (Fig 17, compare lanes 1 and 4), which was similar to that observed for DNA (see above). Thus, the chromosomal proteins had little or no effect on the efficiency of hybridization or matrix retention. However, it was uncertain as to why the efficiency of hybridization for both DNA and chromatin, was only in the order of 50 %, as 1000 fold excess probe 5 was added. Increasing the probe ratio failed to increase the hybridization efficiency, thus it was concluded that the stringency of hybridization was the controlling factor (data not shown).

Digestion of the matrix bound chromatin hybrids with P1 nuclease showed only a partial release (Fig 17, lane 2) (reproducibility of the P1 nuclease release was highly variable ranging from no release to partial release). This differed from the results obtained for DNA and would suggest that the chromatin proteins directly inhibited P1 nuclease catalysed release. This inhibition may be due to a number of factors: 1) Binding of nuclear proteins to the single stranded probe domain may inhibit P1 nuclease activity. 2) Cation induced chromosomal condensation may inhibit the accessibility of the single stranded domain. 3) Non-specific binding of the histone quintet chromatin to the solid phase via the associated proteins may prevent the specific release catalysed by P1 nuclease digestion, even though non-specific binding could not be demonstrated in the presence of excess biotin.

The binding of nuclear protein to the single stranded probe would not only influence the digestibility but would also inhibit matrix retention. Because the efficiency of retention is similar to that obtained with DNA it is proposed that this would not be an inhibiting factor.

The ionic conditions during P1 nuclease digestion may enhance chromatin condensation. To minimize this effect the magnesium concentration was decreased to 0.25 mM, however, the yield remained constantly low. Lowering of the NaCl concentration (<100 mM) appeared to inhibit the release, which may be a function of enhancing non-specific binding, due to the reduced charge neutralization.

Thus, it would appear that non-specific binding of the hybrids to the solid phase was the major inhibiting factor preventing the release via P1 nuclease digestion. To demonstrate this, the matrix bound chromatin hybrids were digested with P1 nuclease, followed by

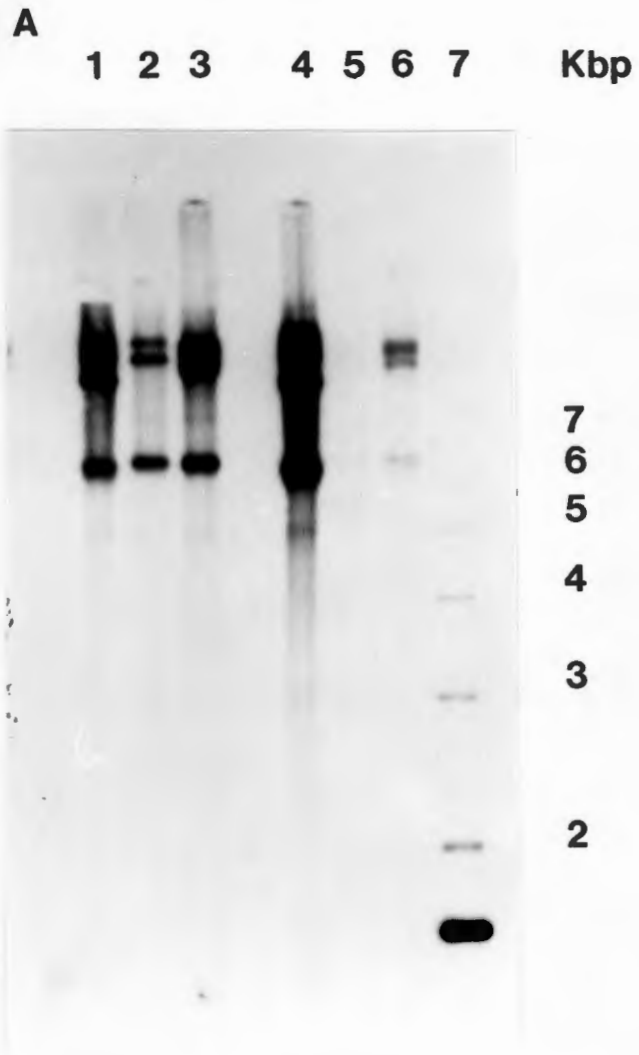
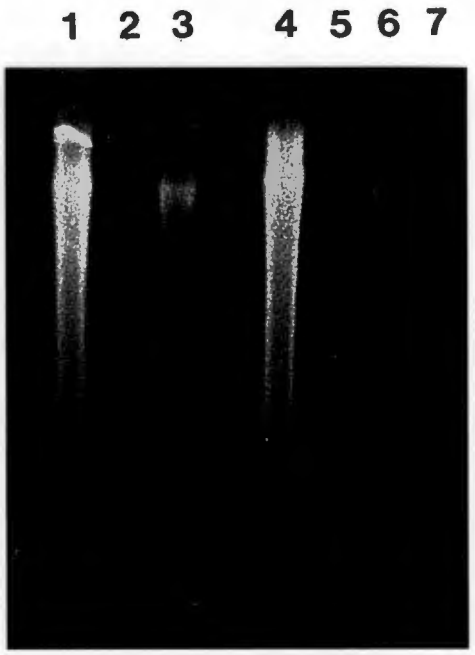


Figure 17. P1 nuclease directed release of the hybrid [probe 5-BamHI-T7 gene 6 exonuclease digested sea urchin embryo chromatin] from streptavidin-hexaglycyl-microspheres.

A) 5'-^{32}P labelled probe 5 was hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin as described in figure 16. Tween 20, BSA and glycerol were then added to the hybridization mixture to give a final concentration of 0.25 %, 250 µg/ml and 5 %, respectively. Aliquots of the hybridization mixtures were then incubated for 2 hours at 24° C with streptavidin-hexaglycyl-microspheres (120 µl microspheres per 30 µg chromatin; with or without pre-quenching with excess biotin), equilibrated in the above blocking buffer. The matrices were then pelleted by gentle centrifugation and the supernatants retained (unbound fraction). The matrices were then resuspended in 200 µl P1 nuclease buffer (0.25 % Tween, 5 % glycerol, 250 µg/ml BSA, 0.25 mM $MgCl_2$, 100 mM NaCl and 10 mM Tris-HCl pH 7.5) and the hybrids were digested with 3 units P1 nuclease at 37° C for 45 min. The matrices were again gently pelleted and the supernatants retained (P1 released fraction). All fractions were then Proteinase K

digested (including the matrices), phenol/chloroform extracted and ethanol precipitated. The DNA was then fractionated by electrophoresis in 0.7 % agarose, Southern transferred to Hybond N+ and the histone gene quintets were visualized by autoradiography after hybridization with ^{32}P labelled pPA(HQ).

Lane 1. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin.
Lane 2. Hybrids release by P1 nuclease digestion from streptavidin-hexaglycyl-microspheres.
Lane 3. Non-specifically bound hybrids released from streptavidin-hexaglycyl-microspheres by Proteinase K digestion.
Lane 4. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin released from streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.
Lane 5. P1 nuclease released hybrids from streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.
Lane 6. Non-specifically bound chromatin released by Proteinase K digestion from streptavidin-hexaglycyl-microspheres pre-quenched with excess biotin.
Lane 7. DNA marker X (Boehringer Mannheim).

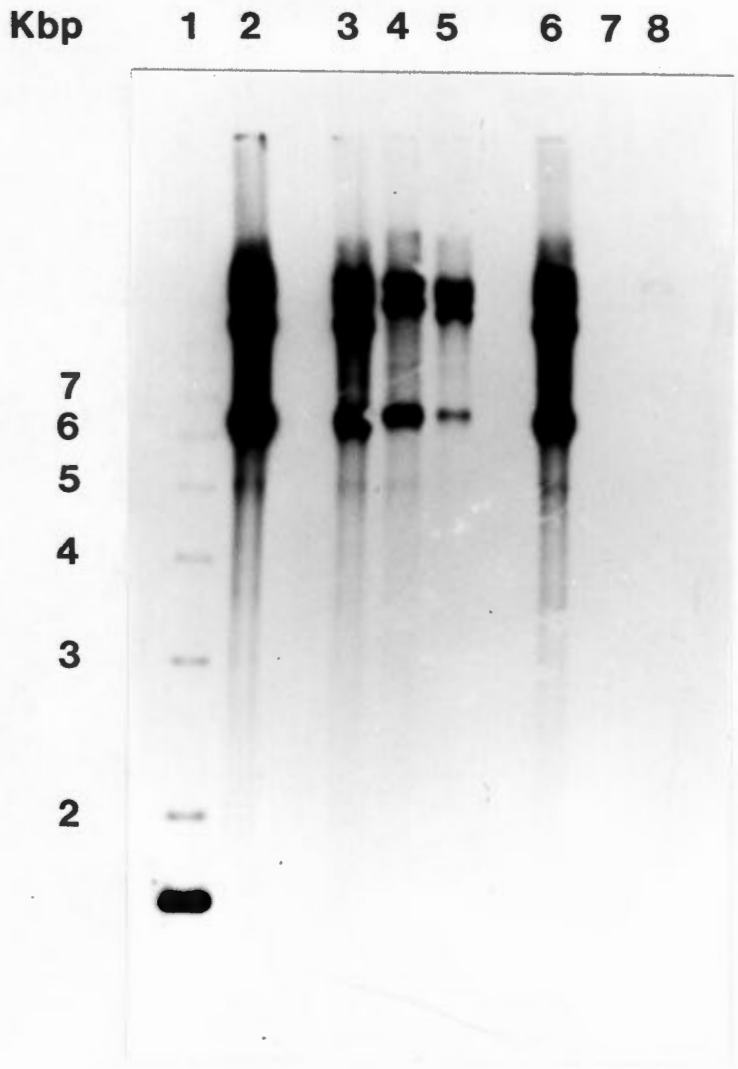


B) DNA (as in part A), visualized by ethidium bromide staining prior to southern transfer.

gentle centrifugation. The unreleased matrix hybrids were then incubated with 0.5 % SDS and Proteinase K (100 μg/ml) for 2 hours, followed by gentle centrifugation. Hybridization analysis of this fraction showed that the histone gene quintet chromatin was released under these conditions (shown by an enrichment when compared to matrices pre-quenched with biotin) (Fig 17A, compare lanes 3 and 6). This non-specific release was not due to the digestion ofstreptavidin, as the 5'-^{32}P label originating from probe 5 remained bound to the matrix (the 5' terminal nucleotide can not be cleaved from the biotinyl nucleotide when complexed with streptavidin due to sterical hindrance). Thus, it was concluded that the P1 nuclease had digested the single stranded domain of probe 5, but the non-hybridizing domain of the histone gene quintet chromatin remained bound to the matrix via non-specific interactions. These interaction could only be reversed by partial protein digestion and the denaturing effect of SDS.

The reason for the observed preferential non-specific binding of the histone gene quintet chromatin is uncertain, particularly as these chromatin fragments only constitute a minor fraction the input chromatin. The high affinity of streptavidin for biotin may be the controlling factor, as the hybrids would be preferentially positioned onto the matrix surface before the non-specific binding was quenched by the bulk of the chromatin. Because non-specific binding was quenched in the presence of excess biotin it must be assumed that the matrix bound unhybridized probe was responsible for this binding rather than the matrix composition. In an attempt to quench the non-specific binding, the streptavidin-hexaglycyl-microspheres were pre-incubated with BamHI solubilized chromatin or mono nucleosomes. However, no improvement of the hybrid release catalysed by P1 nuclease could be demonstrated (data not shown), which may indicate that the hybrids positioned on the matrix surface displaced the non-specifically bound competitor chromatin and thereby became preferentially bound.

Because the non-specific binding shown by the streptavidin-hexaglycyl-microspheres, could not be fully quenched, alternative streptavidin matrices were investigated. Initial screening involved the incubation of commercial matrices (pre-incubated with blocking agents) with BamHI solubilized chromatin to determine the degree of non-specific binding. Both the avidin-D beads (Vectrex) and the streptavidin magnetic beads (Promega) immediately caused clumping, suggesting a high degree of non-specific interactions and were therefore excluded from further investigation. Under the same conditions no visible clumping was observed with the addition of streptavidin magnetic beads from Advanced Magnetics and Dynal. These matrices were then assayed to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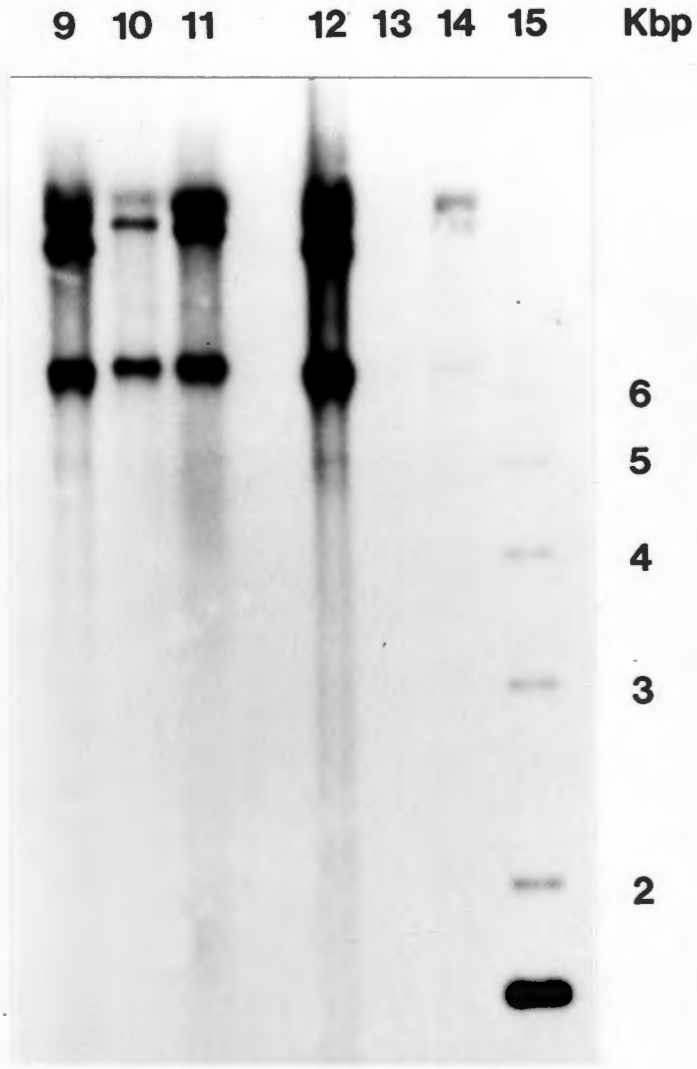
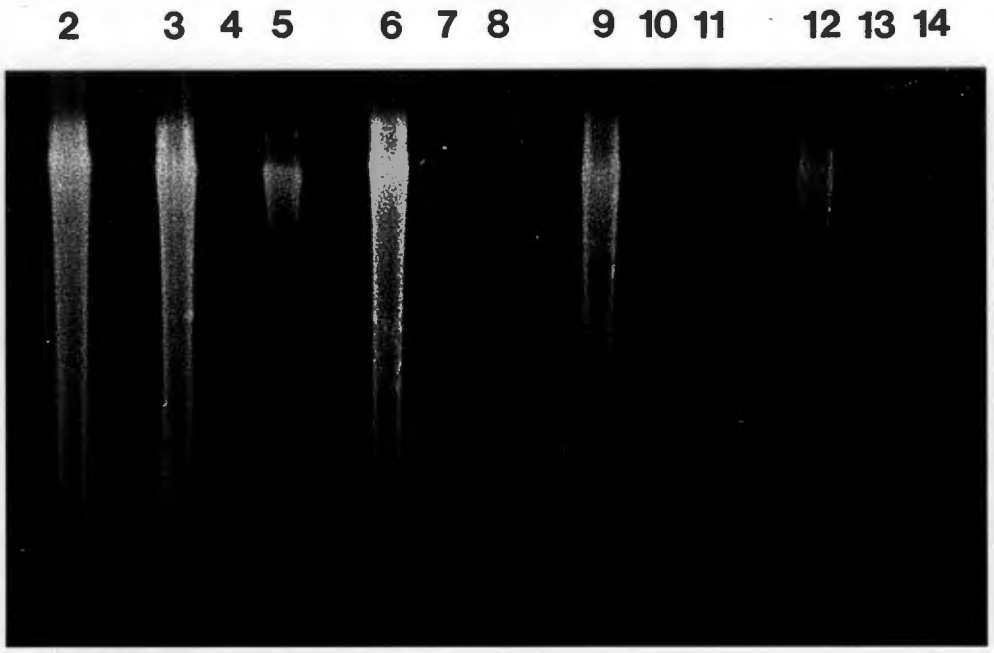


Figure 18. P1 nuclease directed release of the hybrid [probe 5-BamHI-T7 gene 6 exonuclease digested sea urchin embryo chromatin] from streptavidin-magnetic beads (from Advanced Magnetics and Dynal).

5'-^{32}P labelled probe 5 was hybridized to [BamHI-T7 gene 6 exonuclease] digested chromatin and incubated with blocking buffer as described in figure 17. Aliquots of the hybridization mixture were then incubated streptavidin-magnetic beads (Advanced Magnetics or Dynal) (80 µl matrix per 30 µg chromatin; with or without pre-quenching with excess biotin). The respective matrices were then washed, digested with P1 nuclease followed by Proteinase K digestion as described in figure 17.
The purified DNA was then fractionated by electrophoresis in 0.7 % agarose, Southern transferred to Hybond N+ and the histone quintets were visualized by autoradiography after hybridization with ^{32}P labelled pPA(HQ).

Lane 1. DNA marker X (Boehringer Mannheim).

Lane 2. [BamHI-T7 gene 6 exonuclease] digested chromatin incubated in the absence of a matrix.
Lane 3. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin (streptavidin-magnetic beads (Dynal)).
Lane 4. Hybrids released by P1 nuclease digestion from streptavidin-magnetic beads (Dynal).
Lane 5. Non-specifically bound hybrids released by Proteinase K digestion from streptavidin-magnetic beads (Dynal).
Lane 6. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin (streptavidin-magnetic beads (Dynal) pre-quenched with excess biotin).
Lane 7. Hybrids released by P1 nuclease digestion from streptavidin-magnetic beads (Dynal) pre-quenched with excess biotin.
Lane 8. Non-specifically bound hybrids released by Proteinase K digestion, from streptavidin-magnetic beads (Dynal) pre-quenched with excess biotin.
Lane 9. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin (streptavidin-magnetic beads (Advanced Magnetics)).
Lane 10. Hybrids released by P1 nuclease digestion, from streptavidin-magnetic beads (Advanced Magnetics).
Lane 11. Non-specifically bound hybrids released by Proteinase K digestion from streptavidin-magnetic beads (Advanced magnetics).
Lane 12. Unbound [BamHI-T7 gene 6 exonuclease] digested chromatin (streptavidin-magnetic beads (Advanced Magnetics) pre-quenched with excess biotin).
Lane 13. Hybrids released by P1 nuclease digestion from streptavidin-magnetic beads (Advanced Magnetics) pre-quenched with excess biotin.
Lane 14. Non-specifically bound hybrids released by Proteinase K digestion from streptavidin-magnetic beads (Advanced Magnetics) pre-quenched with excess biotin.
Lane 15. DNA marker X (Boehringer Mannheim).



B) DNA (as in part A), visualized by ethidium bromide staining prior to Southern transfer.

determine their respective enrichment efficiencies for isolation of the histone gene quintet chromatin fragments. The Advanced Magnetics matrix showed a similar degree of non-specific binding as was observed with the streptavidin-hexaglycyl-microspheres (Fig 18A, lane 11). This binding was only be reversed with the addition of SDS and Proteinase K. The Dynal matrix showed a very low degree of non-specific binding (Fig 18A, lane 5), thus, the histone gene quintet chromatin hybrids were preferentially solubilized when digested with P1 nuclease (Fig 18A, lane 4) (It remains unexplained as to why the Dynal matrix in combination with excess probe shows reduced non-specific binding). Comparison of the hybridization signal and the ethidium bromide stain of the DNA released, clearly demonstrates the specific enrichment of the histone gene quintet chromatin (compare Fig 18A and Fig 18B). Also this experiment demonstrates that chromatin specifically bound, can be specifically released according to the probe design by P1 nuclease. This novel release mechanism circumvents the requirement for the chemical modification of the chromatin prior to nucleoprotein hybridization, as described by Workman and Langmore (1985b), and Vincenz *et al.* (1991).

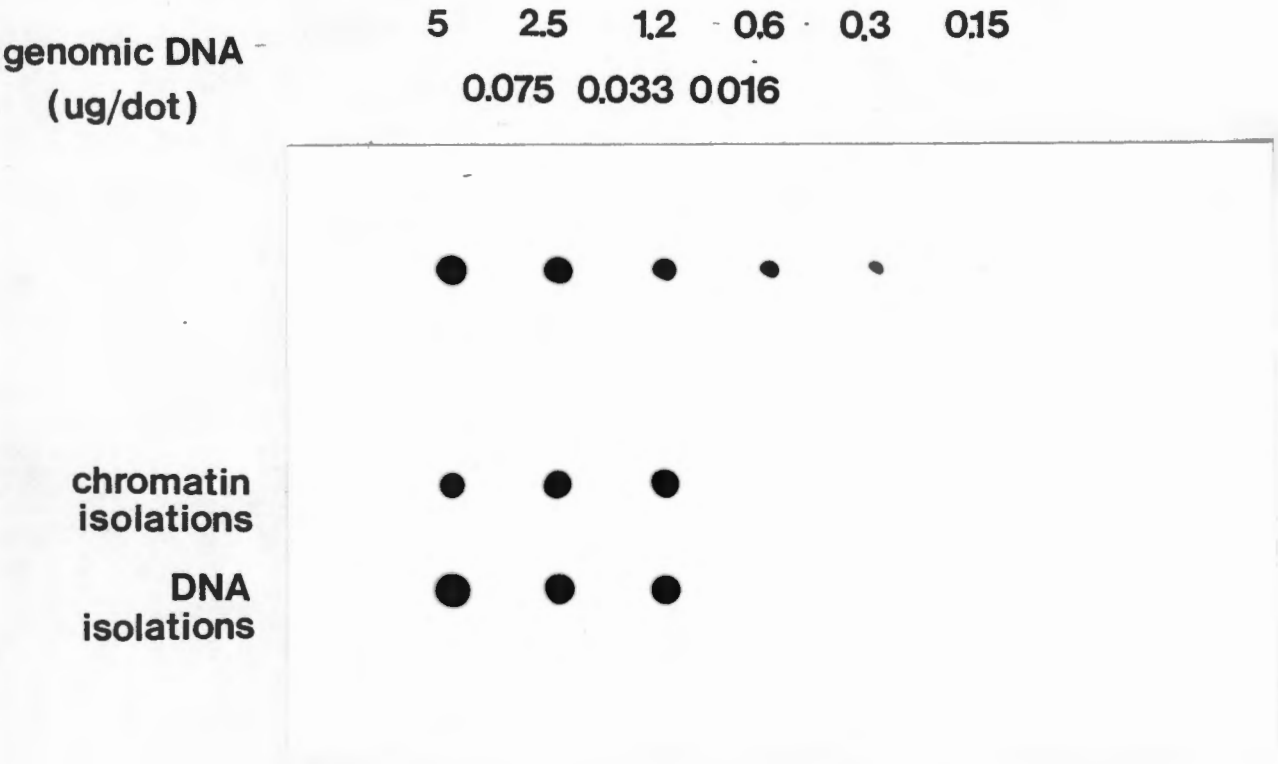
Dot blot hybridization was used to calculate an enrichment factor of the histone quintet chromatin or DNA released from streptavidin-magnetic beads (Dynal) by P1 nuclease digestion. The DNA from the P1 released fractions together with a series dilution of total sea urchin embryo genomic DNA were spotted onto a Hybond N+ membrane and hybridized with ^{32}P labelled pPA(HQ). Comparison of the respective hybridization signals shows that the content of histone quintet DNA in the P1 nuclease released fraction was equivalent to the content of histone gene quintets in approximately 5 μg total genomic DNA (Fig. 19A). This implies that approximately 25-40 ng of histone gene quintet was released by P1 nuclease digestion (calculated using a histone gene quintet content of 0.5-0.8 % for total genomic DNA, Birnsteil *et al.*, (1974)). Rehybridization of the stripped dot blot with ^{32}P labelled total sea urchin embryo genomic DNA shows that the DNA content of the P1 nuclease release fraction was approximately 40-80 ng (Fig 19B). The purity of the P1 nuclease released fraction can thus be calculated to be between 30 and 100 %, which represents a maximal enrichment of 200 fold. This result was similar to that reported by Vincenz *et al.* (1991), who demonstrated a that the histone gene quintet chromatin could be enriched to a purity of >80 %.

This methodology demonstrates, in principle, that the histone gene quintet chromatin can be specifically enriched. However, due to a number technicalities this methodology requires refinement to optimize reproducibility, enrichment and yield. The following

modifications may be suggested. 1) Due to the chromatin length, entanglement with the matrix was observed which results in a carry over of bulk chromatin thereby reducing the enrichment factor. This may be avoided by reducing the chromatin length by a second restriction endonuclease digestion, which inturn would require the addition of two probes complimentary to the 5' ends, but may enhance the accessibility of matrix binding. 2) Prior enrichment of the histone gene quintet chromatin hybrids by density gradient ultracentrifugation would remove the bulk chromatin and excess probe (Fig 20), thereby reducing the non-specific effects as described above. These methodological modification were not investigated for this study.

Bulk isolations of the histone gene quintet chromatin (with possible modifications as described above) can be envisaged to facilitate the characterization of the associated the chromosomal proteins. Characterization of these proteins and their modification may give an insight into their role in maintaining a transcriptionally active chromatin conformation, if the chromatin was isolated at a stage of embryonic development when these genes were actively expressed.

A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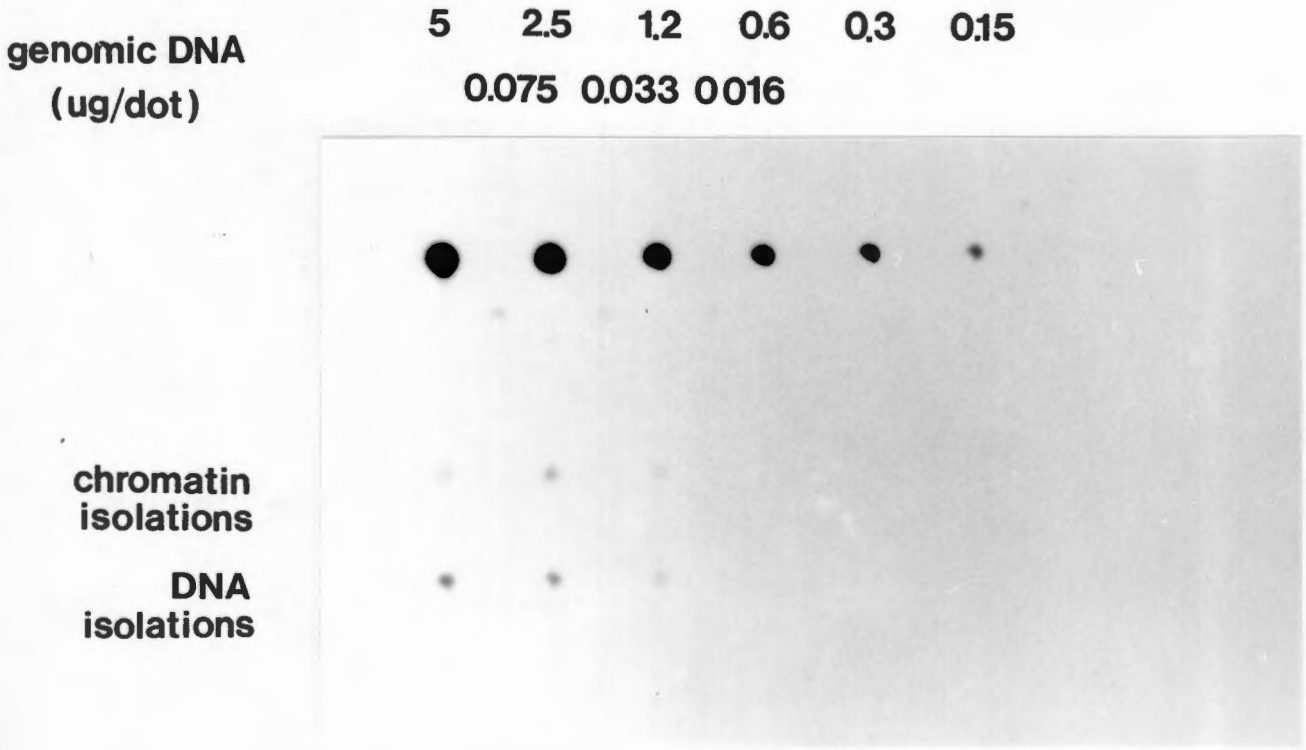
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Figure 19. Dot blot hybridization of P1 nuclease released hybrids to evaluate enrichment.

The hybrids formed between probe 5 and [BamHI-T7 gene 6 exonuclease] digested sea urchin embryo chromatin or DNA were released from streptavidin-magnetic beads (Dynal) as described in figure 18. The chromatin or DNA was then deproteinized by digestion with Proteinase K, phenol/chloroform extracted and ethanol precipitated. The DNA was redissolved in 100 µl 10x SSC pH 7.0, heat denatured at 100° C for 10 min and spotted onto Hybond N+ using a dot blot apparatus. (A) The DNA was fixed by alkaline treatment and the histone gene quintet content determined by hybridization with ^{32}P labelled pPA(HQ). (B) The membrane was then stripped by alkaline treatment and the total DNA content was determined by hybridization with ^{32}P labelled total sea urchin embryo DNA.

Row 1 and 2. Doubling dilution of total sea urchin embryo DNA ranging from 5 µg to 0.002 µg.
Row 3. Chromatin hybrids release by P1 nuclease digestion (three separate isolations).
Row 4. DNA hybrids release by P1 nuclease digestion (three separate isolations).

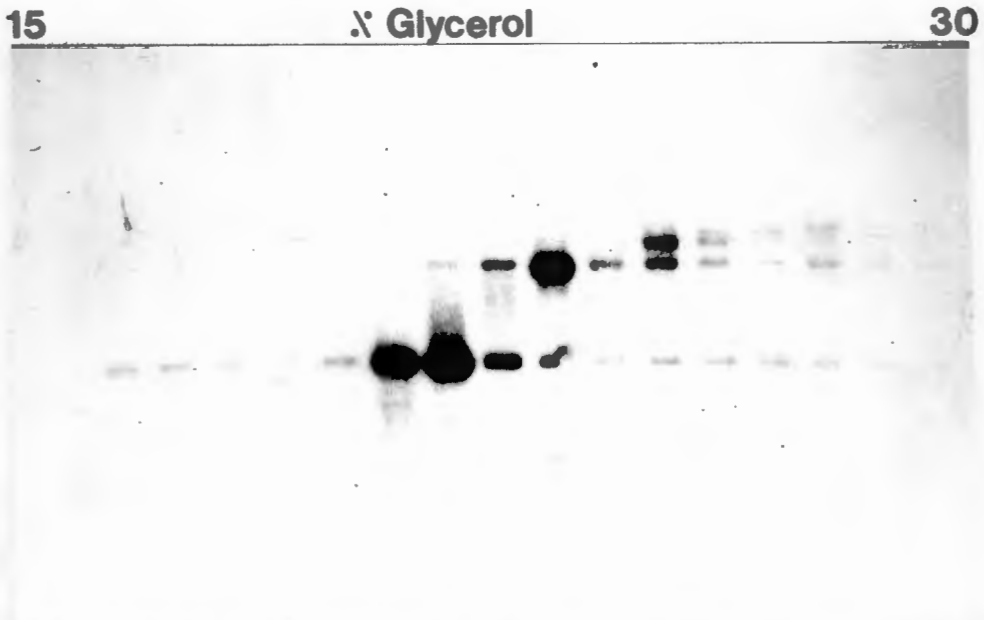


Figure 20. Enrichment of the histone gene quintet chromatin fragments by glycerol gradient ultracentrifugation.

BamHI solubilized sea urchin embryo chromatin was prepared as described in methods and materials. Aliquots of the soluble chromatin (approximately 40 µg) were overlayered on a 11 ml (SW40Ti) 15-30 % glycerol gradient containing TE pH 8.0 and 0.1 % PMSF, followed by centrifugation at 39000 rpm for 200 min at 4° C. The gradients were then fractionated by displacement into 0.6 ml fractions. The respective chromatin fractions were then deproteinized by digestion with Proteinase K, phenol/chloroform extraction followed by ethanol precipitation. The purified DNA was refractionated by electrophoresis in 0.7 % agarose and the histone gene quintet fragments were visualized by autoradiography after Southern transfer to Hybond N (Amersham) and hybridization with ^{32}P labelled pPA(HQ).

CHAPTER 4.

NASCENT RNA: A POTENTIAL TARGET FOR THE AFFINITY ISOLATION OF TRANSCRIPTION COMPLEXES.

INTRODUCTION:

The product of transcription is described as nascent RNA. This nascent RNA forms an integral component of the transcription complex, while transcription is proceeding, forming the ternary transcription complex [RNA-RNA polymerase-DNA]. Due to the specific association of the nascent RNA with the initiated RNA polymerase transcription complex, it could be suggested that the nascent RNA is directly linked to transcriptionally active chromatin. This unique characteristic could therefore be proposed as a target for the specific labelling of the transcription complex, thus enabelling the affinity purification of transcriptionally active chromatin.

The incorporation of affinity ligands into the nascent RNA would be a function of *in vitro* transcription in the presence of derivatized nucleotide triphosphates. However, due to the stringency of nucleotide triphosphate recognition, eucaryotic RNA polymerases only recognize derivatives with small functional groups. This was demonstrated by the fact that the biotinylated UTP (Bio-11-UTP) derivative described by Langer *et al.* (1981), which is a substrate for procaryotic transcription was not recognized by eucaryotic RNA polymerases. In contrast, the precursor to biotinylated UTP, allylamine-UTP (Langer *et al.*, 1981) has been shown to be incorporated into RNA by eucaryotic RNA polymerases (personal observation). However, this ligand would require a second derivatization step to incorporate an affinity ligand (i.e. post-transcriptional biotinylation with biotin-N-hydroxysuccinimide), making it unfavorable for nascent RNA labelling. The smaller nucleotide triphosphate derivative, Hg-UTP (Dale *et al.*, 1973), has also been shown to be incorporated in RNA by eucaryotic *in vitro* run off transcription (Beebee and Butterworth, 1976). This method of RNA labelling has facilitated the specific purification of the mercurated RNA transcripts by affinity chromatography on sulfhydryl-Sepharose. Thus, suggesting that Hg-UTP could label the transcriptionally active chromatin via the ternary transcription complex.

This chapter investigates the mercuration of the ternary transcription complexes generated by *in vitro* transcription and the affinity of these complexes towards immobilized sulfhydryl groups.

METHODS AND MATERIALS:

Synthesis of Hg-UTP.

(according to Dale *et al.* (1973) and (1975), with modifications).

Uridine triphosphate (UTP) (22 mg; 50 µmol) dissolved in 2 ml 100 mM sodium acetate pH 6.0, was added to mercuric acetate (63.5 mg; 200 µmol) dissolved in 2 ml 100 mM sodium acetate pH 6.0. The reaction mixture was incubated at 50° C for 3 hours and then allowed to cool to room temperature. The unincorporated Hg^{2+} ions were removed by exclusion chromatography on Sephadex G10 equilibrated in 20 mM Tris-HCl pH 7.0. The void volume peak was pooled and applied to DE52 cellulose (chloride form) and the unbound reactants were eluted by extensive washing with 20 mM Tris-HCl pH 7.0. The products were eluted with a linear gradient of LiCl (0-4.0 M). The fractions containing the Hg-UTP were identified by the characteristic absorbance spectrum, λ_{max}= 267. These fractions were pooled and the Hg-UTP was precipitated with the addition of 3 volumes of cold acetone followed by incubation at -20° C for 16 hours. The precipitate was collected by slow speed centrifugation and the solvent was removed under vacuum. The Hg-UTP was shown to be pure, as only a single spot was observed when resolved by thin layer chromatography on PEI-cellulose F_{254} plates, developed with 1.8 M LiCl (R_f = 0.1). Partial demercuration of the Hg-UTP was only observed when incubated with a large (10 fold) excess of ß-mercaptoethanol, in contrast Hg-CTP showed total demercuration under similar conditions, as was previously reported by Dale *et al.*, (1975).

Mercuration of the ternary transcription complex.

14 hour sea urchin embryo nuclei were digested with micrococcal nuclease (160 units/mg DNA) in nuclei storage buffer containing 1 mM $CaCl_2$ on ice for 10 min. The digestion was stopped by chelation of the calcium ions with the addition of EDTA (final concentration 2 mM) and the soluble chromatin was extracted by dialysis against 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1 mM PMSF at 4° C.

Run on *in vitro* transcription was initiated with the addition of 2 volumes of transcription buffer (135 mM KCl, 7.5 mM NaCl, 25 mM Tris-HCL pH 8.0, 15.5 mM $MgCl_2$, 0.075 mM spermine, 0.25 mM spermidine, 0.1 mM EDTA, 0.1 mM EGTA, 12.5 % glycerol, 0.6 mM ATP, 0.6 mM CTP, 0.6 mM Hg-UTP or UTP, 0.015 mM ^{32}P GTP (10 µCi/nmol) and 6 mM ß-mercaptoethanol) to 1 volume of

soluble chromatin, followed by incubation at 25° C. Transcription was arrested during the linear incorporation phase, with the addition of EDTA (final concentration 10.5 mM). The ternary transcription complexes were then desalted at 4°C by exclusion chromatography on Sephadex G50 equilibrated in desalting buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA; see figure legends for addition of detergents). The void volume peak fractions were pooled and incubated by gentle rolling with activated sulfhydryl-agarose (3 mole equivalents sulfhydryl groups (23-27 μmol SH/ml packed beads) per mol Hg-UTP), pre-equilibrated in the desalting buffer. This binding mixture was then packed into a column and washed (desalting buffer) until no further radiation eluted. The bound fraction was eluted with addition of 500 mM ß-mercaptoethanol to the buffer and the efficiency of retention was described as the percentage Cherenkov counts specifically eluted with ß-mercaptoethanol versus total counts in the flow through and eluate.

RESULTS AND DISCUSSION:

To investigate the incorporation of Hg-UTP into nascent RNA and the formation of mercurated ternary transcription complexes, a soluble sea urchin embryo chromatin *in vitro* transcription system was employed. This system had the advantage in that soluble ternary transcription complexes would be formed during the transcription process, thereby eliminating post-transcription nuclease digestion, which could result in the degradation of the nascent RNA.

In vitro transcription on soluble chromatin templates demonstrated that the rate of incorporation of ^{32}P-GMP into RNA was initially linear (approximately 4 minutes) and plateaued after 10 minutes (Fig 1). This differs from nuclei *in vitro* transcription where linear incorporation was maintained for approximately 15 minutes (see chapter 1). The early termination was proposed to be a function of the chromatin template length and or loss of polymerase activity due to the method of chromatin extraction. Substitution of UTP with Hg-UTP resulted in a 75 % decrease in the rate of transcription (Fig 1), demonstrating that Hg-UTP was not a favored substrate. Schäfer (1977), suggested that this inhibition of transcription was as a result of premature termination. The observed low level of transcription was not a function of endogenous UTP as no transcription could be shown in the absence of exogenous UTP. Furthermore, RNA mercuration was demonstrated by the fact that partially purified Hg-RNA transcripts were specifically retained on sulfhydryl-Sepharose and eluted with ß-mercaptoethanol (Fig 2).

The formation of a mercurated ternary transcription complex would be dependent on transcription being inhibited prior to termination. *In vitro* transcription was arrested during the linear incorporation phase by one of two method. 1) Chelation of the magnesium ions with EDTA. 2) Rapid desalting at 4° C, to remove both the salts and the nucleotide triphosphates. Fractionation of the putative mercurated ternary transcription complexes by agarose gel electrophoresis (Chelm and Geiduschek, 1979) showed that the nascent RNA (^{32}P labelled) co-migrated with the chromatin and this co-migration was transcription dependent (Fig 3A and B). Similarly, the nascent RNA was shown to co-migrate with the chromatin when fractionated by sucrose density gradient ultracentrifugation (Fig 3C and D). Together these results suggested that a soluble mercurated ternary transcription was formed *in vitro*.

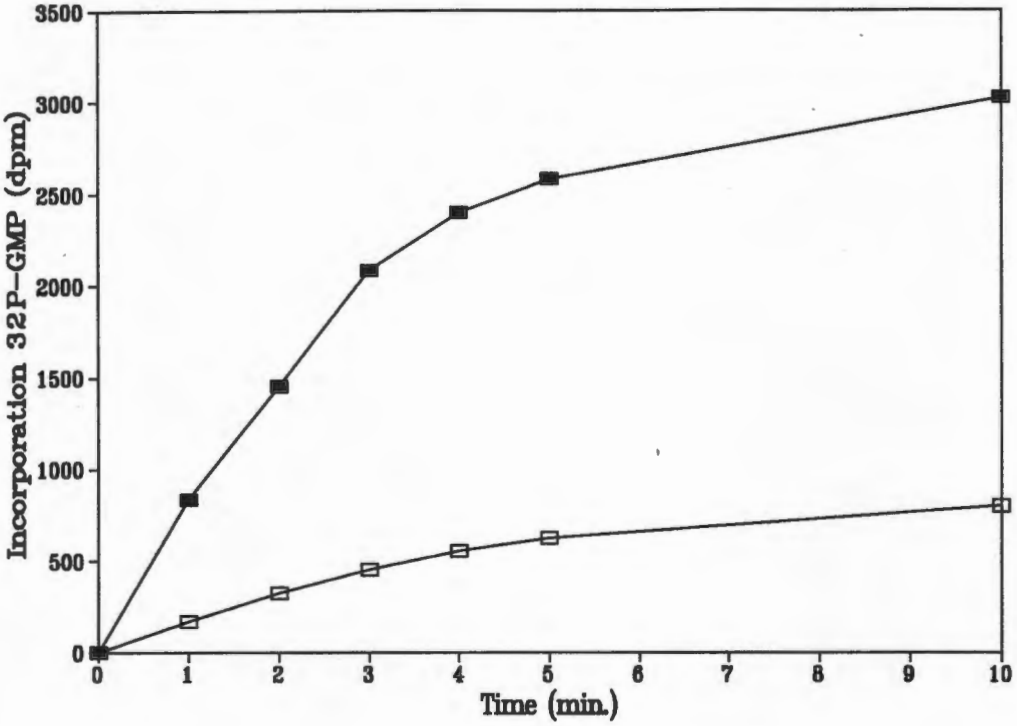


Figure 1. Incorporation of ^{32}P-GMP into RNA as a function of run on *in vitro* transcription using solubilized chromatin as a template.

Soluble sea urchin embryo chromatin was prepared as described in methods and materials. Two volumes of transcription buffer containing either UTP (*filled squares*) or Hg-UTP (*open squares*) was added to the soluble chromatin and *in vitro* transcription was allowed to proceed by incubating at 25° C for various time intervals (see figure). Transcription was terminated with the addition of 15 volumes stop buffer (1 % SDS, 10 mM EDTA pH 8.0) and aliquots were spotted in duplicate onto GFC filters. Unincorporated ^{32}P-GTP was removed by washing the filters with cold 10 % TCA, 5 % TCA and 100 % ethanol and the incorporated ^{32}P-GMP was determined by liquid scintillation counting.

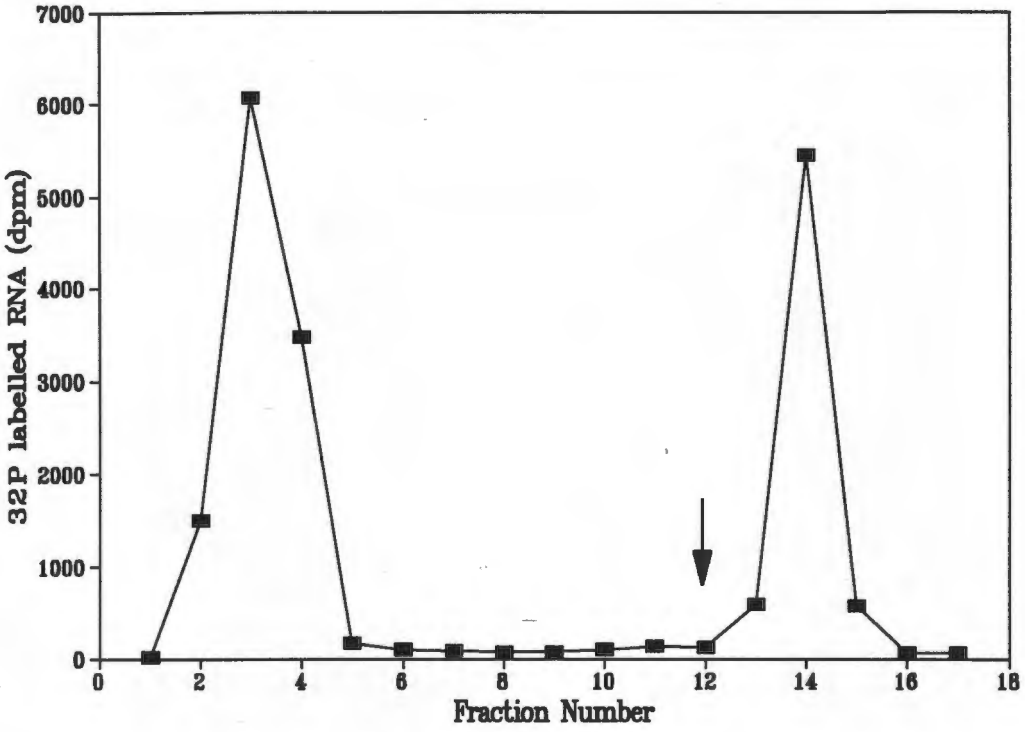


Figure 2. Retention of mercurated nascent RNA on sulfhydryl-Sepharose.

Mercurated nascent RNA (labelled with ^{32}P-GMP) was prepared by run off *in vitro* transcription (10 min) using solubilized chromatin as the template. The total transcription reaction was desalted by Sephadex G50 exclusion chromatography (equilibrated in 10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The void volume fractions were pooled and deproteinized by phenol extraction followed by ethanol precipitation. The crude mercurated RNA (containing DNA) was dissolved in 1 % SDS, 0.2 M NaCl and 10 mM Tris-HCl pH 8.0 and applied to an activated sulfhydryl-agarose column equilibrated in the same buffer. The unbound fraction was eluted with the above buffer and the bound fraction was eluted with above buffer containing 500 mM ß-mercaptoethanol (arrow indicates initial addition).

A

1 2 3 4 5 6 7

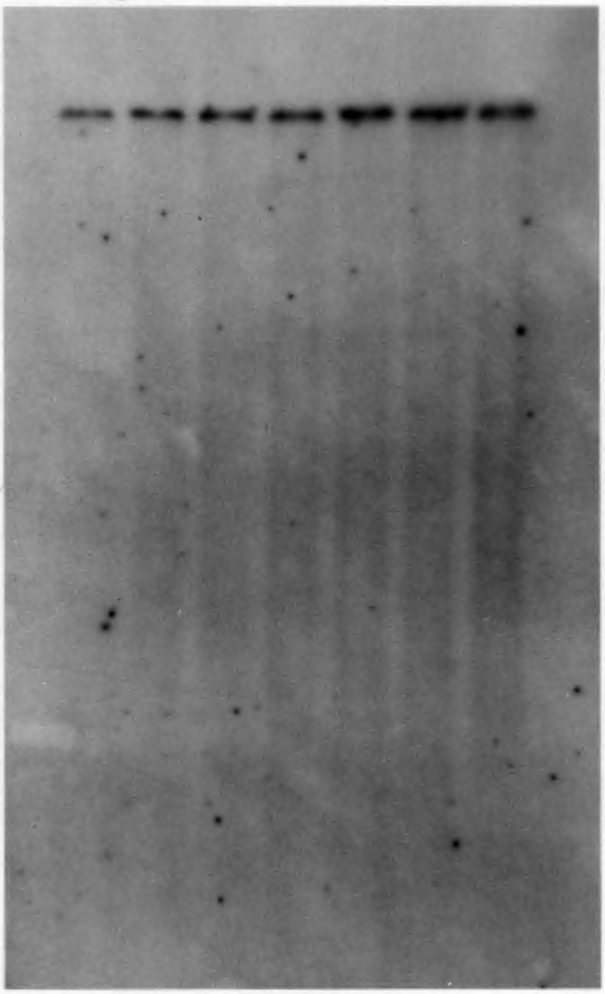
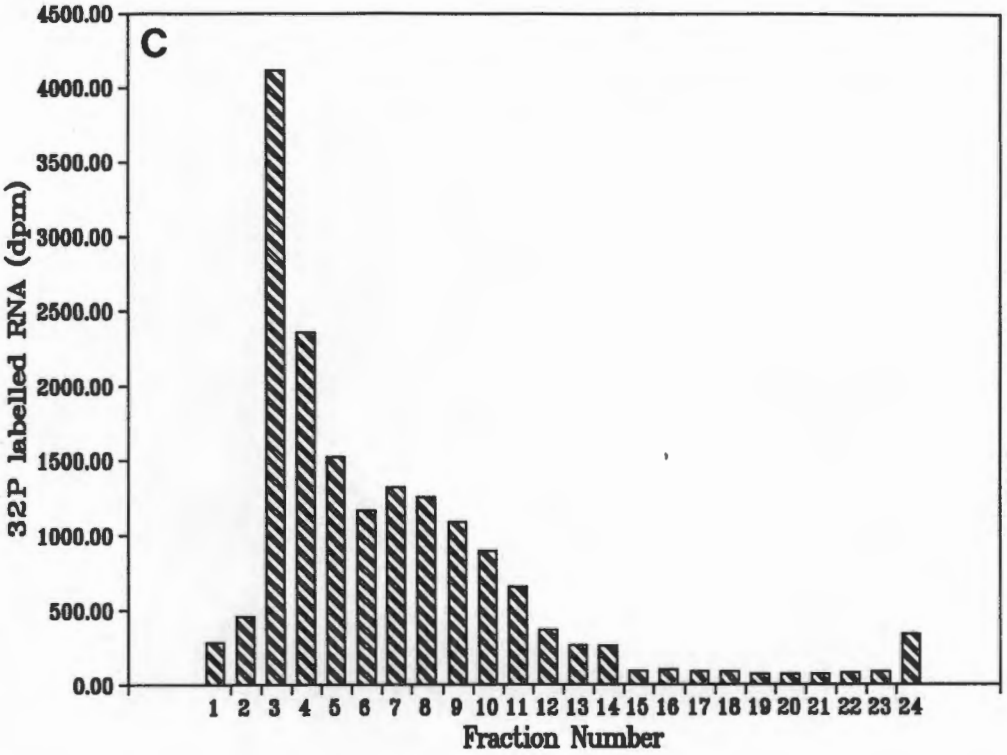


Figure 3. Fractionation of putative mercurated ternary transcription complexes.

A) Mercurated ternary transcription complexes were formed by run on *in vitro* transcription for various time intervals (ranging from 0 to 5 min). Transcription was stopped with the addition of EDTA and the respective ternary transcription complexes were fractionated by electrophoresis in 1 % agarose (40 mM Tris-acetate pH 7.9, 20 mM sodium acetate and 1 mM EDTA, 1.5 V/cm). The gel was dried and the transcription complexes were visualized by autoradiography.

Lane 1. t = 0 min.
Lane 2. t = 0.5 min.
Lane 3. t = 1.0 min.
Lane 4. t = 2.0 min.
Lane 5. t = 3.0 min.
Lane 6. t = 4.0 min.
Lane 7. t = 5.0 min.

B) Ethidium bromide stain of nucleoprotein complexes, fractionated as in figure 3A.


C
4500.00
4000.00
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
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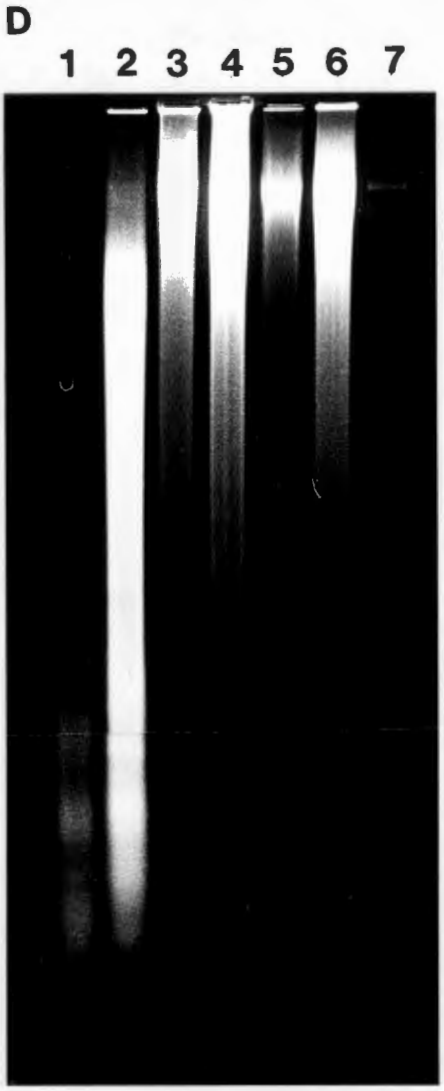
32P labelled RNA (dpm)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fraction Number


D
1 2 3 4 5 6 7

C) The mercurated ternary transcription complexes were formed by run on *in vitro* transcription (1 min), cooled to 4° C and rapidly desalted by Sephadex G50 exclusion chromatography. The void volume fractions were pooled and fractionated by gradient density ultracentrifugation (20-60 % sucrose containing 10 mM TEA pH 7.6, 2 mM EDTA, 0.1 % PMSF and 10 % glycerol (centrifugation, SW40ti rotor at 25000 rpm for 16 hours at 4° C)). The gradients were fractionated by displacement and aliquots of the individual fractions were assayed for acid insoluble ^{32}P labelled RNA (liquid scintillation).

D) The fractions from the sucrose gradient were deproteinized and refractionated by electrophoresis in 1 % agarose (1x TBE pH 8.3) and the DNA was visualized by staining with ethidium bromide.

Lane 1. Fractions 3 and 4.
Lane 2. Fractions 5, 6 and 7.
Lane 3. Fractions 8, 9, 10 and 11.
Lane 4. Fractions 12, 13, 14 and 15.
Lane 5. Fractions 16, 17, 18 and 19.
Lane 6. Fractions 20, 21, 22, 23 and 24.
Lane 8. EcoRI and HindIII digest of λ.

The high affinity of mercurated compounds for sulfhydryl groups was proposed as a means to affinity purify the mercurated ternary transcription complex. To test this, mercurated ternary transcription complexes were generated by brief *in vitro* transcription and desalted over Sephadex G50 to remove excess Hg-UTP (Fig 4). The outer volume fraction was then incubated with sulfhydryl-agarose, followed by washing to elute unbound fraction. The bound fraction was eluted in the presence of an excess of sulfhydryl groups (500 mM ß-mercaptoethanol). The elution profiles showed that only 1 % of the ^{32}P labelled mercurated nascent RNA (putative ternary transcription complexes) was specifically retained (Fig 5). This was in contrast to the observation that >40 % of the ^{32}P labelled mercurated nascent RNA (purified by phenol extraction) could be specifically retained by sulfhydryl-agarose (Fig 2). Thus, the inability of the mercurated ternary transcription to bind to sulfhydryl-agarose must be a function of the following factors. 1) Complexing of nuclear proteins with the nascent RNA, sterically hindering the access to the mercury groups. 2) Direct interaction between the sulfhydryl groups of the nuclear proteins and the mercury groups on the RNA, inhibiting sulfhydryl exchange with the sulfhydryl-agarose. 3) Length of the nascent RNA transcript, sterically preventing accessibility of the mercury groups.

To further investigate the complexing of nuclear proteins with the nascent RNA, the ternary transcription complexes were pre-incubated in the presence of detergents. The addition of the non-ionic detergent Triton X100 failed to show any increase in binding suggesting that the protein-RNA interactions were not hydrophobic (Fig 6). The addition of the ionic detergent Sarkosyl (0.2 % v/v) resulted in a 25 % increase in binding of the ^{32}P labelled mercurated RNA to the sulfhydryl-agarose (Fig 6). This result demonstrated that the displacement of proteins ionically associated with either the DNA or the nascent RNA increased accessibility of the mercury groups. The bound fraction constituted the ternary transcription complexes rather than free mercurated RNA because the addition of 0.2 % Sarkosyl does not lead to the dissociation of the transcription complex, as demonstrated by the absence of inhibition of transcription in the presence of Sarkosyl (Table 1). Thus, it was concluded that the nuclear protein became complexed with the nascent mercurated RNA and thereby inhibited access of the sulfhydryl-agarose to the mercury groups.

The binding of sulfhydryl nuclear proteins to the RNA mercury groups is presumably the result of the low molecular weight reducing agents being removed during the desalting step, thus deprotecting the mercury in the RNA and allowing the sulfhydryl protein to react. In an attempt to quench the formation of these complexes excess

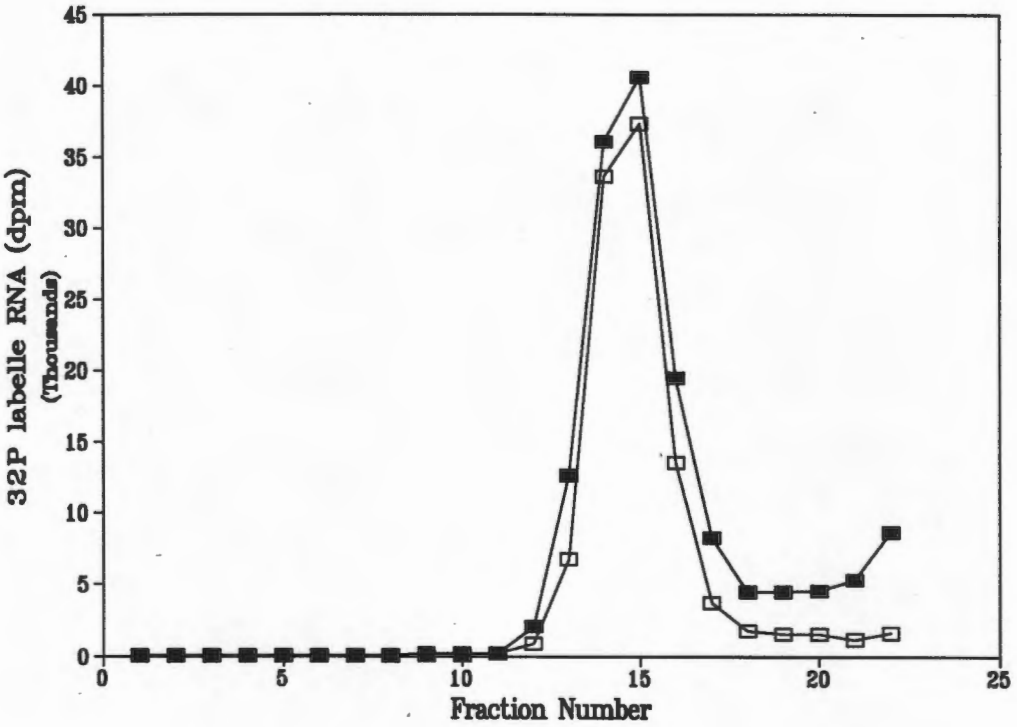


Figure 4. Desalting of the mercurated ternary transcription complexes by gel exclusion chromatography on Sephadex G50.

Mercurated (*filled squares*) and non mercurated (*open squares*) ternary transcription complexes were formed by run on *in vitro* transcription as described previously and desalted by exclusion chromatography on Sephadex G50 equilibrated in KTE pH 8.0 (50 mM KCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

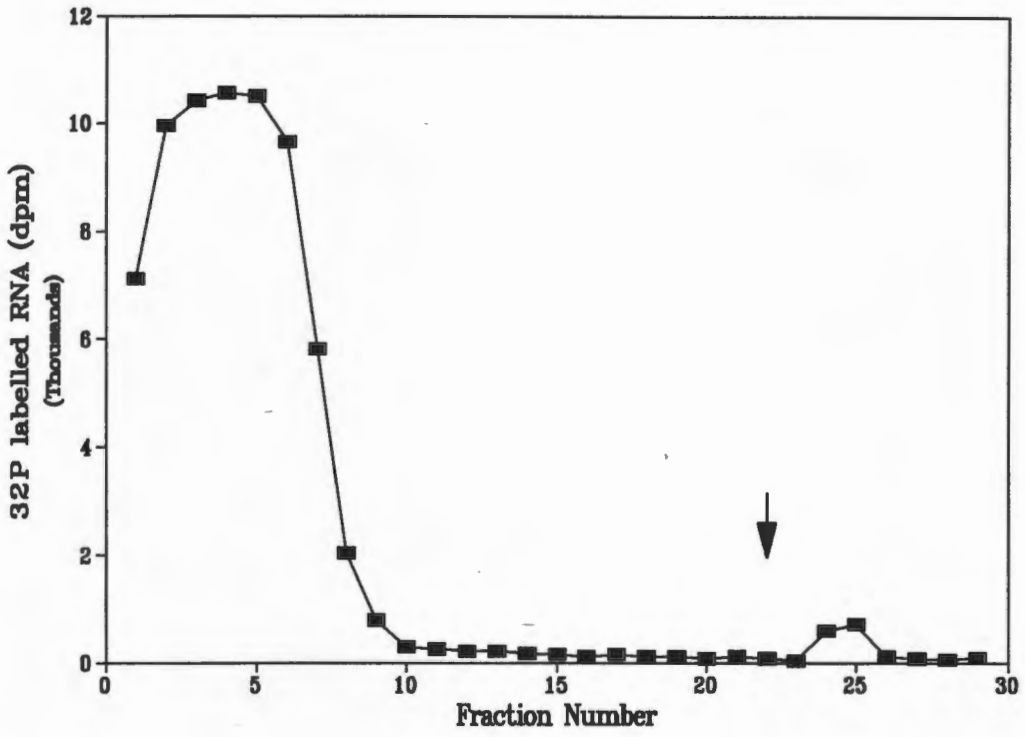


Figure 5. Sulfhydryl affinity chromatography of the mercurated ternary transcription complexes.

Mercurated ternary transcription complexes were formed by run on *in vitro* transcription as described previously and desalted by exclusion chromatography on Sephadex G50 equilibrated in KTE pH 8.0 (50 mM KCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The void volume fractions were pooled and added to activated sulfhydryl-agarose equilibrated in the KTE pH 8.0. Binding was allowed to occur by gently rolling the matrix suspension at 24° C for 30 min, the matrix was then transferred to a column and the unbound fraction was eluted by washing with the KTE pH 8.0. The bound fraction was eluted with KTE pH 8.0 containing 500 mM ß-mercaptoethanol (arrow indicates initial addition).

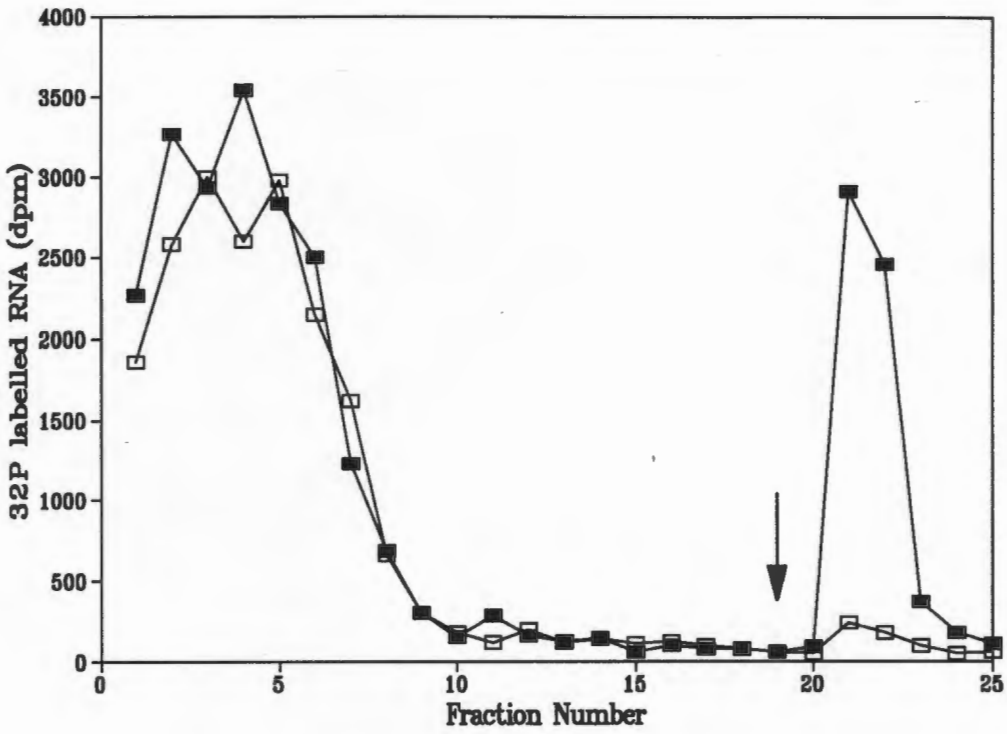


Figure 6. Sulfhydryl affinity chromatography of the mercurated ternary transcription complexes in the presence of detergents.

Mercurated ternary transcription complexes were formed by run on *in vitro* transcription as described previously. The transcription mixture was then desalted over Sephadex G50 equilibrated in KTE pH 8.0 containing either 0.1 % Triton X100 (*open squares*) or 0.2 % Sarkosyl (*filled squares*). The void volume fractions were pooled and added to activated sulfhydryl-agarose equilibrated in the respective buffer and incubated at 24° C for 30 min. The unbound fraction was eluted with the respective buffer and the bound fraction was eluted with the respective buffer containing 500 mM ß-mercaptoethanol (arrow indicates initial addition).

Table 1. Effect of various detergents on the rate of run on *in vitro* transcription.

Detergent added	^{23}P-GMP incorporated (pmol)
Without detergent	0.057
0.1 % Triton X100	0.055
0.1 % Deoxycholate	0.052
0.2 % Sarkosyl	0.055

14 hour sea urchin embryo nuclei were added to 2 volumes of transcription buffer (see methods and materials chapter 1) containing the respective detergents and UTP. *In vitro* transcription was initiated by warming the nuclei to 25° C. Transcription was allowed to proceed for 5 min followed by the addition of 15 volumes of stop buffer. Aliquots were spotted in duplicate of GFC filters and washed as described previously. The incorporation of ^{32}P-GMP was determined by liquid scintillation counting.

Hg-UTP was added to the transcription mixture prior to desalting, thereby effectively blocking all the sulfhydryl groups. Subsequent sulfhydryl-agarose affinity chromatography showed that the binding to the matrix improved, as approximately 10 % of the ^{32}P label were retained (Fig 7). In conclusion, this series of experiments suggests strongly that the nascent RNA mercury groups are in part blocked by sulfhydryl interaction with nuclear protein sulfhydryl groups.

The complexing of the nuclear proteins with the nascent RNA implies that the nascent RNA did not any longer constitute a target for the affinity purification of transcriptionally active chromatin. Though treatment with detergents appears to improve the enrichment on the affinity matrix, this would limit the application of this methodology, because the aim was to develop methodology for the isolation and characterization of the chromatin associated proteins, some or many could well be lost as the result of detergent promoted dissociation.

Analysis of the proteins (^{125}I labelled) associated with the bound fraction, as assayed by SDS polyacrylamide gel electrophoresis, demonstrated that specific proteins were enriched (Fig 8). The proteins enriched were not identified. The histone content was dramatically reduced by detergent displacement.

Due to the complex problems associated with the above technique, it was not further pursued.

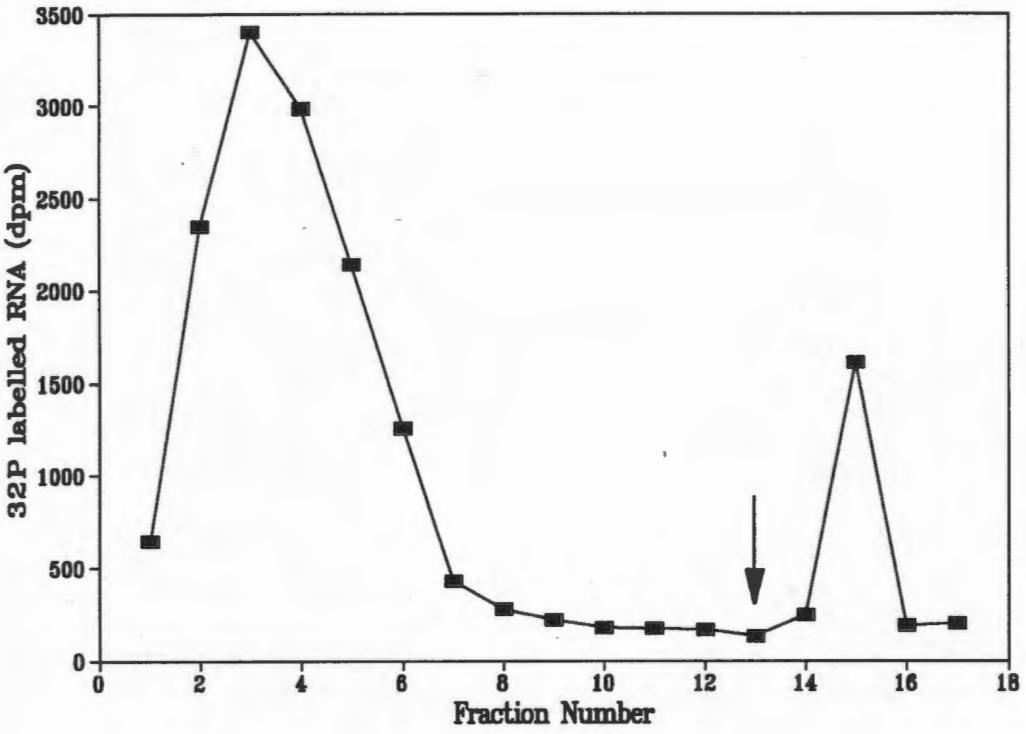


Figure 7. Sulfhydryl affinity chromatography of the mercurated ternary transcription complexes after pre-quenching of the nuclear protein sulfhydryl groups with Hg-UTP.

Mercurated ternary transcription complexes were formed by run on *in vitro* transcription as described previously. Excess Hg-UTP (molar ratio Hg-UTP:ß-mercaptoethanol, 10:1) was added to the transcription mixture and allowed to react at 4° C for 10 min, followed by desalting over Sephadex G50 equilibrated in KTE pH 8.0. Activated sulfhydryl-agarose was then added to the pooled void volume fraction and mixed at 24° C for 30 min. The unbound fraction was eluted with KTE pH 8.0 and the bound fraction was eluted with KTE pH 8.0 containing 500 mM ß-mercaptoethanol (arrow).

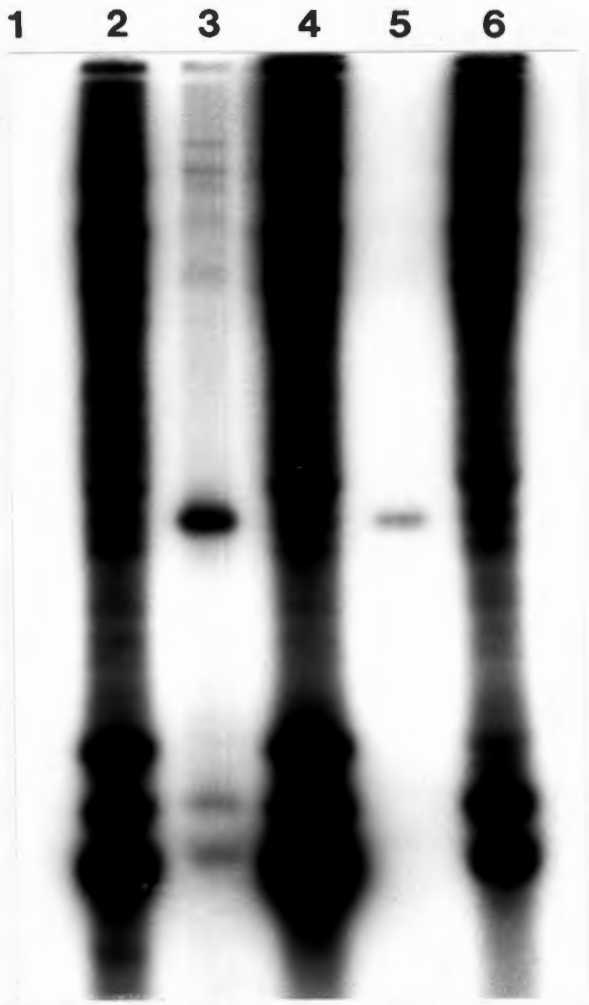


Figure 8. SDS polyacrylamide gel electrophoresis of the nuclear proteins eluted from the sulfhydryl affinity matrix.

The unbound and bound fractions eluted from the sulfhydryl-agarose without and with ß-mercaptoethanol, respectively, were dialysed against H_2O, concentrated by freeze drying and redissolved in H_2O. The proteins were iodinated with ^{125}I using Iodogen coated tubes and the excess ^{125}I was removed by dialysis. The ^{125}I labelled protein were then fractionated by electrophoresis in 15 % SDS polyacrylamide and visualized by autoradiography.

Lane 1. Nuclear proteins associated with the bound fraction (without detergent).
Lane 2. Nuclear proteins associated with the unbound fraction (without detergent).
Lane 3. Nuclear proteins associated with the bound fraction (with 0.2 % Sarkosyl).
Lane 4. Nuclear proteins associated with the unbound fraction (with 0.2 % Sarkosyl).
Lane 5. Nuclear proteins associated with the bound fraction (with 0.1 % Deoxycholate).
Lane 6. Nuclear proteins associated with the unbound fraction (with 0.1 % Deoxycholate).

CHAPTER 5.

INVESTIGATION OF SOLID PHASE MATRICES FOR THE AFFINITY PURIFICATION OF MACROMOLECULAR WEIGHT BIOTIN.

INTRODUCTION:

The efficiency of binding a macromolecular weight molecule to an immobilized ligand, is directly related to the accessibility of the ligand. Accessibility is limited by two factors: 1) The spacer arm length, which positions the ligand away from the solid phase surface, thereby facilitating binding without steric hindrance. 2) The surface area seen by the macromolecular weight molecule, which is determined by the porosity and surface topology of the solid phase. Taken together these criteria would suggest that the spacer arm and ligand should be covalently coupled to a non-porous solid phase with a large surface area (ie. microspheres). A further advantage of this type of solid phase is that the concentration of the ligand would be maximal and therefore the binding equilibrium would be driven towards binding, particularly for low affinity ligands.

When constructing an affinity matrix the chemical composition of the solid phase should also be considered, as the non-specific binding may be greater than specific binding. This effect can be reduced by pre-incubation with a variety of blocking agents, which include non-ionic detergents, competitor proteins and competitor nucleic acid. The incorporation of a chemical feature for a specific release mechanism may overcome the contaminating effect of non-specific binding, because only the specifically retained target molecules will be released (provided that the non-binding domains of the target are not non-specifically bound). This is of particular relevance when the target is found only in very low concentrations.

The aim of this section was to systematically construct a number streptavidin or avidin affinity matrices, with the aim to produce the ideal matrix that could be applied for the affinity isolation of complex biotinylated macromolecular weight complexes. The suitability of the following matrices has been investigated; 1) fragmented Sephadex; 2) liposomes; 3) microcrystaline cellulose; 4) polyacrylamide microspheres, with avidin/streptavidin and or biotin as ligands.

METHODS AND MATERIALS:

Chemical 5' biotinylation of DNA.
(according to Chollet *et al.*, 1985)

EcoRI linearized pPA(H2B-H3S) plasmid (133 µg; 100 pmol 5' ends) was dissolved in 100 µl activation buffer (0.25 M ethylene diamine, 0.1 M N-methylimidazole pH 6.0 and 0.1 M EDC) and incubated at 24° C for 16 hours. A further 100 µl of the above activation reagent solution was then added and the incubation was continued for a further 6 hours. The 5'amino modified DNA was ethanol precipitate with the addition of 0.1 volumes 3 M sodium acetate pH 5.5 and 3 volumes 100 % ethanol, incubated at -20° C for 16 hours to complete the precipitation.

The 5'-amino modified DNA was collected by centrifugation (48000 xg; 30 min) and washed twice with 70 % ethanol. The vacuum dried DNA was dissolved in 100 mM NaBorate pH 8.5 and 0.2 % SDS (200 µl), and biotinylaminocaproyl N-hydroxysuccinimide (400 µg; 0.88 µmol) dissolved in 40 µl dimethylformamide was added. The mixture was incubated at 24° C for 16 hours, followed by ethanol precipitation as above and the biotinylated DNA was washed 3 times with 70 % ethanol to remove all unincorporated biotin. The biotinylated DNA was dissolved in 100 µl TE pH 8.0 and stored ad -20° C.

The efficiency of biotinylation was determined using a gel retardation assay. Briefly, the biotinylated DNA was digested with a restriction endonuclease (Hind III), thereby generating two smaller DNA fragments. These fragments were then incubated with a excess of streptavidin and fractionated by electrophoresis in a 1 % agarose gel (Fig 1). Biotinylation was shown by the specific retardation of the DNA fragments when compared to DNA fractionated in the absence of streptavidin. 100 % biotinylation was shown at each 5' end for each modification reaction performed.

Figure 1. Electrophoretic retardation of 5'-biotinylated DNA as a function of streptavidin binding.

5'-biotinyalated DNA was prepared and digested with Hind III as described in methods and materials. Approximately 1 μg of the biotinylated DNA was incubated in the absence or presence of streptavidin (1 μg) at 24° C for 1 hour. The complexes were then fractionated by electrophoresis in 1 % agarose (1x TBE pH 8.3) and the DNA was visualized after staining with ethidium bromide.

Lane 1. 5'-Biotinylated DNA (Hind III digested).
Lane 2. 5'-Biotinylated DNA (Hind III digested) complexed with streptavidin.

Preparation of Sephadex particles.

Washed Sephadex beads (G50, G25, G15) were resuspended in two volumes of H_2O (10 ml), an equal volume of glass balls (1 mm) were then added. The suspension was shaken at maximum rpm in a CO_2 cooled ball mill at 24° C. The degree of fragmentation was monitored by light microscopy (the absence of bead structure was considered as complete fragmentation).

The Sephadex particles were then collected, washed repeatedly with H_2O by centrifugation (8000 xg for 2 min) and stored at 4° C.

Coupling to Sephadex particles via periodate oxidation.

The Sephadex particles were washed extensively with H_2O and then with 100 mM sodium acetate pH 6.0. The glucose residues were oxidized by the addition of sodium periodate (12 mM) followed by incubation in the dark at 24° C for 1 hour (higher concentrations of sodium periodate resulted in the solubilization of the particles). Excess ethylene glycol was added to prevent further oxidation and the reactants were removed by dialysis against H_2O.

The aldehydic particles were then washed with 100 mM $NaHCO_3$ pH 9.0 and resuspended in an equal volume of this buffer containing 1 mg/ml avidin. The suspension was mixed at 24° C for 6 hours and the resulting Schiff bases and non-reacted aldehyde groups were reduced by the addition of $NaBH_4$ (final concentration 3.0 mg/ml), followed by incubation at 4° C for 16 hours. Uncoupled avidin was removed by washing with 100 mM NaH_2PO_4 pH 7.0 and avidin particles were then stored at 4° C.

Non-specific binding could be lowered by acetylation of the avidin and pre-incubation with BSA (100 µg/ml). Acetylation of the avidin was achieved by incubating the avidin matrix or avidin in half saturated sodium acetate containing 2 µl acetic anhydride at 4° C for 5 min. Three further additions of acetic anhydride were added at 5 min intervals, followed by a further incubation at 4° C for 1 hour. The matrices were then washed extensively with H_2O and avidin-matrix was dialysed against H_2O followed by freeze drying.

CNBr activation of Sephadex particles.

Spacer arms were coupled to Sephadex particles via the CNBr activation (protocol described in chapter 1). Activation of the spacer arm carboxyl groups and coupling of avidin was performed according to the protocol described in chapter 2.

Purification of lecithin.

(according New, 1990 with modifications).

Egg yolk (150 ml) was initially mixed with an equal volume of methanol, followed by the addition of two volumes of chloroform and mixed at 4° C for 1 hour. The emulsion was then allowed to stand until complete phase separation had occurred. The organic phase was collected and the solvents were removed by rotary evaporation. The residue was then dissolved in dry diethyl ether and filtered to remove any insoluble material and the solvent was removed from the filtrate by rotary evaporation. This residue was then dissolved in 50 ml dry chloroform after which 500 ml acetone was added and the mixture was stirred at 4° for 2 hours. The resulting precipitate was collected by filtration and the above step was repeated until a white precipitate was obtained. The precipitate was dissolved in 50 % chloroform, 50 % methanol and passed though neutral aluminium oxide equilibrated in the above solvents. The flow through was collected and the solvent removed by rotary evaporation. The lecithin was then purified by reverse phase chromatography on silica gel, eluted by a stepwise methanol/chloroform gradient (ranging from 0 to 50 % methanol). The eluted fractions were assayed by thin layer chromatography on silica gel developed with chloroform:methanol:H_2O (65:25:4). The fractions containing pure lecithin were pooled, the solvent was removed by rotary evaporation and the purified lecithin was redissolved in chloroform (100 µmol/ml) and stored under nitrogen at -20° C.

Synthesis of biotinaminocaproyl-aminoethanol-phosphotidyl dipalmitoyl (BIO-CAP-PED).

Phosphotidylethanolamine dipalmitoyl (5 mg; 7.22 µmol) was dissolved in 1 ml dry chloroform by gentle heating at 50° C for 10 min. Solid biotinylaminocaproyl N-hydroxysuccinimide (6.8 mg; 14.45 µmol), together with triethylamine (10.8 µmol) was then added and mixed at 24° C for 16 hours. The mixture was washed three times with an equal volume of 1 % NaCl to remove the excess biotinylcaproic acid.

The solvent was removed under a gentle stream of nitrogen and the efficiency of biotinylation was determined by thin layer chromatography on silica gel F_{254} developed with chloroform:methanol:H_2O (65:25:4). The R_f of the biotinylated lipid (BIO-CAP-PED) was found to be 0.34, visualized with iodine vapor and acidic cinnemaldehyde.

The BIO-CAP-PED derivative was purified by reverse phase chromatography on silica, eluted with a stepwise methanol/chloroform. The solvent was removed in vacuo and the residue was redissolved in dry chloroform and stored under nitrogen at -20° C.

Preparation of biotinyl-liposomes.

Liposomes were prepared using the reverse evaporation technique as described previously (New, 1990). Briefly, lecithin (10 μmol), dicetylphosphate (0.1 μmol) and BIO-CAP-PED (100 nmol) were mixed and dried under a stream of N_2. The residue was dissolved in 1 ml peroxide free diethyl ether and 0.33 ml STE pH 8.0 was added. The mixture was gently sonicated to form an emulsion and the organic phase was slowly evaporated under vacuum with constant mixing. The resulting liposomes were purified by buoyant density centrifugation though 5 % Ficoll 400 in STE pH 8.0, overlayered with STE (0.5 ml) (3000 xg for 5 min), and collected from the meniscus and stored at 4° C under N_2.

Synthesis of 4-p-maleimidophenyl-butyrylaminoethanol-phospotidyl dipalmitoyl (MPB-PED).

Phosphotidylethanolamine dipalmitoyl (21 mg; 30 μmol) was dissolved in 4 ml dry chloroform by gentle heating to 50° C. 4-p-maleimidophenyl-butroyl-N-hydroxysuccinimide (SMPB) (14.25 mg; 40 μmole) dissolved in 1 ml dry dimethylformamide, together with triethylamine (40 μmol) were then added and the coupling reaction was allowed to proceed at 24° C for 1.5 hours. The reaction was shown to be complete by TLC analysis and the product was purified by reverse phase chromatography on silica as described above.

Modification of streptavidin with N-succinimidyl pyridyl dithio propionate (SPDP) to yield PDP-streptavidin.

SPDP (50 μg; 160 nmol) dissolved in 50 μl ethanol was added to streptavidin (1.75 mg; 29 nmol) dissolved in 350 μl 100 mM $NaHCO_3$ pH 8.0 and incubated at 24° C for 16 hours. The uncoupled reactants were removed by extensive dialysis against H_2O, the PDP-streptavidin was freeze dried and stored at -20° C. The degree of modification was determined spectrophotometrically after reduction with DTT (the released 2-thiopyridine group has a characteristic extinction coefficient at 343 nm of 8300). The observed molar ratio of modification was found to be approximately 4:1, suggesting an average of a single modification per subunit. This degree of modification had no effect on the binding of biotin within the binding pocket.

Coupling of PDP-streptavidin to maleimido-liposomes.

PDP-streptavidin (200 μg) was reduced by incubation in 400 μl 500 mM sodium acetate pH 5.5 and 50 mM DTT, at 24° C for 1 hour. The SH-streptavidin derivative was then extensively dialysed against 100 mM NaH_2PO_4 pH 6.5 saturated with N_2, to remove all traces of reducing agents. On completion of dialysis the SH-streptavidin was added to freshly prepared maleimido-liposomes (lipid ratio, lecithin:cholesterol:dicetylphosphate: MPB-PED (1:1:0.01:0.01)) and mixed at 24° C for 16 hours. The streptavidin liposomes were then purified by repeated centrifugation though 5 % Ficoll 400 gradient, concentrated and stored under N_2 at 4°C.

Avidin liposomes were prepared using essentially the same procedure except that cholesterol was excluded from the liposomes and the PDP-avidin was acetylated (see above) prior to reduction with DTT.

RESULTS AND DISCUSSION:

5' Biotinylated DNA (Chollet *et al.*, 1985) was used as the model macromolecular weight biotinyl complex, due to the anomalous size of DNA in solution. However, this does not represent the true complexity of the macromolecular weight molecules we wished to isolate (chromatin) and therefore could only determine accessibility of the immobilized streptavidin or avidin.

The efficiency of streptavidin or avidin immobilization was determined by titration with ^{14}C-biotin (specific activity 114.4 dpms/pmol, see methods and materials chapter 1). The efficiency of macromolecular weight biotin retention was shown by fractionating either the total binding reaction (including matrix) or matrix supernatant by electrophoresis in agarose and visualizing the DNA by staining with ethidium bromide (Fig 2). The results are summarized as follows.

Sephadex particles.

The immobilization of avidin or streptavidin on macroporous matrices (Sepharose 6B-CL and AcA 54) failed to generate a solid phase accessible biotinylated macromolecules, presumably due to immobilization of avidin or streptavidin within the pores (see Table 1).

To increase the accessibility of the immobilized avidin or streptavidin, Sephadex beads (G50, G25 and G15) were investigated as a solid phase, because the avidin or streptavidin molecules should mainly be immobilized on the outer surface and thus accessible the macromolecular weight biotin. However, the covalent coupling of avidin to aminocaproic acid Sephadex beads G50 (Cuatrecasas, 1970), via anhydrous activation (Cuatrecasas, 1972), was found to be very low (see Table 1). Presumably due to the limited surface area accessible to the avidin.

To increase the surface area, Sephadex particles were prepared by fragmenting pre-swollen Sephadex beads in a ball mill. These particles were then extensively washed by repeated centrifugation in order to purify only those particles with a pelletable density (8000 xg for 2 min).

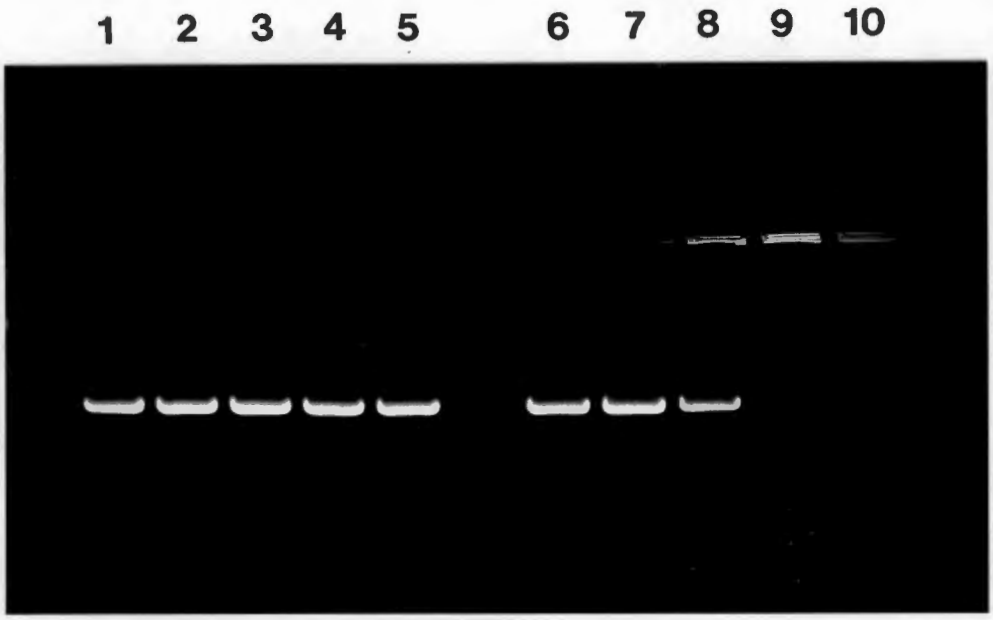


Figure 2. Assay to determine the efficiency of 5'-biotinylated DNA retention by immobilized avidin or streptavidin matrices.

5'-Biotinylated DNA (1 µg; 0.38 pmol) was incubated with increasing volumes of avidin or streptavidin matrices at 24° C for 1 hour (example shown, avidin-C6-Sephadex G25). To determine the level of non-specific binding, the avidin or streptavidin matrices were pre-incubated with an excess of biotin prior to the addition of 5'-biotinylated DNA to quench all the biotin binding activity. (Non-specific binding was reduced by acetylation of the avidin and pre incubating the matrices with BSA 100 µg/ml). The binding reactions (including matrices) were then electrophoretically fractionated in 1 % agarose (1x TBE pH 8.3, 1.5 V/cm) and the DNA was visualized after staining with ethidium bromide. The efficiency of retention (accessibility) was expressed as the volume of matrix required to bind 1 µg 5'-biotinylated DNA.

Lane 1. 0.25 µl matrix, quenched with excess biotin.
Lane 2. 0.50 µl matrix, quenched with excess biotin.
Lane 3. 1.00 µl matrix, quenched with excess biotin.
Lane 4. 2.00 µl matrix, quenched with excess biotin.
Lane 5. 4.00 µl matrix, quenched with excess biotin.
Lane 6. 0.25 µl matrix.
Lane 7. 0.50 µl matrix.
Lane 8. 1.00 µl matrix.
Lane 9. 2.00 µl matrix.
Lane 10. 4.00 µl matrix.

Table 1. Immobilization of avidin on various matrices and the assessment of the accessibility of immobilized avidin to biotinylated macromolecular molecules.

Matrix	Activation	Spacer arm	Avidin (acetylated)[a] (per 100 µl matrix)	Accessibility[c] (µl matrix/µg Bio-DNA)
Sephadex G50	CNBr	C6	15 pmol	ND
Sephadex G50[p]	$NaIO_4$	-	800 pmol	40.0 µl
Sephadex G50[p]	CNBr	C6	820 pmol	10.0 µl
Sephadex G25[p]	CNBr	C6	800 pmol	1.5 µl
Sephadex G25[p]	CNBr	C8	750 pmol	1.0 µl
Sephadex G25[p]	CNBr	polyserine	200 pmol	2.0 µl
Sephadex G25[p]	CNBr	hexaglycine	700 pmol	2.5 µl
Sephadex CM25[p]	-	C6	1.0 nmol	4.0 µl
Sephadex G15[p]	CNBr	C6	100 pmol	4 µl
Sephadex G15[p]	CNBr	C8	160 pmol	ND
Sephadex G15[p]	CNBr	polyserine	50 pmol	4 µ
Avicell	CNBr	C6	900 pmol	5 µl
Sepharose 6B-CL	CNBr	-	500 pmol[b]	> 50 µl
AcA 54	CNBr		450 pmol[b]	>50 µl

a) The quantification of avidin or streptavidin immobilized on the matrix surface was determined by incubating an aliquot (10 µl packed matrix) of the respective matrices (in duplicate) with an excess of ^{14}C-biotin (specific activity 114 dpm/pmol) at 24° C for 1 hour. Bound ^{14}C-biotin was determined liquid scintillation after extensive washing. Non-specific binding was determined by pre-quenching the biotin binding activity with an excess of biotin prior to the addition of ^{14}C-biotin. (pmol avidin bound = [dpm unquenched - dpm quenched/114 dpm/pmol])
b) Streptavidin.
c) Assayed as described in figure 2.
p) Sephadex particles.

Sephadex G50 particles were efficiently activated by sodium periodate oxidation and avidin was covalently coupled at a ratio of 800 pmol/100 µl packed particles after reduction with $NaBH_4$ (see Table 1). (Sodium periodate oxidation of both Sephadex G15 and G25 particles resulted in a >10 fold decrease in coupling efficiency, which appears to be related to the degree of dextran crosslinking within the respective particles). The avidin-Sephadex G50 particles bound low molecular weight biotin (^{125}I-poly-biotinylated BSA) with a high efficiency (binding ratio BSA:avidin, 1.6:1), but showed poor binding of biotinylated DNA (binding ratio DNA:avidin, 0.0001:1), suggesting inaccessibility.

The efficiency of coupling avidin to aminocaproic acid Sephadex G50 particles (Cuatrecasas 1972) was similar to that observed for periodate oxidation (820 pmol/100 µl packed matrix). ^{125}I-poly-biotinylated BSA was retained with a high efficiency (binding ratio BSA:avidin 0.72:1). However, the accessibility of the immobilized avidin to macromolecular weight biotin was again found to be poor (binding ratio DNA:avidin, 0.0005:1) (see Table 1). Thus, it was concluded that the surface topology of the particle was the main inhibiting factor.

The CNBr activation method (Cuatrecasas, 1970) proved to be advantageous in that the activation did not destabilize the crosslinked dextran. Thus, subsequent activation of immobilized spacer arms (carboxyl groups) under anhydrous conditions (Cuatrecasas, 1972) could be performed without solubilization of the particles. In contrast, periodate activated particles were destroyed under these conditions.

It appears reasonable to assume that the surface topology of the Sephadex particles is related to the degree of dextran crosslinking. Thus, avidin was coupled to Sephadex G25 particles via the immobilized spacer arms (C6, C8 and hexaglycine). The efficiency of avidin coupling was 800, 750 and 700 pmol/100 µl packed particles, respectively, which was similar to that of G50. The accessibility of the avidin molecules was increased by 6 fold (binding ratio DNA:avidin, 0.003) (see Table 1).

To further determine the relationship between the degree of particle crosslinking (surface topology) and accessibility, avidin was coupled to Sephadex G15 particles via a C6 spacer. The efficiency of coupling was reduced by a factor 8 (100 pmol/100 µl packed particle), however, the accessibility increased by 3 fold (binding ratio DNA:avidin, 0.01:1). The inclusion of a longer spacer arm (polyserine) further enhanced the accessibility by 2 fold (see Table 1).

Together these results demonstrate that an accessible avidin-Sephadex particle could be synthesized for the affinity isolation of biotinylated macromolecules. Non-specific binding could be reduced by pre-incubation with BSA (100 μg/ml) and 0.1 % Nonidet LE. However, in the context of my prime investigation, the isolation of a specific chromatin fraction, non-specific binding remained high. One of the reasons for the observed high non-specific binding may well be the irregular surface of the fragmented Sephadex particles.

Biotinylated liposomes.

The liposome would be the ideal matrix for the affinity isolation of macromolecular weight biotin due to the uniform surface structure and high surface area. To test this, biotinylated liposomes were prepared (Bayer *et al.*, 1979) with the aim to bind biotinylated DNA via the tetrameric biotin binding proteins, streptavidin and avidin. Titration of biotinylated liposomes with the [^{14}C-biotin-streptavidin] complex (molar ratio 0.6:1), demonstrated that approximately 80% of the ^{14}C biotin was specifically bound as assayed by buoyant density centrifugation though a 5 % Ficoll 400 gradient or agglutination (precipitation) via streptavidin crosslinking (see Table 2). The efficiency of binding could not be increased with the further addition of liposomes to the unbound fraction. This result was unexplained at the time because the to binding constant of streptavidin suggested a virtually irreversible bond.

Titration of biotinylated liposomes with ^{125}I-poly-biotinylated-BSA (ratio 10:1) complexed with excess avidin, demonstrated that >95 % of the BSA was specifically associated with the liposomes (see Table 2). These results suggested that liposomes should constitute a suitable a matrix for affinity purification. However, the binding of 5' biotinylated DNA to biotinylated liposomes via streptavidin could not be demonstrated (see Table 2). Thus, it was concluded that the liposome-biotin displaced the DNA-biotin from the streptavidin binding pocket as a function of the high local concentration of biotin on the liposome surface (a similar observation was made when the [^{125}I-biotin-streptavidin] complex was titrated with poly-biotinylated BSA, see chapter 2). These results demonstrate that the binding of biotin in the binding pockets of avidin/streptavidin is reversible, as it should be. However, this can only be demonstrated with matrix bound poly-biotin or soluble poly-biotinylated macromolecules. In solution the low solubility of biotin does not allow the high concentrations necessary to dissociate the ligand. It is only on the surface of a poly-biotinylated matrix or on the surface of a poly-biotinylated macromolecule, where

Table 2. Retention of streptavidin/avidin-biotinyl complexes on biotinyl-liposomes.

Biotinylated molecule	S/avidin[a] (pmol)	Avidin[a] (pmol)	Bio-liposomes (µl)	Assay method[b] (% bound) ppt	buoyancy	agarose electrophoresis
^{14}C-biotin (90 pmol)	-	150	20	80	-	-
^{14}C-biotin (90 pmol)	-	150	20	-	81	-
^{125}I-BSA (75 pmol)	-	150	20	-	96	-
Bio-DNA (0.185 pmol)	16	-	10	-	-	0
poly-Bio-DNA (0.185 pmol)	3.3	-	10	-	-	100

a) Streptavidin/avidin-biotinyl complexes were preformed prior to the addition of biotinyl-liposomes.
b) Maximum binding as determined by incubating increasing amounts of the streptavidin/avidin-biotinyl complex with a fixed volume of biotinyl liposomes at 24° C for 1 hour. The resulting complexes were purified. 1) Precipitated by gentle centrifugation (8000xg for 2 min). 2) Buoyant density centrifugation through 5 % Ficoll. 3) Electrophoresis in 1 % agarose (as described in figure 2). The efficiency of binding was determined by liquid scintillation (^{14}C-biotin), gamma counting (^{125}I-Bio-BSA) or staining with ethidium bromide (DNA).

Non-specific binding was determined by pre-quenching the vacant biotin binding sites of the streptavidin/avidin-biotinyl complex with an excess of biotin. These results demonstrated that <2 % non-specific binding occurred.

biotin can be assembled in high concentrations, that reversibility of binding can be demonstrated.

To circumvent this effect, the target DNA was 3'-poly-biotinylated with terminal transferase and Bio-11-dUTP. Incubation of the poly-biotinylated-DNA-streptavidin complex with biotinyl liposomes demonstrated the complete and specific retention of the DNA as assayed by agarose gel electrophoresis (see Table 2). Non-specific binding could not be detected at the sensitivity of ethidium bromide staining.

The disadvantages of biotinyl-liposomes as an affinity matrix can be summarized as follows: 1) Only poly-biotinylated molecules could be efficiently retained. The displacement effect could not be reduced by decreasing the liposome-biotin concentration and may only be overcome by covalently coupling streptavidin to liposome surface (see below). 2) The osmotic instability of liposomes required that an osmotic balance be maintained, which is unfavorable when applied to affinity purification of chromatin. Attempts to prepare liposomes in low ionic strength buffers failed, furthermore, dialysis to low ionic strength resulted in liposome disruption.

Streptavidin liposomes.

Streptavidin and avidin liposomes were prepared with the aim to bind mono-biotinylated macromolecular biotin which could not be demonstrated with biotinylated liposomes (see above). Initially avidin-liposomes were constructed and the efficiency of avidin immobilization was found to be high (1.76 nmol/40 μmol lecithin), however, non-specific binding of DNA was also high. Acetylation of the avidin amino groups lowered the non-specific binding and biotinylated DNA was shown to be specifically retained (see Table 3). In contrast streptavidin liposomes prepared using similar conditions failed to show any biotin binding activity, which was interpreted to indicate that the streptavidin coupling to the surface of the liposomes had not taken place. This was possibly as a result of the fluidity of the liposomes, which prevented the reactive groups reaching each other. The inclusion of cholesterol which stabilizes the lipid bilayer, facilitated the coupling of streptavidin (0.12 nmol/40 μmol lecithin). Specific retention of biotinylated DNA was demonstrated, however the efficiency of retention was low (see Table 3). Futhermore, the lack of osmotic stability makes the liposomes an unsuitable matrix

Table 3. Retention of biotinylated DNA on avidin-liposomes or streptavidin-liposomes.

	pmol immobilized[b] per 40 µmol lecithin	Accessibility[c] µl liposomes/µg Bio-DNA
Avidin[a]-liposomes	1.76 nmol	7.5
Streptavidin-liposomes (Cholesterol stabilized)	0.12 nmol	>20

a) Acetylated.
b) Efficiency of immobilization determined by titration with ^{14}C-biotin (see Table 1).
c) Determined as described in figure 2.

for chromatin isolation. Though, for target molecules insensitive to an ionic environment (100 mM), the liposome must be considered as a ideal solid phase.

Microcrystaline cellulose.

Biotinyl-cellulose had been previously applied for the affinity isolation of biotinylated DNA via the binding of avidin (Dawson *et al.*, 1989).

Avidin was covalently linked to Avicell (Merck) by CNBr activation and the coupling efficiency was shown to be 900 pmol avidin per 100 µl packed matrix. However, the binding of biotinylated DNA suggested that only 0.09 % of the immobilized avidin was accessible (biding ratio Bio-DNA:avidin 0.0009:1) (see Table 1). In an attempt to improve the accessibility, a biotinyl-Avicell construct was prepared. Diaminopropylamin was coupled to the matrix surface after bisoxirane activation (Sundberg and Porath, 1973), followed by biotinylation with biotinylhexaglycyl N-hydroxysuccinimide. Biotinylation was demonstrated by acidic cinnemaldehyde, however, the binding of [^{125}I-biotinyl-streptavidin] was found to be poor (44 pmol per 100 µl matrix) and no retention of biotinylated DNA could be demonstrated.

Streptavidin microspheres.

Microspheres offer the advantage over the above matrices in that the surface topology is uniform and they are stable over a large range of low ionic strengths. However, the chemical composition is often not favorable and non-specific binding is a major factor against their use. Streptavidin-hexaglycyl-microspheres were constructed as described in chapter 2 and were shown to efficiently bind biotinylated DNA (binding ratio, Bio-DNA:streptavidin 0.16:1) (see Table 4). But non-specific binding posed a major problem in their successful application for the affinity isolation of biotinylated chromatin (see chapter 3). Furthermore, the introduction of a long and flexible spacer arm was essential to promote the accessibility of the immobilized streptavidin (see chapter 2).

In conclusion, the solid supports investigated were unable to simultaneously fulfill satisfactorily all the criteria postulated for the successful affinity purification of multi-component macromolecular weight biotinylated complexes. In the course of

Table 4. Immobilization of streptavidin on oxirane acrylic microspheres and the assessment of the accessibility of the immobilized streptavidin to biotinylated macromolecular molecules.

Spacer arm	Streptavidin[a] (pmol/mg matrix)	Accessibility[b] (mg matrix/μg Bio-DNA)
-	1.0	2.5
Hexaglycine	0.45	2.0
Insulin A chain	0.39	1.5

a) Efficiency of streptavidin immobilization determined by titration with ^{14}C-biotin binding (see Table 1).
b) Determined as described in figure 2.

my investigations several commercial avidin/streptavidin matrices became available. I found that most of them exhibit similar binding efficiencies. Only one of the magnetic type of matrices showed characteristics suitable for the enrichment of biotinylated chromatin (see chapter 3).

ABBREVIATIONS.

Bio.	Biotin.
Boc.	N-tert-butoxy-carbonyl.
BSA.	Bovine serum albumin.
DEX.	Dextran.
DTT.	Dithiothreitol.
EDTA.	Ethylenediaminetetraacetic acid.
EGTA.	Ethylene glycol-bis-(ß-aminoethyl ether) N,N,N,N-tetraacetic acid.
EM.	Electron microscopy.
GFC.	Glassfibre circles.
HPLC.	High performance liquid chromatography.
LM.	Light microscopy.
NSB.	Nuclei storage buffer.
PMSF.	Phenylmethylsulfonylfluoride.
ppt.	Precipitate.
SA.	Streptavidin.
SDS.	Sodium dodecyl phosphate.
SH.	Sulfhydryl.
SSC.	Standard saline citrate.
SSPE.	Standard saline phosphate EDTA.
TAE.	Tris-Acetate-EDTA.
TBE.	Tris-borate-EDTA.
TCA.	Trichloroacetic acid.
TFA.	Trifluoroacetic acid.
Tris.	Tri(hydroxymethyl) aminomethane.

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