DEOXYRIBONUCLEASE PROBING OF SEA URCHIN EMBRYO CHROMATIN

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SUMMARY

The role that the sea urchin, Parechinus angulosus, embryo and sperm histone variants play in chromatin structure has been investigated. Chromatin structure has been determined at different levels of resolution in sperm and in developing embryos using micrococcal nuclease, pancreatic deoxyribonuclease (DNase I) and restriction endonucleases.

Micrococcal nuclease and restriction endonuclease digestions of sea urchin gastrula chromatin have been analysed and it is shown that it is not possible to isolate large polynucleosomal chromatin complexes which are soluble in low ionic strength buffers. The nucleosomal DNA repeat lengths for sea urchin blastula, gastrula and sperm have been determined using micrococcal nuclease. The repeat length for sperm is significantly larger than blastula and gastrula repeat lengths whereas blastula and gastrula repeat lengths are not significantly different.

Nucleosomal core particles have been isolated from early blastula, gastrula and sperm of sea urchins. After DNase I digestion of 5'-labelled core particles the rate constants of cutting of the DNA at the susceptible sites on these core particles have been determined. The DNase I digestion kinetics of blastula and gastrula core particles are similar whereas sperm core particles are digested at a slower rate, mainly at the sites which are closest to the ends of the core particle DNA. Also, a site, which is 5 bases on the outside of the core particle and which is partially protected from nuclease attack, has been identified. The implications of these findings in relation to the histone variants in embryos and sperm of sea urchins are discussed.

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ABBREVIATIONS

b.p.	- base pair
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
DNase I	- pancreatic deoxyribonuclease
EDTA	- ethylene diamine tetra-acetic acid
EGTA	- ethyleneglycol-bis-(2-amino-ethyl ether) N,N'-tetra-acetic acid
PCA	- perchloric acid
PMSF	- phenylmethylsulfonyl fluoride
SDS	- sodium dodecyl sulfate
TRIS	- tris (hydroxymethyl)-aminomethane

BUFFERS

buffer A - 60 mM KCl
15 mM NaCl
0.15 mM spermine
0.5 mM spermidine
5 mM 2-mercaptoethanol
15 mM TRIS-HCl (pH 7.4)

buffer B - 60 mM KCl
65 mM NaCl
0.15 mM spermine
0.5 mM spermidine
5 mM 2-mercaptoethanol
15 mM TRIS-HCl (pH 7.4)

TBE - 90 mM TRIS-borate (pH 8.3)
2.5 mM EDTA

1. INTRODUCTION

1.1 CHROMATIN STRUCTURE

In 1974, Kornberg proposed that an octamer of two of each of the histones H3, H4, H2A and H2B existed in chromatin and that these octamers, together with histone H1 and about 200 base pairs (b.p.) of DNA constitute the repeating unit observed in electron micrographs of chromatin in low ionic strengths (Olins and Olins, 1974) and upon digestion of chromatin with an endodeoxyribonuclease (Hewish and Burgoyne, 1973). These repeating units are called nucleosomes and are the basic units of structure in almost all eucaryotic chromatins. (The exceptions being certain sperm chromatins which are packaged by protamines which are small, basic proteins).

Prolonged digestion of chromatin by micrococcal nuclease demonstrated that there is a stretch of DNA about 140 b.p. long, in chromatin which is particularly resistant to attack by this enzyme (Sollner-Webb and Felsenfeld 1975; Shaw, et al, 1976 and Noll and Kornberg, 1977). This unit has been called the core particle and is connected to adjacent particles by a stretch of DNA called the linker which is more easily digested by nucleases. The amount of DNA which is associated with the core particle is always about 146 b.p. whereas the linker length differs between species, between different tissues and even within the same stretch of chromatin (Kornberg, 1977; Chambon, 1977 and Strauss and Prunell, 1982).

The primary unit of structure of chromatin has been characterised. Core particles from rat liver chromatin have been crystallised (Finch, et al, 1977). One and three quarters turns of a left-handed superhelix of DNA are wrapped around the octamer of histones. The pitch of this superhelix is

2.8 nm and the DNA is smoothly bent around the protein core. The overall dimensions of the core particle are 11 x 11 x 5.7 nm. Higher resolution crystal structural determinations will show the exact protein-DNA and protein-protein interactions.

In 1976, Finch and Klug proposed a solenoidal model for the next higher structure of chromatin. In low concentrations of $MgCl_2$ the "beads-on-a-string" nucleofilament, observed by Olins and Olins (1974), forms specific aggregates which are proposed to be helices of nucleosomes with a pitch of about 11 nm and diameter of about 30 nm containing about 6 nucleosomes per turn. Hozier et al. (1977) have proposed a superbead model of similar dimensions which consists of about 8 nucleosomes in total. This can be reconciled with the solenoidal model as the superbead may represent a short segment of solenoid. Three fundamentally different models have expanded the idea of a solenoid of nucleosomes in a 25-30 nm fiber (Thoma, et al., 1979; Worcel and Benyajati, 1977 and McGhee, et al., 1980). Thoma, et al (1979) have shown the ionic strength dependence of the formation of this fiber in the presence of H1 and conclude that H1 is positioned at the entry and exit points of the DNA from the nucleosome and that nucleosomes are radially arranged around a central core of H1 molecules with the linker DNA on the inside of the solenoid. Labhart, et al (1982) have shown that similar chromatin structures also exist in metaphase chromosomes of Chinese hamster ovary cells. The essential differences between this model and those of Worcel and Benyajati (1977) and McGhee, et al. (1980) are the location of H1 relative to the solenoid and the positioning of nucleosome linker DNA. Maintenance of the solenoidal structure may not only be the function of histone H1 but also of the inner histones because tryptic removal of the N-terminal, basic peptides of the core histones inhibits solenoid formation under favourable conditions which allow its formation with

intact nucleosomes. (Allan, et al, 1982). The significance of these experiments is, however, debatable as partial removal and possible migration of cleaved peptides before reconstitution with whole H1 could inhibit the H1-DNA interactions.

Various proposals have been made for the next higher structure of chromatin (i.e. packaging of the 30 nm fiber) but little that is definitive is known. Laemmli, et al (1978) have proposed that there is a protein "scaffold" around which metaphase chromosomes are formed. Electron micrographs of these protein "scaffolds" show a remarkable resemblence to a metaphase chromosome. However, Okada and Comings (1980) have shown that mild treatment of metaphase chromosomes do not produce this "scaffold" structure and conclude that it is an artefact produced by the histone depletion method which is necessary for the "scaffold" to be observed in the electron microscope. Benyajati and Worcel (1976) and Igo-Kemenes and Zachau (1978) have proposed that chromatin domains of variable size exist. This concept of giant loop structures has been supported by biochemical analyses. Ethidium bromide intercalation and DNase I nicking, relaxed these supercoiled DNA loops. (Benyajati and Worcel, 1976). Mild micrococcal nuclease and restriction endonuclease digestion of the nuclei produced soluble chromatin segments varying in size from 34000 to 75000 base pairs of DNA (Igo-Kemenes and Zachau, 1978).

One of the objectives of chromatin structure determination is to ascertain the structural prerequisites for regulation of gene expression. This problem has been approached by determining the digestability of particular genes with different endonucleases. Other lines of investigation have also been followed (for review see Weisbrod, 1982). It is well established that active genes possess an altered structure which allows greater acces-

sibility of the DNA to some nucleases. This has been shown for a K-type light chain protein of a mouse leukemia cell line. (Parslow and Granner, 1982). Similarly, the ovalbumin gene in hen, and hormone-treated chick, oviduct nuclei was preferentially attacked by micrococcal nuclease (Anderson, et al, 1983).

Investigation of the chromatin structure of specific genes has led to the question of the location of nucleosomes on genes. Numerous possibilities can be envisaged but some researchers have hypothesised that there is a phase relationship in nucleosome placement at particular sites on genes. Levy and Noll (1980) have proposed that on the heat shock protein gene (hsp 70) in Drosophila melanogaster nucleosomes are precisely phased in at least three frames. 5S RNA genes of D. melanogaster (Louis, et al., 1980) and Xenopus laevis (Gottesfeld and Bloomer, 1980) are non-randomly aligned in some phase relationships. A direct phase relationship has been observed in component α DNA of African green monkey cells (Musich, et al., 1982) and in tRNA genes of X.laevis in transcriptionally inactive erythrocytes. In transcriptionally active liver and cultured kidney cells phasing of tRNA genes is absent. (Bryan, et al., 1981). Similarly, in the histone gene battery of D. melanogaster, non-transcribed spacers display an arrangement of nucleosomes precisely positioned on the underlying DNA sequence in contrast to the transcribed regions which are irregularly spaced (Samal, et al., 1981) while the α subtype histone genes of the sea urchin P. lividus show an altered nucleosomal organization which is dependent on the expression of these genes (Spinelli, et al., 1982).

Kornberg (1981) levelled criticisms against these experiments. The major objection is that micrococcal nuclease shows a preference for cleaving certain DNA sequences (Hörz and Altenburger, 1981; Dingwall, et al, 1981;

Keene and Elgin, 1981 and Pauli, et al, 1982; McGhee and Felsenfeld, 1983). Thus, preferentially digested DNA would be the result of sequence preference of the nuclease rather than of higher order structure. Also a statistical analysis of the products of digestion of chicken erythrocyte chromatin by micrococcal nuclease has shown non-randomness of cleavage (La Fond, et al, 1981). Kornberg (1981) hypothesises that regulatory proteins at the 5' end of genes could allow nucleosomes to form at non-random locations in the chromatin. As to precise phasing of nucleosomes in relation to a specific nucleotide sequence over long stretches of DNA there is no conclusive evidence.

1.2 THE HISTONE CONTENT AND SYNTHESIS IN SEA URCHIN EMBRYOS AND SPERM

By polyacrylamide gel electrophoresis it was demonstrated that histones with different electrophoretic mobility exist in sperm, morula, blastula and prism nuclei of sea urchins (Benttinen and Comb, 1971 and Easton and Chalkley, 1972). By combining electrophoresis with the incorporation of radioactive amino acids, histones H2A, H2B, H3, H4 and H1 were found to be present from the 16-cell stage through to pluteus stage (Ruderman and Gross, 1974). Histones isolated from sea urchin embryos prior to the first S-phase are electrophoretically similar to egg histones (Imschenetzky, et al., 1980). Zygote histones isolated after the first S-phase are also electrophoretically similar to egg histones (Carrol and Ozaki, 1979).

Heterogeneity within one subgroup of the histones was first established in 1974, for the sea urchin sperm histones H2B (Strickland et al., 1974). The existence of such variants could therefore cause different chromatin struc-

tures with different functional properties (e.g. one or more permutations of histone combinations may be associated with actively transcribed genes whereas other permutations may associate with genes which are suppressed).

A stage specific synthesis of histones and the subsequent appearance of these histone variants in the chromatin of developing sea urchin embryos are well-documented (Ruderman and Gross, 1974; Cohen, et al., 1975; Arceci et al., 1976; Newrock et al., 1978; Senger et al., 1978; Arceci and Gross, 1980; Brandt et al., 1979; von Holt, et al., 1983).

During the early stages of embryogenesis, the sea urchin embryo uses maternally synthesised mRNA stored in the egg as inactive messenger ribonucleoproteins. These are released for translation after fertilization and embryological development initially continues in the absence of new mRNA synthesis (Raff, 1980). It was found that maternal histone mRNA, transcribed during oogenesis and stored in the unfertilized egg, was utilized for translation during early development. (Gross, et al., 1973 and Galau, et al., 1976). It has been shown that maternal histone mRNA appears in the polysomes just prior to the first cell division. (Woods and Fitschen, 1978). A potential mechanism for this regulation of translation has been hypothesised to result from a message-specific sequestration of maternal mRNA in the sea urchin egg. (Showman, et al., 1982). Other evidence has shown that at least histone H1 synthesis takes place during the first cell cycle. (Ruderman and Gross, 1974). Also, Herlands, et al. (1982) have shown that cleavage stage and early histones H1, H2A and H2B are synthesised before the first cell division and von Holt, et al. (1983) have also shown early histone synthesis during the first S-phase prior to the first cleavage. Stage-specific changes in synthesis of histone H1, H2A and H2B which depended on differential mRNA content in the polysomes was shown by

Ruderman and Gross (1974), and Ruderman et al. (1974).

Changes in synthesis of the various forms of three histone classes, H1, H2A and H2B have been demonstrated in numerous species of sea urchin embryos. (e.g. Strongylocentrotus purpuratus, (Cohen, et al., 1975 and Newrock et al., 1978) Arbacia punctulata, (Ruderman and Gross, 1974) Lytechinus pictus (Arceci et al., 1976; Senger et al., 1978 and Arceci and Gross, 1980) and Parechinus angulosus (Brandt and von Holt, 1978; Brandt et al., 1979 and von Holt, et al., 1983).

Three stages of histone variant synthesis have been observed in S. purpuratus (Cohen, et al., 1975 and Newrock et al., 1978). The cleavage stage histones (CS histones) are the first set of histones which are synthesised. The production of these histones decreases in the post-morula preblastula period at which stage the α subtype, early or embryonic histone synthesis is increased. Synthesis of the early histones decreases prior to mesenchyme blastula formation when increased synthesis of the β , γ and δ or late or larval histones occurs. In P. angulosus, it has not been possible to show the presence of CS histones. (von Holt, et al., 1983). The synthesis of the first set of histones (i.e. the early subtypes) occurs already 20 minutes after fertilisation (i.e. during the first S-phase prior to the first cleavage) and decreases during early blastulation at which stage synthesis of the second set of histone subtypes increases. (von Holt, et al., 1983). Also, it has been shown that in embryos of S. purpuratus that late H2B gene expression is activated by events occurring at fertilization and that late H2B mRNA synthesis is detectable already at the 16-cell stage but at a low level. This rate of late H2B mRNA synthesis increases 15-fold during a defined period towards the end of blastulation. (Maxson, et al., 1983). This abrupt rise in late H2B gene expression coincides with

a decreased rate of early gene transcription. (Maxson and Wilt, 1981). The pulse-label experiments which demonstrated the stage-specific synthesis of the histone variants also showed that the histones synthesised earlier are retained in the chromatin until at least the prism stage (Newrock et al., 1978; Arceci and Gross, 1980; von Holt, et al., 1983). It is probable that the specialised function that each histone might perform on a particular part of the genome, persists through at least part of the life cycle of the sea urchin embryo. Also, it is probable that histone variants could maintain a state of terminal cell differentiation.

The possibility that the stage-specific histones are products of post-translational enzymatic modifications can be dispelled. "In vitro" protein synthesis from polysomal mRNA from sea urchin blastula, mesenchyme blastula and gastrula in a wheat germ cell-free translation system, yielded the stage-specific histone variants (Newrock et al., 1978). Several histone H2A, H2B and H1 variants isolated from early gastrula, late gastrula and from fully differentiated gut cells of the sea urchin P. angulosus, have been characterised by amino acid composition and partial amino acid sequencing (Brandt et al., 1979). Sea urchin embryo variants are therefore proteins differing in their primary structure and not the result of various permutations of amino acid modifications (e.g. acetylation, phosphorylation, methylation and ADP-ribosylation).

1.3 DEOXYRIBONUCLEASE DIGESTION OF THE CHROMATIN FROM SEA URCHIN EMBRYOS AND SPERM

Various nucleases have been exploited in an attempt to ascertain the structure of the packaged DNA in the nucleus. Consequently, the sea urchin sperm and developing embryo nuclei have been probed by such enzymes as

micrococcal nuclease, (Spadafora and Geraci, 1975; Spadafora and Geraci, 1976; Spadafora et al., 1976(b); Keichline and Wassarman, 1977 and 1979; Cognetti and Shaw, 1981; Savic et al., 1981; Shaw et al., 1981 and Arceci and Gross, 1980) pancreatic deoxyribonuclease (DNase I) (Spadafora and Geraci, 1975; Spadafora et al., 1976(a); Arceci and Gross, 1980(a) and Arceci and Gross, 1980) and spleen acid deoxyribonuclease (DNase II). (Keichline and Wassarman, 1979).

After nuclease digestion for various times, structural differences in chromatin can be analysed by two methods. Firstly, the release of acid soluble, small oligonucleotides with respect to time gives some gross indication of how available the substrate is to the enzyme. Secondly, nucleosomal DNA repeat lengths can be calculated by comparison of data to restriction endonuclease digestions of DNA of known sequence.

Sea urchin sperm chromatin is digested by micrococcal nuclease slower than blastula chromatin (Spadafora et al., 1976(a)). Also, a higher percentage of DNA is hydrolysed in blastula chromatin as compared to sperm. DNase I digestion gives similar results (Spadafora and Geraci, 1975 and Spadafora et al., 1976(a)). Similar comparative experiments have shown that the rate and extent of digestion of sea urchin chromatin decrease progressively as development proceeds from morula through mesenchyme blastula, gastrula, pluteus and larva. (Keichline and Wassarman, 1977; Arceci and Gross, 1980). Blastula chromatin is digested by micrococcal nuclease 4.55 times, gastrula chromatin 3.66 times, pluteus 3.00 times and 11-day larva 1.58 times faster than sperm chromatin. (Arceci and Gross, 1980). Thus when late histone variants begin to accumulate, the rate of enzyme digestibility decreases.

The stage-specific histone synthesis programme during early sea urchin embryogenesis is characterised by switches of histone production just prior to blastulation and at the end of blastulation (Newrock et al., 1978; Arceci and Gross, 1980 and von Holt, et al., 1983). The rate and extent of digestion of chromatin by micrococcal nuclease during the same period of development of the sea urchin embryo show a progressive decrease as development proceeds. (Keichline and Wassarman, 1977 and Arceci and Gross, 1980). These changes in susceptibility could be related to the alterations of the chromatin which probably occur, at least partially, as a result of the synthesis and incorporation of the new histone variants in the chromatin.

Sea urchin sperm and embryo chromatin contains nucleosomes. The nucleosomal DNA repeat lengths of various developmental stages of different sea urchin species are summarised in Table 1.1. In each species the sperm chromatin repeat length is the longest. The repeat lengths determined for A. punctulata embryo (Keichline and Wassarman, 1977), A. lixula embryo (Spadafora, et al., 1976(b)) and S. purpuratus embryos (Keichline and Wassarman, 1979) are very similar (220-223 b.p.), whereas those determined for L. pictus (Arceci and Gross, 1980 and Savic, et al., 1981) and S. purpuratus (Savic, et al., 1981) are interpreted to increase as development takes place. Savic, et al., (1981) correlate these changes in repeat length with the three general classes of histone variants (i.e. sperm histones, cleavage stage histones and the later stage histones).

SPECIES	DEVELOPMENTAL STAGE	REPEAT LENGTH (b.p.)	REFERENCE
Arbacia punctulata	sperm hatching blastula pluteus	260 ± 26 220 ± 22 220 ± 22	Keichline & Wassarman 1977
Arbacia lixula	sperm gastrula	242 223	Spadafora et al., 1976 (b)
Strongylocentrotus purpuratus	sperm morula mesenchyme blastula pluteus gut	250 ± 12 222 ± 10 222 ± 10 222 ± 10 222 ± 12	Keichline & Wassarman 1979
Lytechinus pictus	sperm morula blastula gastrula larva	247 ± 3 213 ± 3 213 ± 3 217 ± 3 230 ± 3	Arceci and Gross, 1980
Strongylocentrotus purpuratus	sperm 8 - cell 16 - cell blastula gastrula	243 ± 3 196 203 ± 1 218 ± 4 215 ± 3	Savic et al., 1981
Lytechinus pictus	sperm 4 - cell 16 - cell blastula	239 ± 4 206 209 ± 3 215 ± 4	Savic et al., 1981

TABLE 1.1 SUMMARY OF NUCLEOSOMAL DNA REPEAT LENGTHS OF SEA URCHIN EMBRYO CHROMATIN

I question the significance of these small differences in repeat length. Keichline and Wassarman (1979) and Savic, et al., (1981) determined different repeat lengths for the same species (S. purpuratus) at blastula stage (i.e. 222 b.p. as opposed to 218 b.p. respectively). Also Arceci and Gross (1980) and Savic, et al. (1981) determined different repeat lengths for L. pictus blastula. These differences are similar to those in a single investigation. In order to establish the correlation between repeat length differences and the programmed synthesis of histone variants, nucleosomal repeat lengths have been determined at various stages where the specific variants are incorporated into the chromatin. (Section 2.4.).

Noll (1976) has hypothesised that the overall basicity (i.e. arginine and lysine content) of the whole nucleosome, the core histones plus one H1, determines the DNA repeat length of the nucleosome. The ratio of basic amino acids in Neurospora crassa nucleosomes to the basic amino acids in calf thymus nucleosomes is similar to the ratio of DNA repeat lengths of the two nucleosome species. Noll refines the hypothesis further by postulating that the difference in basicity of only the H1 histones could account for "shorter linkages of adjacent chromatin subunits". A correlation of the changes in histone H1 variant basicity of the sea urchin embryo with differences in the nucleosomal repeat length could support this hypothesis. Cleavage stage H1 (CSH1) is less basic than early or late H1's (Newrock et al., 1978; Brandt et al., 1979). The repeat lengths of the 4-cell, 8-cell and 16-cell stages, during which predominantly CSH1 is being synthesised and incorporated in the chromatin, appear to be shorter than the later stages where the early and late H1's are synthesised (see Table 1.1).

It is probable that histone H1 primary structure and amino acid content are not the sole determinants of nucleosomal DNA repeat lengths. Sea urchin

sperm H1 from Sphaerechinus granularis digested with trypsin gives a limit peptide of about 85 amino acids (Giancotti et al., 1981). This digestion increases the percentage helicity slightly (26.6% to 33.1%) as measured by circular dichroism. Thus a certain amount of helicity is present in the nose and/or tail section of this H1. In contrast, for calf thymus H1 tryptic core peptide, the percentage helicity is more than doubled (12.9% to 27.6%) indicating a considerable degree of helicity in the nose and tail region. Thus, superficially a correlation may exist between helicity and nucleosomal DNA repeat lengths.

Comparing sea urchin sperm and starfish sperm chromatin repeat lengths and histone contents, Zalenskaya et al., (1981) have suggested the possibility that it is not only the variable H1's which are responsible for changes in repeat length. The sperm H2B of a related species of starfish lacks the N-terminal extension of a pentapeptide repeat structure. (Stickland, et al., 1980). Zalenskaya et al., (1981) suggest that the histones of intermediate variability, H2A and H2B, could bind varying amounts of linker DNA which is determined by the variable N-terminal portion of these two histones, especially H2B.

DNase I digestion of swollen sea urchin sperm and blastula chromatin has shown that digestion of the blastula chromatin is twenty times faster than sperm chromatin (Arceci and Gross, 1980(a)). DNase I recognises a novel structural feature of the sperm chromatin. This enzyme cleaves sperm chromatin into fragments which differ from each other by 500 b.p. Blastula chromatin does not show this large repeat length. However, as in the case of other chromatins, DNase I also digests the sperm chromatin into a characteristic 10 base repeat ladder (Arceci and Gross, 1980(a) and Keichline and Wassarman, 1979). DNase II digestion of sperm chromatin exhibits

the characteristic series of fragments at 10 base intervals (Keichline and Wassarman, 1979; Arceci and Gross, 1980(a)). No 500 base pair repeat is observed with DNase II. This large repeat length may be due to the unique sperm histone H1 or possibly the larger H2B's found in the sperm of sea urchins (Strickland et al., 1977(a); Strickland et al., 1977(b); Strickland et al., 1978). Because few proteins other than histones have been observed in these nuclei, (Arceci and Gross, 1980(a)) it is probable that the compaction of DNA and thus the nucleosome repeat length is due solely to the presence of the histones.

1.4 PROPERTIES OF THE SEA URCHIN NUCLEOSOMAL CORE PARTICLE

The physico-chemical properties of the core particle from the sea urchin, Strongylocentrotus purpuratus sperm have been investigated (Simpson and Bergman, 1980). The DNA content of this core particle is 145 base pairs to which is bound the normal histone core. It has the sedimentation coefficient of 11.0S and circular dichroism shows a maximal ellipticity at 282 nm which is similar to chicken erythrocyte core particles. However, sea urchin sperm core particles differ from chicken erythrocyte core particles by two criteria. Firstly, the computed derivative melting curve of the sperm core indicates a greater stability of this core particle to heating. The highest temperature transition occurs at 78.5°C, about 2°C higher than that for the erythrocyte particle. Simpson and Bergman (1980) conclude that this difference is due to the more basic H2B histones in sea urchin sperm core particles. Secondly, DNase I digests chicken erythrocyte core particles faster than sea urchin sperm cores and the latter are less susceptible to hydrolysis by DNase I at specific sites than the former. (Simpson and Bergman, 1980).

Blastula and pluteus core particles have been investigated from the sea urchin Strongylocentrotus purpuratus (Simpson, 1981). The derivative melting curves of these core particles are very similar but the thermal transitions occur at temperatures below those required to disrupt chicken erythrocyte core particles. Blastula core particles expand under the stress of lowered ionic strength at a significantly higher salt concentration in comparison to sperm and pluteus stage core particles which expand similarly to calf thymus at lower ionic strengths. It is hypothesised that there is an increase in accessibility of blastula and pluteus core particles to DNase I at particular sites and blastula cores are allegedly digested by this enzyme roughly twice as fast compared to pluteus core particles. It thus appears that the histones binding DNA in blastula nucleosomes do so with weaker forces than in pluteus.

By two dimensional gel electrophoresis, Zalenskaya et al., (1981(a)) have shown that mononucleosomes which contain a single H2B isotype may exist in sea urchin sperm chromatin of two species (Strongylocentrotus intermedius and Scaphechinus mirabilis), and have concluded that the population of sperm nucleosomes may be heterogeneous. The possibility exists that this heterogeneity of nucleosomes may play a role in supranucleosomal chromatin higher structure in the sperm nucleus.

Pulse-labelling of developing sea urchin embryos and subsequent fractionation of the micrococcal nuclease-digested chromatin on sucrose gradients and analysis of the histone content of each nucleosomal fraction have shown that cleavage stage histones are laid down in the chromatin in the form of nucleosomes, from the two-cell stage onwards (Shaw et al., 1981). The cleavage stage histones persist in nucleosomes right through to the prism stage. Such results imply the existence of different classes of nucleo-

somes, heterogeneous in their content of histone variants. Monomer nucleosome histone content was however shown to parallel the total histone population of the cell (Shaw et al., 1981). However, the separation methods used did not allow differential separation of nucleosomes with differing histone composition.

1.5 OBJECTIVES OF THIS PROJECT

Other workers have used deoxyribonucleases to investigate the packaging of DNA in chromatin. This has resulted in the elucidation of chromatin structure at particular levels of resolution. It is the objective of this thesis to use deoxyribonucleases to investigate the alterations of chromatin structure which are brought about by the isohistones in the sperm and embryo of Parechinus angulosus.

2. RESULTS AND DISCUSSION

2.1 GENERAL PROPERTIES OF SEA URCHIN CHROMATIN

2.1.1 ISOLATION OF NUCLEI

In order to investigate chromatin structure using deoxyribonucleases as analytical probes, it is important to maintain the intactness of the DNA in the nucleus to as high a degree as possible. However, endonucleases are present in the nuclei of actively transcribing cells such as rat liver cells (Mechali and De Recondo, 1975) and sea urchin embryo cells (see Section 2.1.3), are associated with DNA polymerases and are mostly dependent on divalent cations for activity (Mechali and De Recondo, 1975 and Parisi and De Petrocellis, 1972). Thus, when isolating nuclei which are to be used for nuclease experiments, the objective is to inhibit the activity of the endogenous endonucleases. The method of Burgoyne et al. (1970) has been chosen because chromatin isolation without endogenous nuclease activity was achieved by using this method.

2.1.1.1 Rat Liver Nuclei

In order to isolate intact rat liver nuclei which were free of cytoplasmic contaminants, Blobel and Potter (1966) centrifuged disrupted cells through 2.3 M sucrose. This technique yielded nuclei which had no endoplasmic reticulum attached and nuclear membranes were largely intact. However, the chromatin of nuclei isolated according to this method is degraded by the endogenous nucleases present, to a series of nucleosomes of various lengths (i.e. mononucleosomes, dinucleosomes, etc) if the nuclei are incubated in

similar buffers at 37°C, without the addition of an exogenous nuclease. (Figure 2.1.1)

Rat liver nuclei were then isolated by a similar method, (Burgoyne, et al., 1970) except $MgCl_2$- containing buffers were replaced by buffers containing low concentrations of the polyamines, spermine and spermidine. These nuclei were incubated in buffer A, 0.25 M sucrose and 10 mM $MgCl_2$ for various times at 37°C. The reactions were stopped, the DNA isolated and analysed on a 1% agarose gel in TBE. After 2 hours incubation no detectable degradation of the DNA took place. (Figure 2.1.2) Thus it is possible to isolate rat liver nuclei in which endogenous nuclease activity is not detectable.

2.1.1.2 Sea Urchin Embryo Nuclei

Having thus established that the Burgoyne method (Burgoyne, et al., 1970) of isolation yields nuclei with undegraded chromatin, sea urchin gastrula nuclei were isolated according to the same method. Though the final step of centrifuging the nuclei through 2.3 M sucrose resulted in a white pellet, this was only a small fraction of the total of nuclei present (about 25% as determined by DNA content). Most of the nuclei (as identified by phase contrast microscopy) did not penetrate the heavy sucrose and were floating on top of the solution. Lowering the sucrose concentration to 1.75 M did not increase the yield of nuclei in the pellet substantially. It was concluded that the different density of a large proportion of the nuclei may be the result of adhering, low density, cytoplasmic contaminants. It thus became desirable to remove cytoplasmic contaminants from nuclear preparations by using a detergent which would solubilise the lipid part of the nuclear membrane and thus release attached particles. The technique of Keichline and Wassarman (1979) was modified accordingly. The

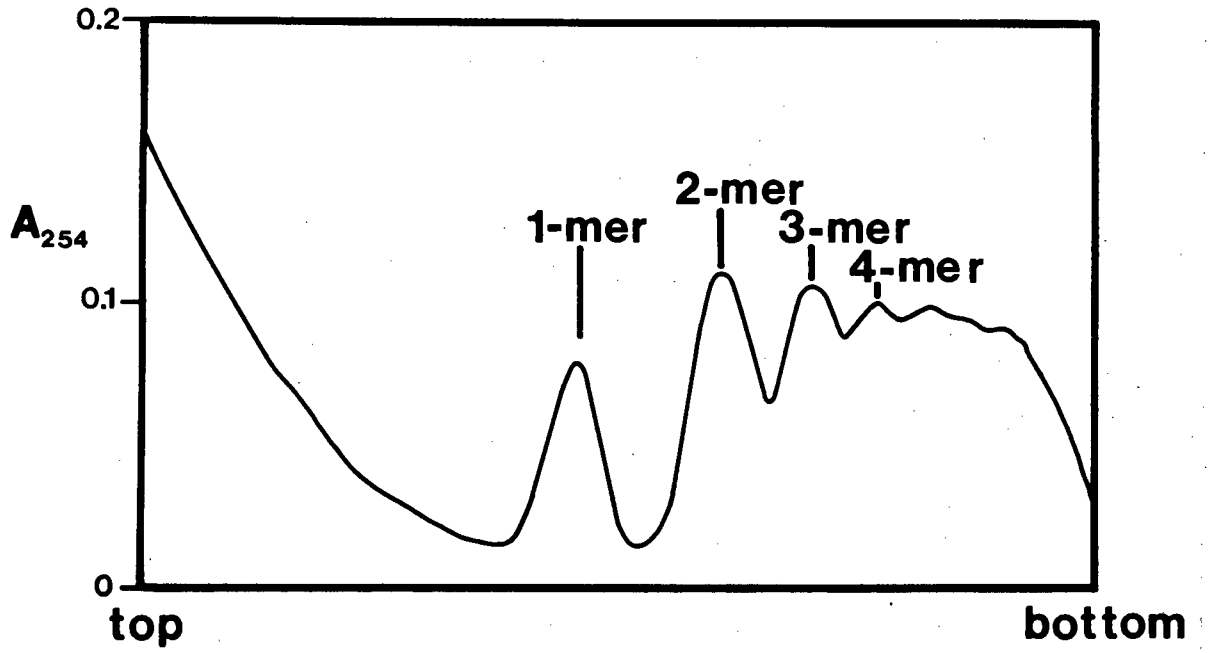


FIGURE 2.1.1 Linear 5 - 20% sucrose density gradient profile of rat liver nuclei isolated in buffers containing 1.5 mM $MgCl_2$ (Blobel and Potter, 1966), incubated at 37°C for 16 minutes in the same buffer and then extracted with 0.2 mM EDTA-TRIS (pH 7.9). Gradients were centrifuged at 170 000 x g for 18 hours at 4°C.

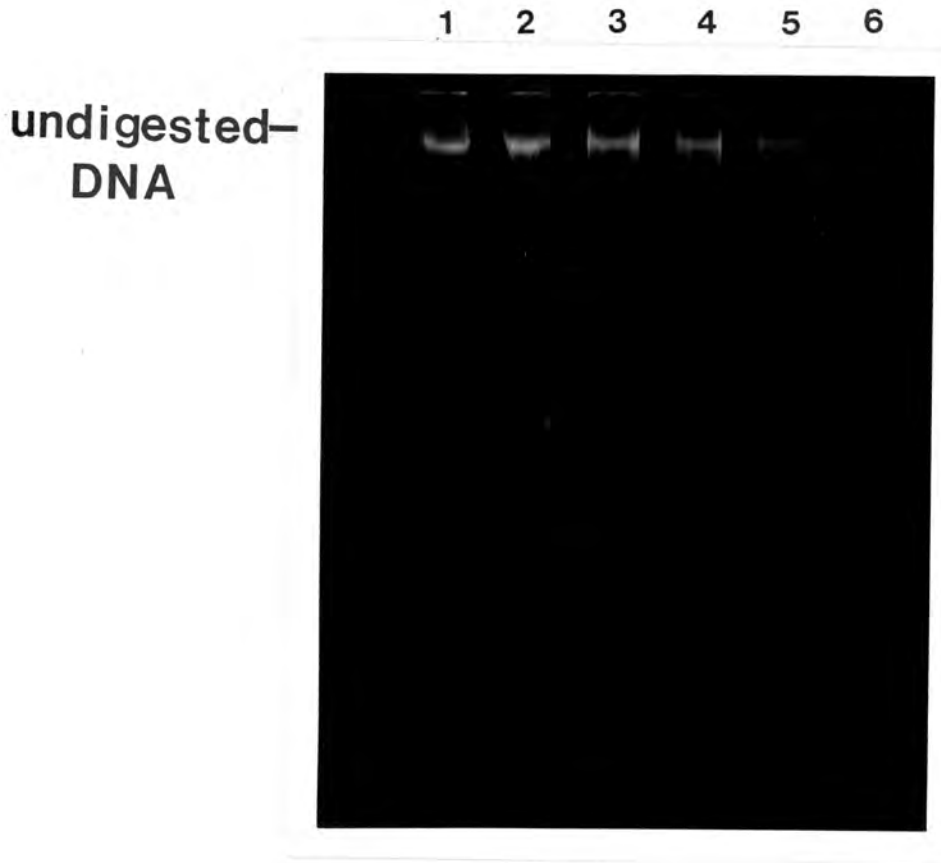


FIGURE 2.1.2 1% agarose gel of DNA extracted from rat liver nuclei isolated in polyamine buffers incubated for various times in the same buffer with 10 mM $MgCl_2$ at 37°C. Lane 1 = zero time, lane 2 = 30 minutes, lane 3 = 1 hour, lane 4 = 2 hours, lane 5 = 4 hours and lane 6 = 7 hours.

modification was that the $MgCl_2$ buffers used by these authors were replaced with Nonidet LE as opposed to Triton-X100. Triton-X100 absorbs strongly at 260 nm whereas Nonidet LE shows no absorbance, thus more accurate DNA determinations can be performed spectrophotometrically. Nuclei isolated by this method were orange-white and seemed intact when viewed under the phase contrast microscope although there was slight clumping indicating partial release of chromatin from the envelope. This technique was used to isolate nuclei from embryo stages as early as blastula to late gastrula with a reproducibly high yield of nuclei. It should be kept in mind that these nuclear pellets represent nuclei in which the nuclear membrane is severely altered by the extraction of the lipid part by the detergent.

2.1.2 ENDOGENOUS ENDONUCLEASES IN SEA URCHIN EMBRYO CHROMATIN

An endodeoxyribonuclease has been described from nuclear extracts of blastulae of the sea urchin, Paracentrotus lividus (Parisi and De Petrocellis, 1972). It has been shown that the activity of this enzyme changes during embryonic development. The activity pattern parallels DNA synthesis because a reduced nuclease activity corresponds to reduced mitotic activity during gastrulation. (De Petrocellis and Parisi, 1972). Also, endonuclease activity has been shown to be associated with, but separable from DNA polymerase activity in nuclei of sea urchin S. purpuratus and S. franciscanus blastulae. (Loeb, 1969).

To test whether chromatin isolated from gastrula embryo of the sea urchin, P. angulosus, according to the method of Keichline and Wassarman (1979) in $MgCl_2$- containing buffers also contains such an endogenous nuclease, gastrula chromatin was isolated. The pellet was incubated at 37°C in the final washing buffer, repelleted and soluble chromatin extracted. This was

analysed by density gradient ultracentrifugation. The sucrose density gradient profile (Figure 2.1.4) shows that the chromatin is digested by an endonuclease to a series of nucleosome monomers and polymers. The fraction marked (1) contains DNA which is mononucleosomal length (216 b.p.) and the fraction marked (2) contains DNA lengths which are multiples of the mononucleosome (432 and 611 b.p. respectively) (Figure 2.1.5).

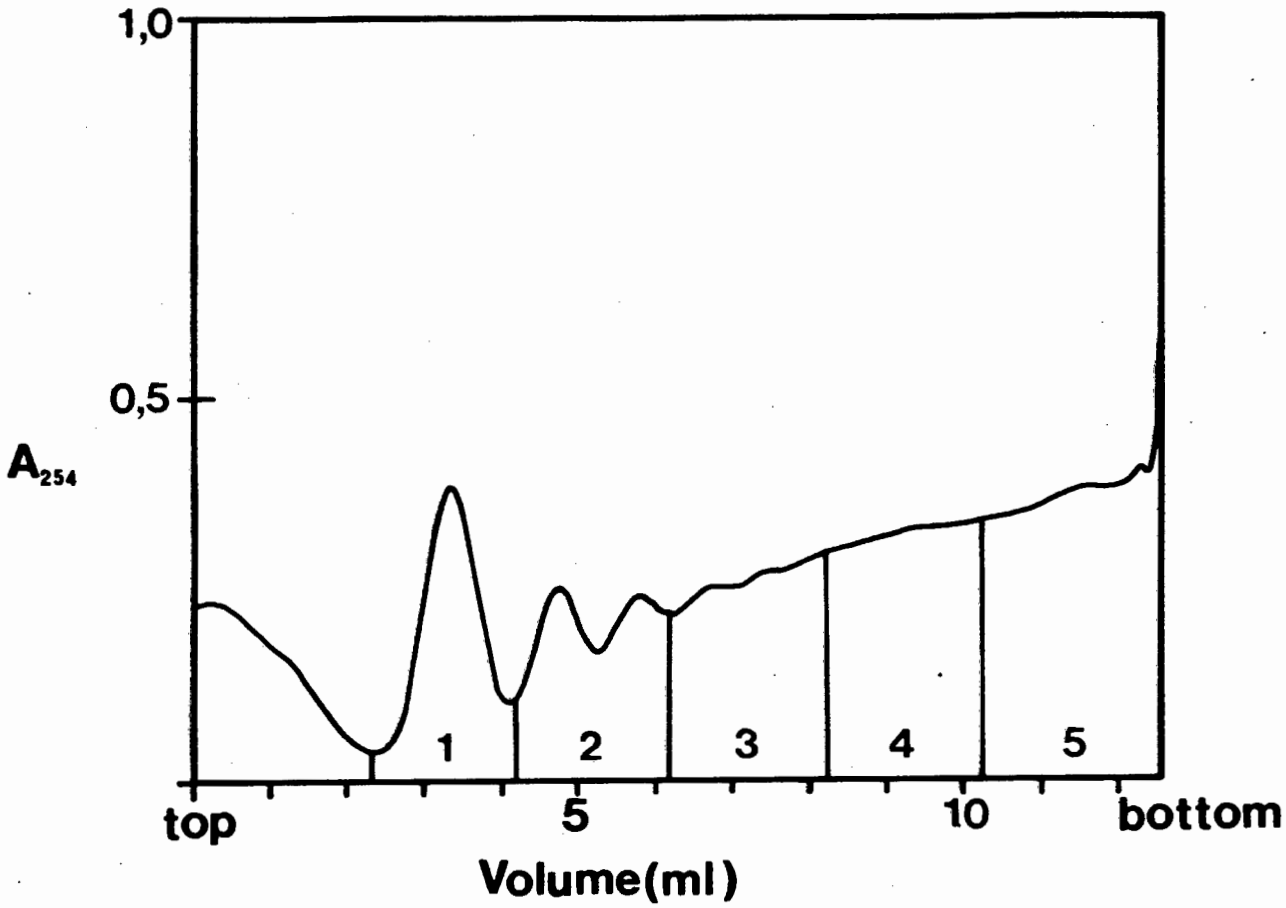


FIGURE 2.1.4 Linear 5 - 20% sucrose density gradient in 2.0 mM EDTA-TRIS (pH 7.5) of sea urchin gastrula chromatin, autodigested in the final washing buffer for 2 minutes and extracted with 2.0 mM EDTA-TRIS (pH 7.5). Gradients were centrifuged at 130 000 xg for 16 hours at 4°C. Five fractions were pooled as indicated.

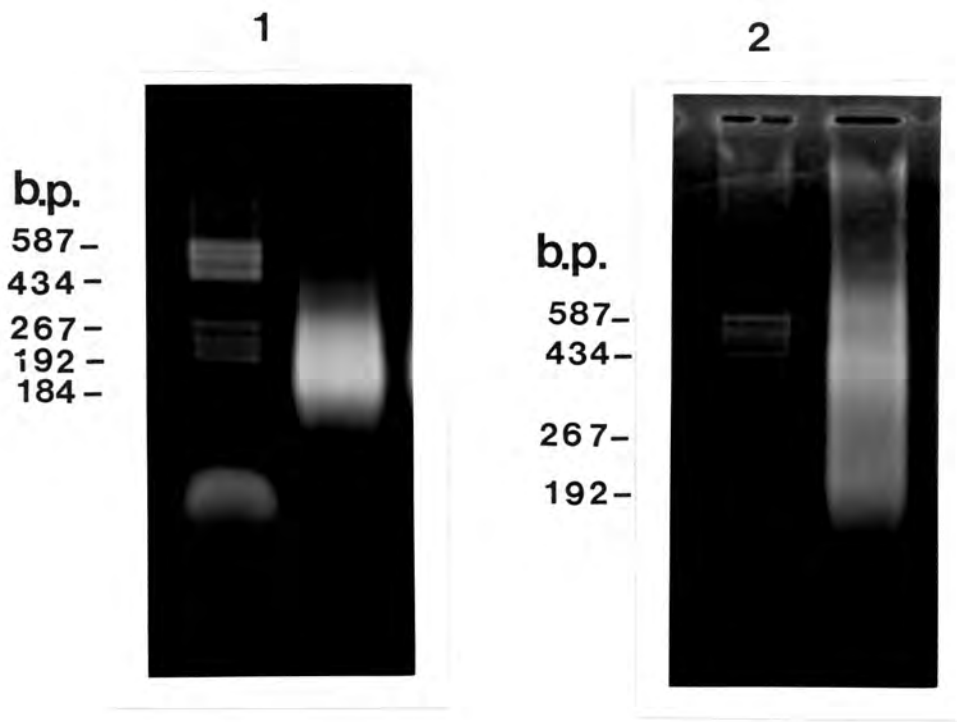


FIGURE 2.1.5 Gel electrophoresis of DNA extracted from fractions 1 and 2 of Figure 2.1.4 analysed on 3% agarose gels in TBE buffer. The standard was a Hae III digest of the plasmid pBR 322.

The mononucleosome fraction from Figure 2.1.4 was concentrated, treated with bacterial alkaline phosphatase to remove the 5'-phosphate group, labelled with ^{32}P at the 5'-end and then digested with DNase I. The products of digestion were analysed by denaturing gel electrophoresis and autoradiography. (Figure 2.1.6).

A nominal 10 base pair ladder is present at zero time and as digestion continues more low molecular weight DNA is produced and high molecular weight DNA disappears with the 10 base pair ladder present throughout. Thus the endogenous nuclease digests the chromatin in a way similar to DNase I. It is therefore a single-stranded nicking enzyme.

The autodigestion of chromatin into a regular series of multiples of the smallest size unit of DNA was first demonstrated by Hewish and Burgoyne (1973) for rat liver nuclei. A similar enzyme has now been shown to exist in the nuclei of P. angulosus gastrulae. The nuclear endonuclease cleaves DNA on single strands and the products are 3'-hydroxyl and 5'-phosphate oligonucleotides. The structure of the 5'-end can be deduced by the fact that 5'-end labelling is only effective after prior bacterial alkaline phosphatase treatment.

Thus when isolating sea urchin embryo chromatin which is to be probed with exogenous nucleases, it is important to inhibit the endogenous nuclease activity. This is possible by isolating the nuclei by a modification of the method of Keichline and Wassarman (1979) as described in Section 2.1.1.2. Chromatin isolated according to this modification is not degraded by endogenous nucleases. (see Figure 2.3.3, lanes 1).

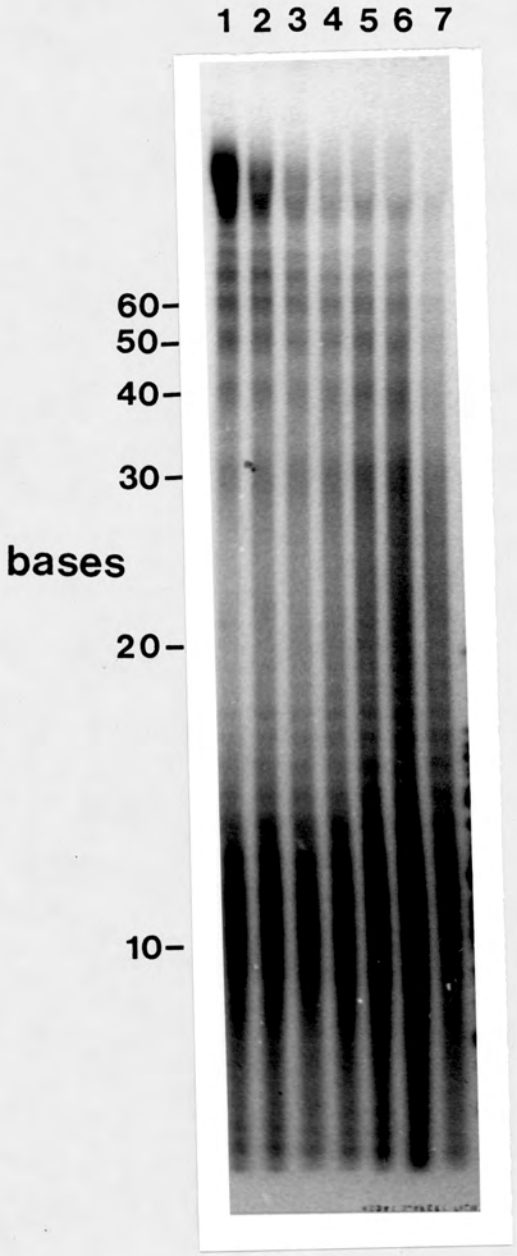


FIGURE 2.1.6 Autoradiograph of the electrophoretogram of DNA from mononucleosomes after digestion with DNase I. Mononucleosomes were labelled with ^{32}P by polynucleotide kinase after bacterial alkaline phosphate treatment. Digestion with DNase I was for zero time (lane 1), 10 seconds (lane 2), 20 seconds (lane 3), 40 seconds (lane 4), 60 seconds (lane 5), 100 seconds (lane 6) and 3 minutes (lane 7). DNA was extracted after the reaction was terminated, analysed on a 20% polyacrylamide denaturing gel and then autoradiographed.

2.1.3 SEA URCHIN CHROMATIN SOLUBILITY

2.1.3.1 Sea Urchin Intercellular and Extracellular Ionic Concentrations

Most marine invertebrates have body fluids with the same osmotic pressure as the sea water that surrounds them. Generally, these animals are called osmoconformers (i.e. they do not regulate osmotic concentration when external changes take place, the osmotic concentration of their body fluids conform with that of the medium). However, body fluids do not necessarily have identical solute composition as the sea water. The sea urchin species are no exception to this general rule (Schmidt-Nielsen, 1979).

The coelomic fluid of Strongylocentrotus droebachiensis has a very similar ionic composition to sea water (Lange, 1964). The sodium and potassium ion coelomic fluid concentrations are not identical to sea water but the overall ionic environment inside and outside the body cavity are essentially similar (see Table 2.1).

Ion	Sea Water	Body Fluid	Intestinal Cells
Ca	10.0	10.1	39.6
Mg	51.9	50.9	
Na	458.0	452.0	143.0
K	9.5	14.0	128.0
Cl	519.0	511.0	270.0

TABLE 2.1 COMPARISON OF IONIC COMPOSITIONS OF SEA WATER, THE BODY FLUID AND INTESTINAL CELLS OF A SEA URCHIN, S. DROEBACHIENSIS (from Lange, 1964). Concentrations are in mmol/l

However, the ionic composition in intestinal cells deviates considerably. These cells must actively maintain the intracellular salt concentrations at different levels to the surrounding sea water and coelomic fluid. Similarly, it has been shown that the ionic composition of unfertilized eggs of Paracentrotus lividus is similar to that in the gut cells of S. droebachiensis. (Rothschild and Barnes, 1953). Tyler, et al (1956) showed that the potassium ion concentration in eggs of the starfish, Asterias forbesii was 17 - 21 times higher than that of sea water and that the sodium ion concentration was one twentieth of that of sea water. Also, Tyler, et al. (1956) showed that a potential difference exists between the inside and the outside of the egg and potential difference changes occur after a sperm cell fertilises an egg. Thus, the sea urchin embryo manages to actively maintain the intracellular ionic strength well below that of the surrounding sea water, the mechanism of which is not well understood.

2.1.3.2 Chromatin Solubility in Buffers containing Monovalent Ions

Solubility measurements of chromatin do not demonstrate fine structural properties. The solubility of chromatin is probably not solely the result of the histone proteins present. The non-histone chromosomal proteins, of which there are many, could also be involved in influencing the solubility properties of the chromatin. However, the histones are the primary structural components present and must therefore play the major role in the manifestation of this property. It is for this reason that solubility will be discussed in terms of histone structure.

The solubility of chromatin preparations from calf thymus (Davies and Walker, 1974), chicken erythrocyte (Muyldermans, et al., 1980) and human placenta (Sahasrabuddhe and Saunders, 1977) decreases to a minimum at a

monovalent cation concentration range of 150-250 mM. Muyldermans, et al. (1980) have demonstrated that this precipitation, in chicken erythrocyte chromatin, is due to the histones H1 because H1-depleted chromatin is soluble between 150 and 250 mM NaCl. Histones dissociate from the chromatin at increasing ionic concentrations. H1 is dissociated above 450 mM, H2A and H2B above 850 mM and H3 and H4 above 1200 mM NaCl in calf thymus chromatin (Burton, et al., 1978). At high ionic concentrations, disruption of the ionic interactions which exist between the basic amino acids, arginine and lysine, of the histones and the phosphate groups on the DNA results in the chromatin solubilisation. Ionic interactions between histones and DNA remain intact in low ionic strengths as nucleosomes have been observed in electron micrographs of chromatin in about 1 mM monovalent salt (Thoma, et al., 1979) and mononucleosomes have a characteristic sedimentation coefficient in low ionic strengths (Strätling, 1979). Disruption of the inter- and intramolecular hydrogen bonds results in the solubilisation of chromatin in low ionic concentration (i.e. below 80 mM).

Sea urchin embryo chromatin inside the cell encounters similar ionic concentrations to those chromatins mentioned above (see Section 2.1.3.1). Thus, it would be expected that similar solubility properties would be displayed. However, initial problems met in the attempts to solubilise sea urchin gastrula chromatin which had been digested by micrococcal nuclease, (see Section 2.3.1) in low ionic concentrations prompted an investigation into the solubility of sea urchin embryo and sperm chromatins (see Section 2.1.3.3).

2.1.3.3 The Solubility of Sea Urchin Gastrula and Sperm Chromatin

Sea urchin gastrula (21 hour), sea urchin sperm and chicken erythrocyte nuclei were prepared using the polyamine buffers of Hewish and Burgoyne (1973). The nuclear suspensions were mildly digested with micrococcal nuclease in order to shear the DNA so that the chromatin solutions are not viscous when resuspended in low ionic strengths. Solubilities over a wide range of NaCl concentrations were determined for each preparation, the results of which are presented in Figure 2.1.3.

Whereas sea urchin gastrula and chicken erythrocyte chromatin show very similar solubilisation at NaCl concentrations greater than 150 mM up to 800 mM, sea urchin sperm chromatin remained insoluble at greater NaCl concentrations. Sea urchin gastrula and chicken erythrocyte chromatins are minimally soluble in the NaCl concentration range of 150-250 mM. Between 250 and 450 mM NaCl, the solubility of these chromatins increases to reach a plateau maximally soluble above 450 mM NaCl. However, sea urchin sperm chromatin is minimally soluble in the concentration range of 0-500 mM NaCl. Above 500 mM NaCl sea urchin sperm chromatin solubility increases to reach also a plateau at about 1M NaCl.

Gastrula chromatin is soluble at very low salt concentrations (i.e. salt concentrations are determined solely by the buffer ions) but becomes increasingly insoluble as the sodium chloride molarity increases to 10 mM and above.

Chicken erythrocyte chromatin, on the other hand, was nearly maximally soluble between ionic strengths 0 - 80 mM. Sodium chloride was dissolved at the concentration given in 0.2 mM EDTA and adjusted to pH 7.5 with 1M TRIS base.

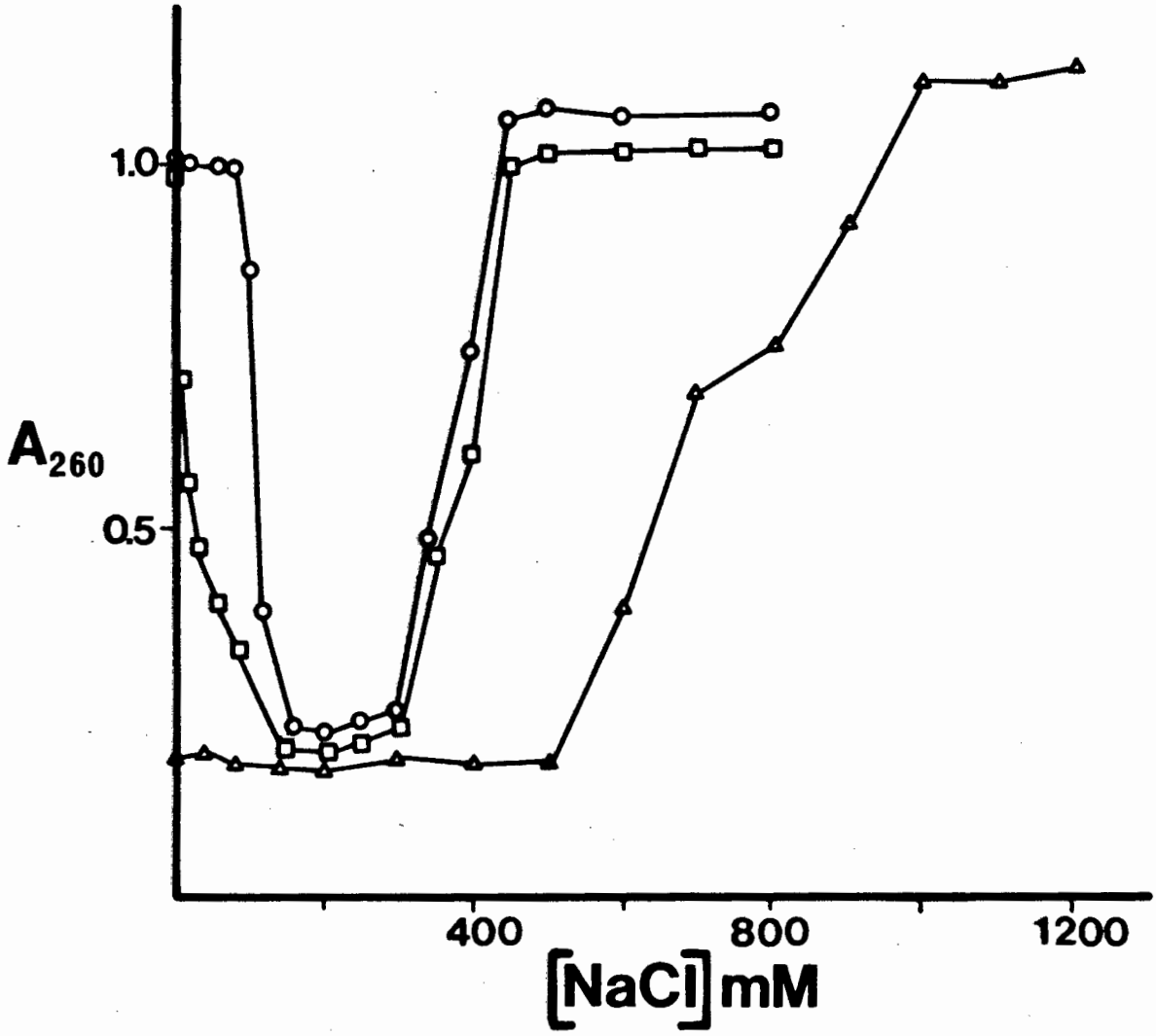


FIGURE 2.1.3 Solubility of chromatin preparations from sea urchin gastrula, sea urchin sperm and chicken erythrocyte as a function of salt molarity at 20°C and pH 7.5. In each experiment the initial DNA concentration was 50 ug/ml.

□ = sea urchin gastrula

△ = sea urchin sperm

○ = chicken erythrocyte

Amino acid analyses of histones from sea urchin gastrula, (Brandt, et al., 1979 and von Holt, et al., 1983) sea urchin sperm (von Holt, et al., 1983) and chicken erythrocyte (Brandt and von Holt, 1974; Laine, et al., 1978 and van Helden, et al., 1982) have been compared. Histones H2A from these cell types do not show great differences in the basic amino acid content. The lysine : arginine ratio is between 1.2 and 1.4. Histones H2B from sperm are more basic than gastrula or chicken erythrocyte H2B's and the arginine content is more than double that of the histones from somatic cells. The lysine : arginine ratio of sperm H2B's is about 0.6 - 0.7 whereas in somatic cell H2B's this ratio is greater than 1.5. Similarly, histones H1 from sperm contain much more arginine and are more basic than the H1's in gastrula and chicken erythrocyte. Thus sea urchin sperm histones are in general more arginine rich than gastrula and chicken erythrocyte.

Leng and Felsenfeld (1966) have shown that polylysine and polyarginine precipitate DNA. However, the polylysine-DNA complex is solubilised by increasing the ionic strength above 1 M whereas greater salt concentrations are required to solubilise polyarginine-DNA complexes. Analogous to this, polylysine-DNA complexes are thermally denatured at a temperature 4.5°C lower than polyarginine-DNA complexes (Olins, et al., 1967). The reason for the lower solubility of the polyarginine-DNA complexes is due to the properties of the guanidinium group of arginine. This can form two hydrogen bonds with oxygen atoms in the same phosphate group while an ionic interaction could also take place between the third nitrogen atom and another phosphate group as seen in crystals of methylguanidium dihydrogenphosphate. (Cotton, et al., 1973). On the other hand, the ϵ -amino group of lysine residues can only participate in a single interaction (i.e. hydrogen bond or ionic, but not both). Thus arginine residues can bind the phosphate groups of the DNA backbone more tightly than lysine residues.

Consequently, histones which have a higher arginine content would be able to maintain DNA-protein interactions in chromatin structures at higher ionic strengths because they would bind more strongly to DNA. This is verified by the arginine-rich histones, H3 and H4, which dissociate from DNA at higher ionic strengths than do the other histones in calf thymus chromatin (Burton, et al., 1978). Assuming that most basic amino acids in the histones interact with DNA, then it would be expected that sperm histones would remain bound to DNA at higher ionic strengths and sea urchin sperm chromatin would thus become soluble only at higher ionic strengths. This is evident in Figure 2.1.3.

As stated above, sea urchin sperm and gastrula chromatin behave differently to each other and to chicken erythrocyte chromatin in low ionic strengths. It can be assumed that a small fraction of the charged groups in chromatin are free to interact with the solvent and would territorially bind their counter-ions. As the ionic strength is decreased, these territorially bound ions diffuse away from their protein or DNA environment. Thus the oppositely charged groups in chromatin are free to interact with each other and, if their proximity is close enough, these interactions would take place. Thus chromatin could remain insoluble even in very low salt concentrations. The solubility of a particular type of chromatin would thus be determined by the location of these oppositely charged ionic groups.

2.2 RESTRICTION ENDONUCLEASE DIGESTION OF SEA URCHIN GASTRULA CHROMATIN

Restriction endonuclease digestion of DNA at specific sites would cleave large pieces of DNA at relatively few unprotected sites in comparison to an enzyme which has a much lower specificity of cleavage, such as DNase I or

micrococcal nuclease. Thus it could be possible to cleave the DNA in chromatin at a few specific sites in order to produce large fragments of chromatin which are manageable in solution. Separation of these fragments, by virtue of their size, after a series of different restriction endonuclease digestions, would permit isolation and, at least partial purification of various elements of the chromatin for further probing. The attempt has here been made to test the feasibility of this approach.

2.2.1 THE SELECTION OF A SERIES OF RESTRICTION ENDONUCLEASES SUITABLE FOR SEA URCHIN DNA CLEAVAGE

DNA was isolated from sea urchin sperm (Kedes, et al, 1975). The purified DNA was dissolved at a concentration of 0.25 mg/ml. Digestion was in the specified restriction endonuclease buffer. 25 units of enzyme were added to 8 µg DNA and incubated for 16 hours at 37°C. The enzymes used were Xba I, Sal I, Hind III, Hind II, Hae III, Eco RI, Bgl II and Bam H1. The reaction was terminated by cooling and adding EDTA in two-fold molar excess. Samples were applied directly to an agarose gel for electrophoretic analysis (Figure 2.2.1)

Comparing the control experiments to the digestions, it can be seen that all the enzymes cleave the sperm DNA but there are fewer sites for certain enzymes. Hind II and Hae III digest the DNA to smallest fragments while Sal I, Eco RI, Bam H1 and Xba I do not cleave the DNA at many sites. Hind III and Bgl II cleave the DNA at an intermediate number of sites. It was thus decided to use Sal I, Eco RI, Bam H1, Xba I, Hae III and Hind III in the digestion of chromatin.

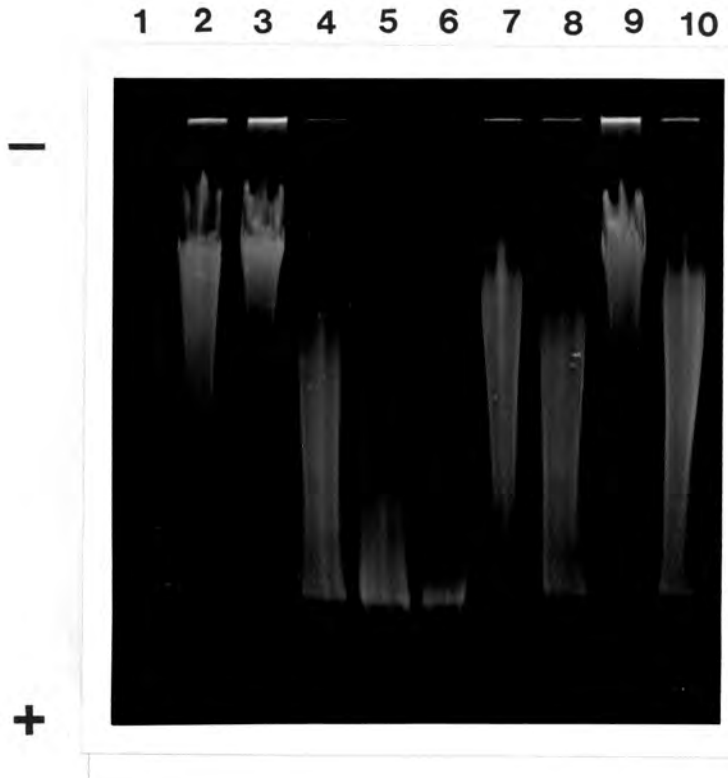


FIGURE 2.2.1: 0.4% agarose gel in TBE of sea urchin sperm DNA digested with restriction endonucleases. Lane 1 - Xba I; lane 2 - Sal I; lane 3 - control; lane 4 - Hind III; lane 5 - Hind II; lane 6 - Hae III; lane 7 - Eco RI; lane 8 - Bgl II; lane 9 - control; lane 10 -Bam H1.

2.2.2 DIGESTION OF CHROMATIN FRACTIONS BY RESTRICTION ENDONUCLEASES

Due to the problems encountered with sea urchin chromatin solubilization in low ionic strengths (2.1.3), it was decided to autodigest the chromatin with its endogenous nuclease (see Section 2.1.2) and then isolate the products in a very low ionic strength buffer. The digestion products were fractionated on a sucrose gradient (see Figure 2.1.4) and the five fractions where pooled, dialysed and concentrated, in 2 mM EDTA-TRIS (pH 7.5), to a final concentration of about 0.25 mg DNA/ml. About 10 µg of each fraction was digested with 15 units of restriction enzyme in the appropriate buffer for 7 hours at 37°C. The reaction was terminated and the DNA was extracted and analysed by gel electrophoresis.

From Figure 2.2.2 it can be deduced that no detectable digestion took place with the restriction enzymes used. Either the complexation of DNA with chromosomal proteins during the isolation procedures for each fraction altered the DNA conformation such that the restriction sites became hidden or buried. Alternatively, the presence of Mg^{++} in the buffers which rendered the chromatin insoluble in most cases made it impossible for the enzymes to attack the DNA. No further investigations in this direction were made. Isolation of large complexes of nucleosomes has also been attempted by mild micrococcal nuclease digestion of the same chromatin (see Section 2.3.1) but this technique gave a low yield of soluble products. Thus, chromatin structure has been investigated at a different level of resolution, the nucleosome and the core particle, in order to ascertain what effect histone variants have on chromatin structure.

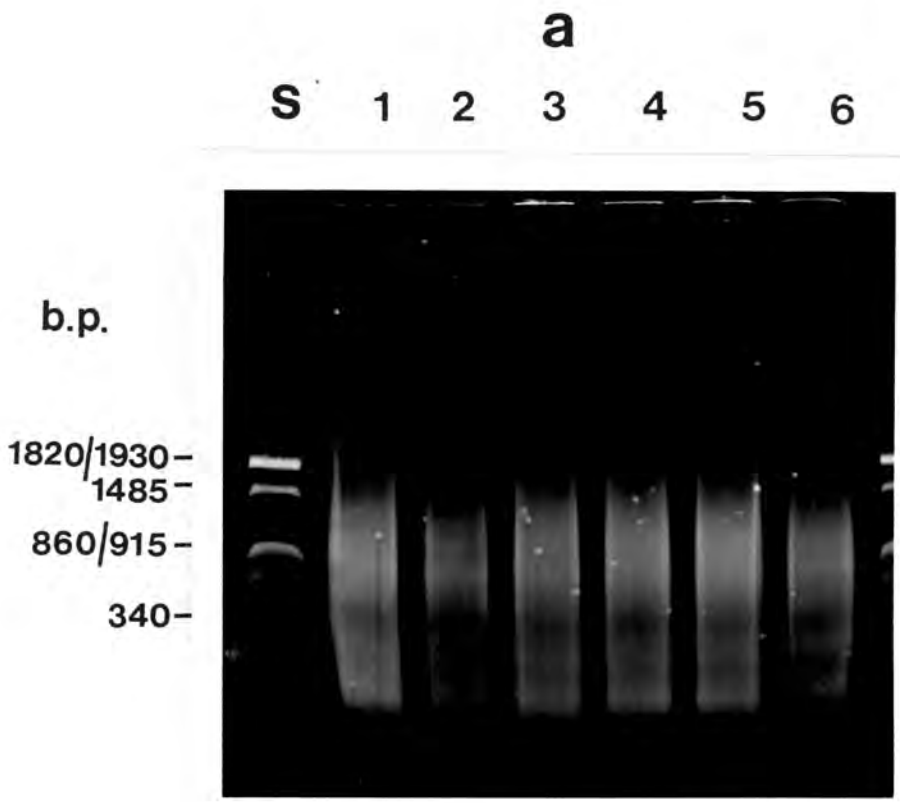


FIGURE 2.2.2 (a) 1% agarose gel in TBE of sea urchin gastrula fraction 3 (Figure 2.1.4) digested with restriction endonucleases.

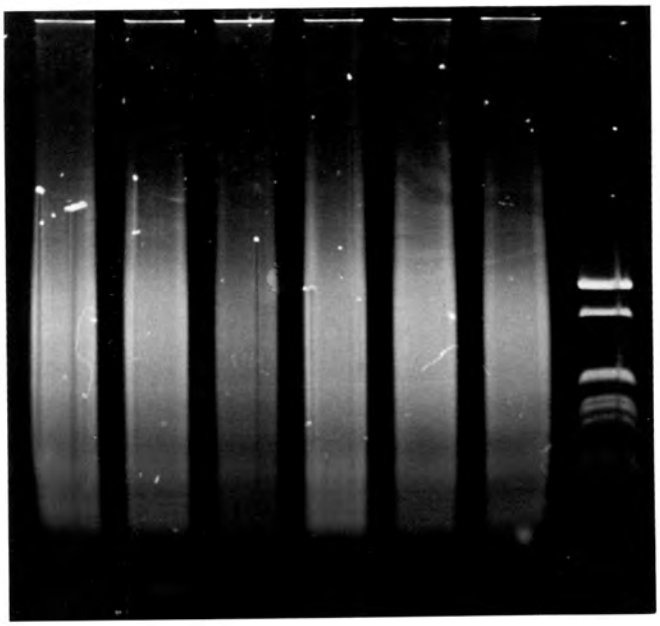
(b) 1% agarose gel in TBE of sea urchin gastrula fraction 4 (Figure 2.1.4) digested with restriction endonucleases.

(c) 2% agarose gel in TBE of sea urchin gastrula fraction 5 (Figure 2.1.4) digested with restriction endonucleases.

lane S - DNA standard: a PM2 - Hae III digest
lane 1 - Bam H1
lane 2 - Eco RI
lane 3 - Hae III
lane 4 - Hind III
lane 5 - Sal I
lane 6 - Xba I

b

1 2 3 4 5 6 S



b.p.

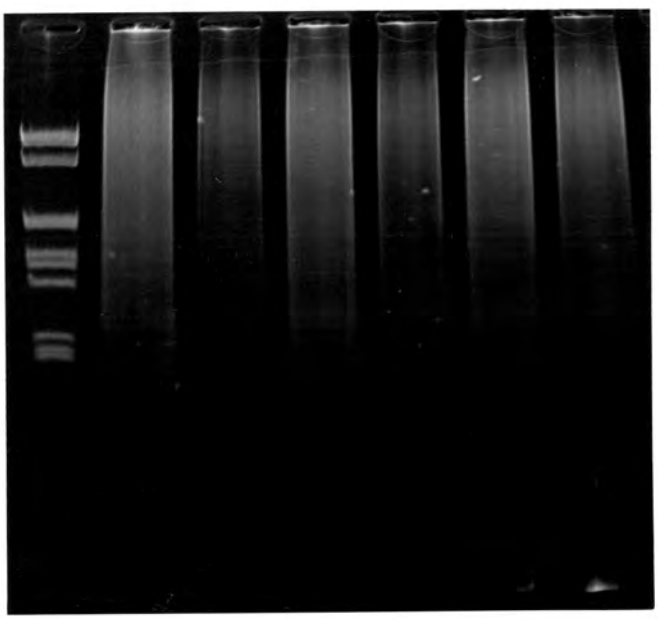
- 1820/1930
- 1485
- 860/915
- 340

c

S 1 2 3 4 5 6

b.p.

1820/1930-
1485-
860/915-
695-
540-
340-



2.3 MICROCOCCAL NUCLEASE DIGESTION OF SEA URCHIN CHROMATIN

2.3.1 DIGESTION KINETICS MEASURED BY DNA SOLUBILISATION AT VARIOUS IONIC STRENGTHS

Ruiz-Carrillo, et al., (1980) and Jorcano, et al., (1980) have isolated a multinucleosome complex from chicken erythrocyte chromatin in low ionic strengths after mild digestion of the chromatin with micrococcal nuclease. Similar complexes have been isolated from rat liver nuclei (Strätling and Klingholz, 1981 and Strätling, et al., 1978). The attempt has here been made to isolate these complexes from sea urchin gastrula chromatin.

Sea urchin gastrula chromatin was isolated according to the modified Keichline and Wassarman (1979) technique using the polyamine buffers of Hewish and Burgoyne (1973). Chicken erythrocyte chromatin was isolated in similar buffers according to Ruiz-Carrillo, et al. (1980). These chromatin preparations were digested with micrococcal nuclease and the chromatin extracted under identical conditions (Figures 2.3.1 and 2.3.2).

A comparison of Figures 2.3.1 and 2.3.2 shows that solubilisation of sea urchin gastrula chromatin by micrococcal nuclease at various ionic strengths is distinctly different to solubilisation of chicken erythrocyte chromatin by the same enzyme. The yield of the products of digestion extracted in 40 and 150 mM NaCl is very similar in gastrula chromatin whereas chicken erythrocyte chromatin digestion products are more soluble in 40 mM NaCl than in 150 mM NaCl. This is consistent with Section 2.1.3 where it is shown that gastrula chromatin is nearly insoluble at 40 mM ionic strength whereas chicken erythrocyte chromatin is soluble. The solubilisation in 150 mM NaCl in chicken erythrocyte chromatin has been shown to be

due to the production of nucleosomal core particles of DNA length 140 b.p., which are soluble at this ionic strength. (Simpson, 1978 and Whittaker, et al., 1979). The amount of DNA solubilised as measured by the 260 nm absorption of the solubilised chromatin represents an over-estimate because non-protein bound oligodeoxyribonucleotides produced by the nuclease have similar solubility properties to the chromatin fractions solubilised at 40 mM and 150 mM NaCl. The large increase in solubilisation at 450 mM NaCl is due to the disruption of H1 interactions. (Burton, et al., 1978 and Muyldermans, et al., 1980). Disruption of H1 interactions at high ionic strengths causes complete solubilisation of the chromatin. (Muyldermans, et al., 1980 and Section 2.1.3.3). Thus, the yield of the products of digestion extracted in 450 mM NaCl would be expected to be greater than the yields of extractions in lower ionic strengths.

It is also evident from Figures 2.3.1 and 2.3.2 that the digestion of chicken erythrocyte chromatin follows different kinetics than sea urchin gastrula chromatin digestion. Digestion to salt soluble products appears to be slower in gastrula chromatin as compared to chicken erythrocyte chromatin. Therefore, either gastrula chromatin must be less accessible to micrococcal nuclease than chicken erythrocyte chromatin or the products of digestion of gastrula chromatin by this enzyme are not as easily solubilised in the various concentrations of buffered NaCl.

2.3.2 ANALYSIS OF DNA PRODUCTS OF MICROCOCCAL NUCLEASE DIGESTION BY DNA GEL ELECTROPHORESIS

In Section 2.3.1 differences were observed in the yield of salt-soluble chromatin after digestion with micrococcal nuclease. The question of whether this differential solubility is the result of the production of chromatin fragments of different sizes will now be investigated.

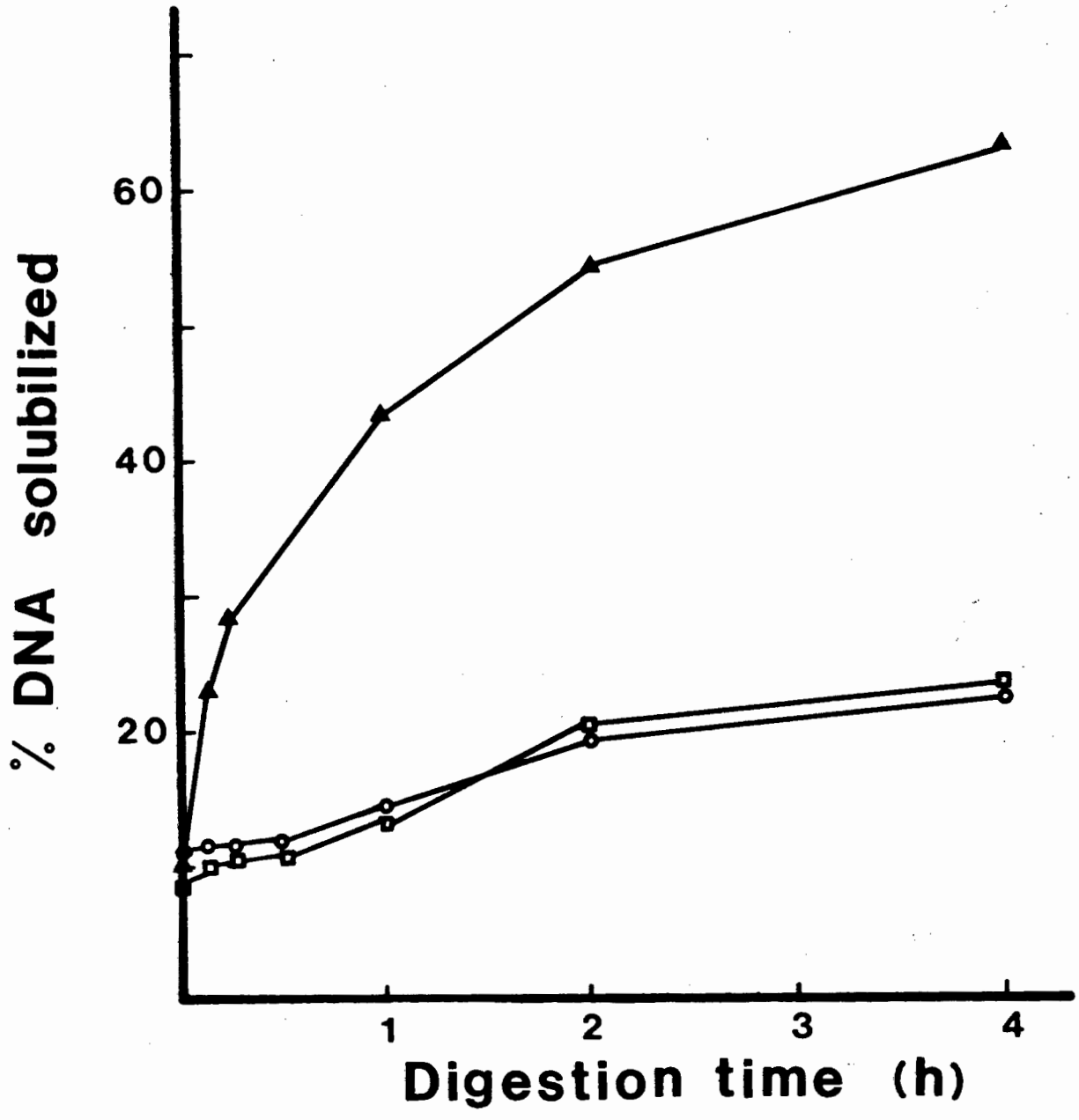


FIGURE 2.3.1 Sea urchin gastrula (21 hour) chromatin digestion by micrococcal nuclease. Digestion was in the washing buffer of the chromatin preparation supplemented with 1.0 mM $CaCl_2$. The DNA concentration was 1 mg/ml and the enzyme concentration was 60 units/mg DNA. Samples were removed at appropriate times and chromatin was extracted in the NaCl concentrations indicated.

(○ = 40 mM extraction buffer, □ = 150 mM extraction buffer and ▲= 450 mM extraction buffer).

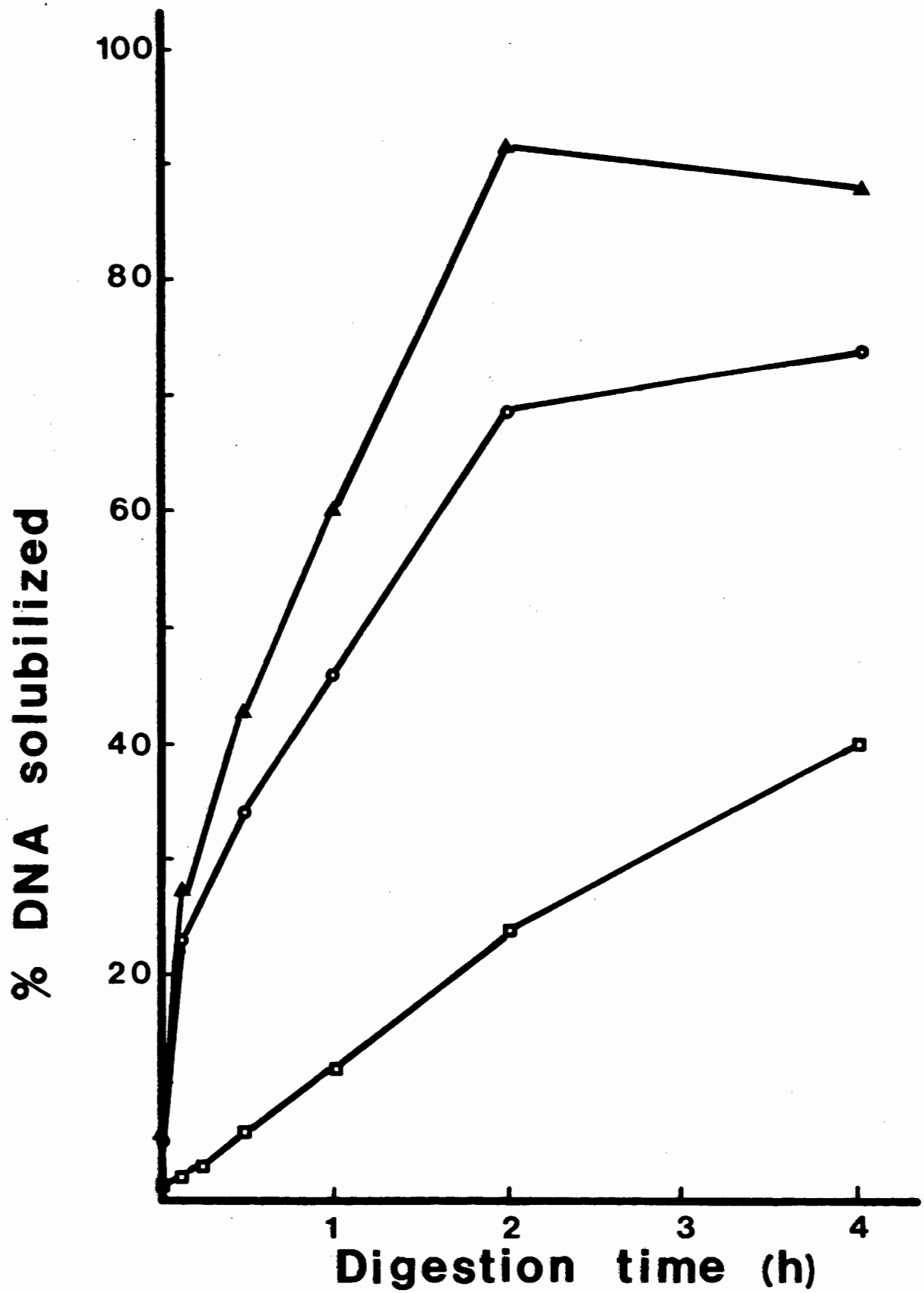


FIGURE 2.3.2 Chicken erythrocyte chromatin digestion by micrococcal nuclease. Digestion and extraction was identical to Figure 2.3.1 (○ = 40 mM extraction buffer, □ = 150 mM extraction buffer and ▲ = 450 mM extraction buffer).

Chromatin was prepared from early (7 hour) and hatched (9 hour) blastula and gastrula (21 hour) embryos in polyamine buffers according to Keichline and Wassarman (1979). Chromatin was digested in the washing buffer supplemented with 1.0 mM $CaCl_2$ and the products were analysed by DNA gel electrophoresis.

Qualitative analysis of Figure 2.3.3 shows that nuclease digestion of chromatin from the various embryological stages is different. A multimeric nucleosomal series exists after digestion for 1 hour in all four cases whereas after 2 hours of digestion predominantly monomeric DNA is present. Comparing lanes 6 (i.e. 1 hour digestions) of all four gels, it can be seen that the multimeric nucleosomal series extends into a much higher molecular weight range in gastrula and sperm than in the two blastula stages. Thus, early and hatched blastula chromatin is digested to monomers faster than gastrula and sperm. In the following section (2.3.3) it is demonstrated that these differences are due to the variable accessibility of chromatins from different embryonic stages to the enzyme.

2.3.3 DIGESTION KINETICS OF CHROMATIN DETERMINED BY THE PRODUCTION OF ACID SOLUBLE PRODUCTS

In order to ascertain a possible role that histone variants perform in chromatin structure of sea urchin embryos, the chromatins of specific embryo stages were probed with micrococcal nuclease. An "early" set of histones has been reported to be synthesised up till the begining of hatching of the blastula at which stage the "late" histone synthesis increases. "Early" histone synthesis decreases towards the end of blastulation. The "late" histones are still being synthesised at the gastrula stage (21 hours) (see Section 1.2).

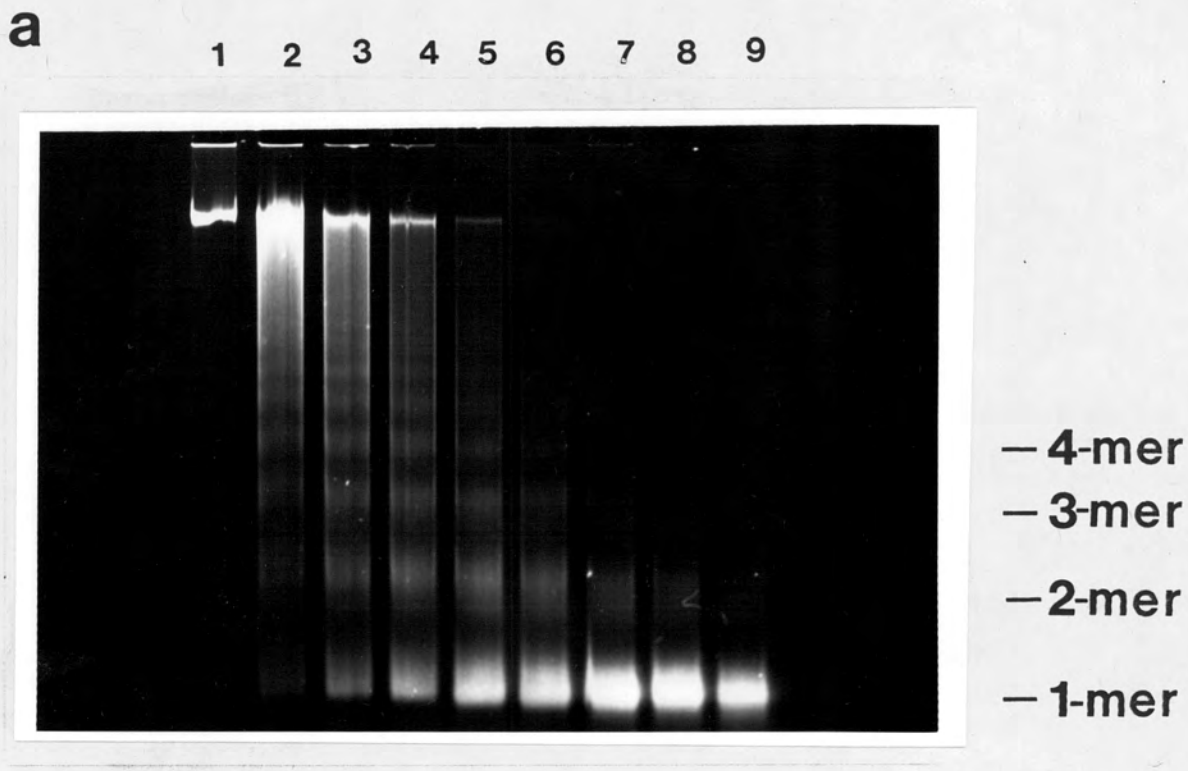


FIGURE 2.3.3 DNA gel electrophoresis of digestion products after micrococcal nuclease digestion of sea urchin embryo and sperm chromatin for various times. Chromatin preparations were digested at 1 mg DNA/ml and 60 units micrococcal nuclease/mg DNA at 37°C. Samples were removed at appropriate times, the DNA extracted and analysed on 2% agarose gels in TBE buffer.

A - early blastula (7 hour)
B - hatched blastula (9 hour)
C - gastrula (21 hour)
D - sperm

lane 1 - zero time
lane 2 - 4 minutes
lane 3 - 8 minutes
lane 4 - 15 minutes
lane 5 - 30 minutes
lane 6 - 1 hour
lane 7 - 2 hours
lane 8 - 4 hours
lane 9 - 8 hours

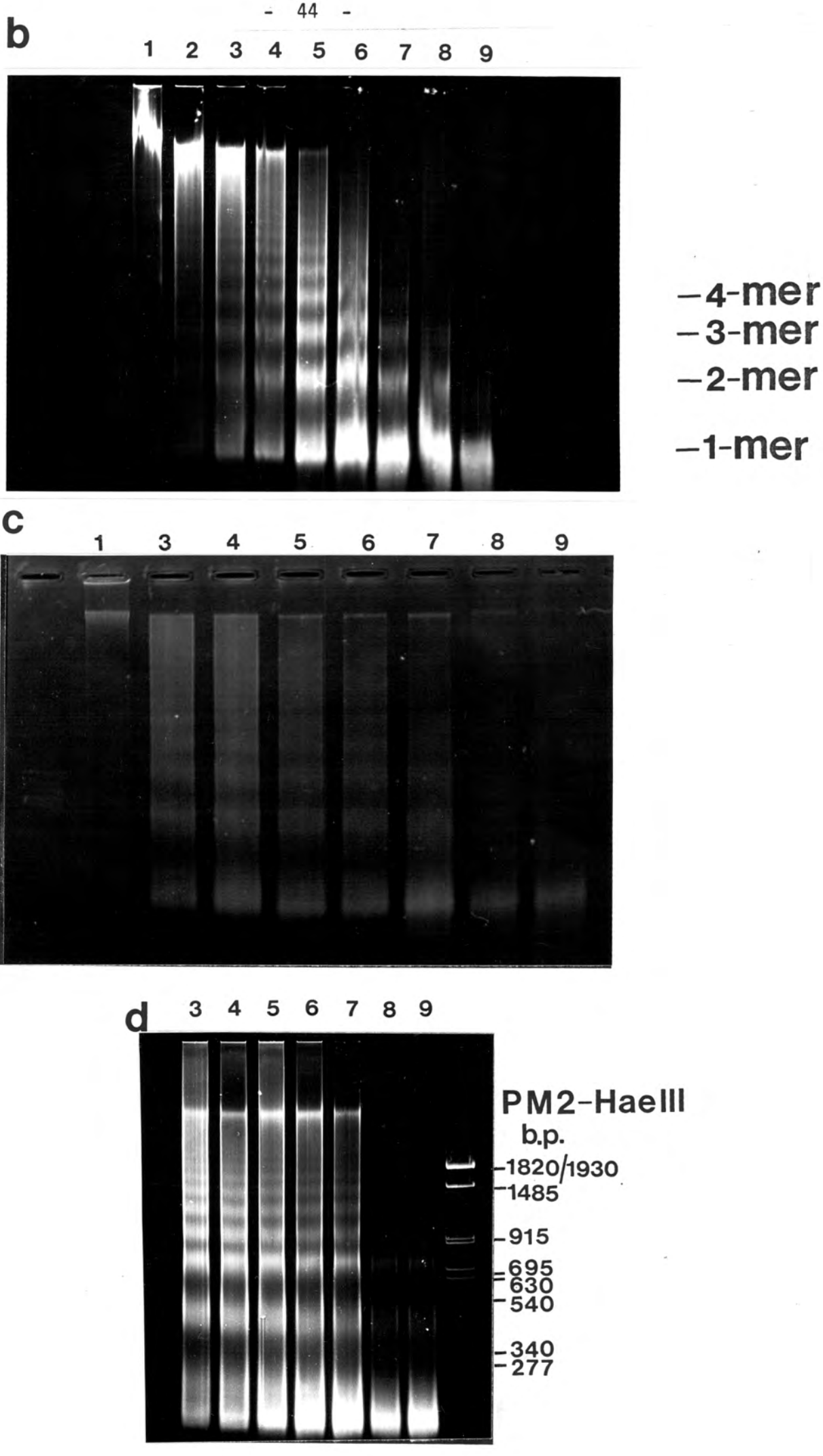

b
1 2 3 4 5 6 7 8 9
4-mer
3-mer
2-mer
1-mer
c
1 3 4 5 6 7 8 9
pBR322
-HaeIII
b.p.
587
434
267
d
3 4 5 6 7 8 9
PM2-HaeIII
b.p.
1820/1930
1485
915
695
630
540
340
277

Figure 2.3.4 shows the rate at which micrococcal nuclease digests sea urchin embryo and sperm chromatin to acid soluble deoxyribonucleotides. The extent (Figure 2.3.4 (a)) and initial slopes (Figure 2.3.4 (b)) of digestion compare well with experiments on other species of sea urchin (Spadafora et al., 1976, Keichline and Wassarman, 1977 and Arceci and Gross, 1980). The early blastula (7 and 8 hour) chromatin is digested most rapidly and close to 80% of the DNA becomes acid soluble. Mesenchyme blastula (12 hour) and gastrula (21 hour) chromatin are digested slower and only about 40% of the DNA becomes acid soluble. Hatched blastula (9 hour) chromatin is the transitional stage where the chromatin is digested at an intermediate rate and about 60% of the DNA becomes acid soluble. Sperm chromatin is digested slowest and only about 20% of the DNA becomes acid soluble.

The transition of the chromatin macrostructure can be observed in the gradual tendency of nuclease digestion kinetics of the early stages to approximate that of the mature blastula and gastrula. Major alterations of the chromatin structure as probed by micrococcal nuclease therefore take place during the blastula stage.

It is well documented that nascent DNA exhibits increased susceptibilty towards micrococcal nuclease. (Levy and Jakob, 1978). Since the cell cycle slows down as the embryo approaches gastrulation (de Groot, 1982) and some cells already reach terminal differentiation with low division frequency, the amount of nascent DNA will become increasingly smaller resulting in a decrease of the digestion plateau reached at the various developmental stages. The very low plateau value displayed by sperm chromatin reflects the higher order packing of the sperm chromatin and the complete absence of nascent DNA.

Mesenchyme blastula, gastrula and pluteus embryos from sea urchins transcribe 28-34% of the total single-copy genome (Hough, et al., 1975 and Kleene and Humphreys, 1977). The single-copy genome represents about 66% of the total genome. Therefore, 18-22% of the total genome is being transcribed during development of the sea urchin blastula and gastrula. Hybridization experiments have demonstrated similar RNA sequences transcribed in blastula and gastrula embryos. (Kleene and Humphreys, 1977). Thus, the relative amount of the genome being expressed is very similar during these stages of development and the actual genes being expressed seem to be similar. The degree of transcribability of chromatin at various developmental stages reported in the literature correlates well with the differential susceptibility towards micrococcal nuclease established in the experiments reported here.

The first major morphologic differentiation step occurs at the 16-cell stage with the formation of mesomeres, micromeres and macromeres (Parisi, et al., 1978). In this work, it has not been possible to isolate reproducibly chromatin digestions from that developmental stage. It is probably at this stage that the structural transition from the cleavage stage type chromatin to the more condensed type takes place. Cell differentiation continues through blastulation (De Petrocellis and Vittorelli, 1975). It is likely that the chromatin in early blastulae and prior to that, represents an open structure and that as blastulation continues and the embryo cells differentiate, so the chromatin assumes a more compact structure. The open structure is more easily accessible to the nuclease and digestion is thus faster and more of the DNA can be digested because the enzyme is less sterically hindered. That the nuclei from early embryos are more fragile and difficult to isolate than nuclei from gastrula or plutei (Keichline and Wassarman, 1979) points in the same direction. Also,

transmission electron micrographs of 4-cell stage embryos from Arbacia punctulata show the chromatin to be finely dispersed whereas chromatin from early pluteus embryos appear to be granular and compact (Arceci and Gross, 1980).

It is therefore concluded that micrococcal nuclease digestion of chromatin to acid soluble products reveals the degree of compaction which is observed under the electron microscope and it is proposed that one of the stages of progressive condensation of embryonic chromatin is initiated during mid-blastula to become completed by the end of blastulation. This condensation is coincident with major morphologic alterations which occur during blastulation.

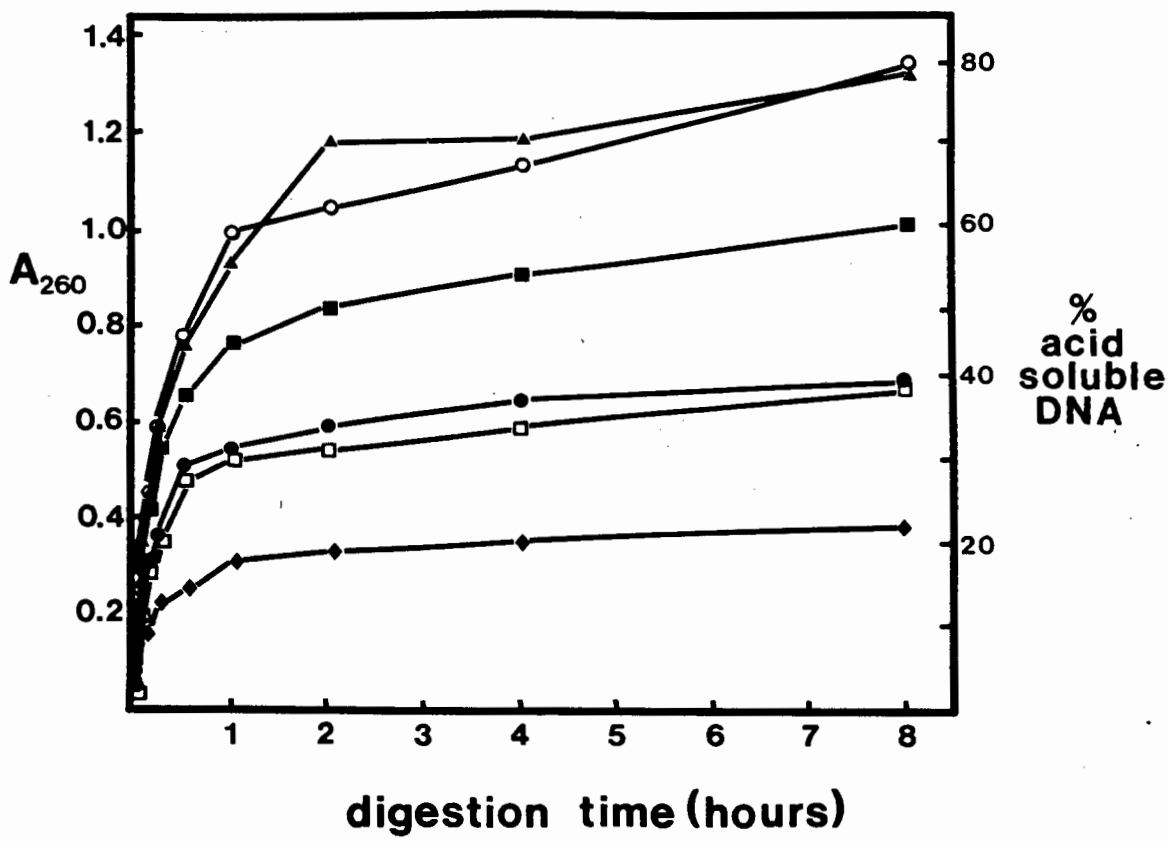
FIGURE 2.3.4 . Micrococcal nuclease digestion kinetics of sea urchin embryo chromatin measured by acid soluble DNA. Chromatin was isolated in polyamine buffers according to the modified Keichline and Wassarman (1979) technique. DNA concentration was 1 mg/ml and micrococcal nuclease concentration was 60 units/mg DNA. Aliquots were removed at the various times into 0.8 M perchloric acid - 0.8 M NaCl (final concentrations). After incubation at 4°C for 10 minutes the insoluble material was pelleted and the supernatant removed. The absorbance at 260 nm of the supernatants was measured. The % acid soluble DNA was calculated according to materials and methods.

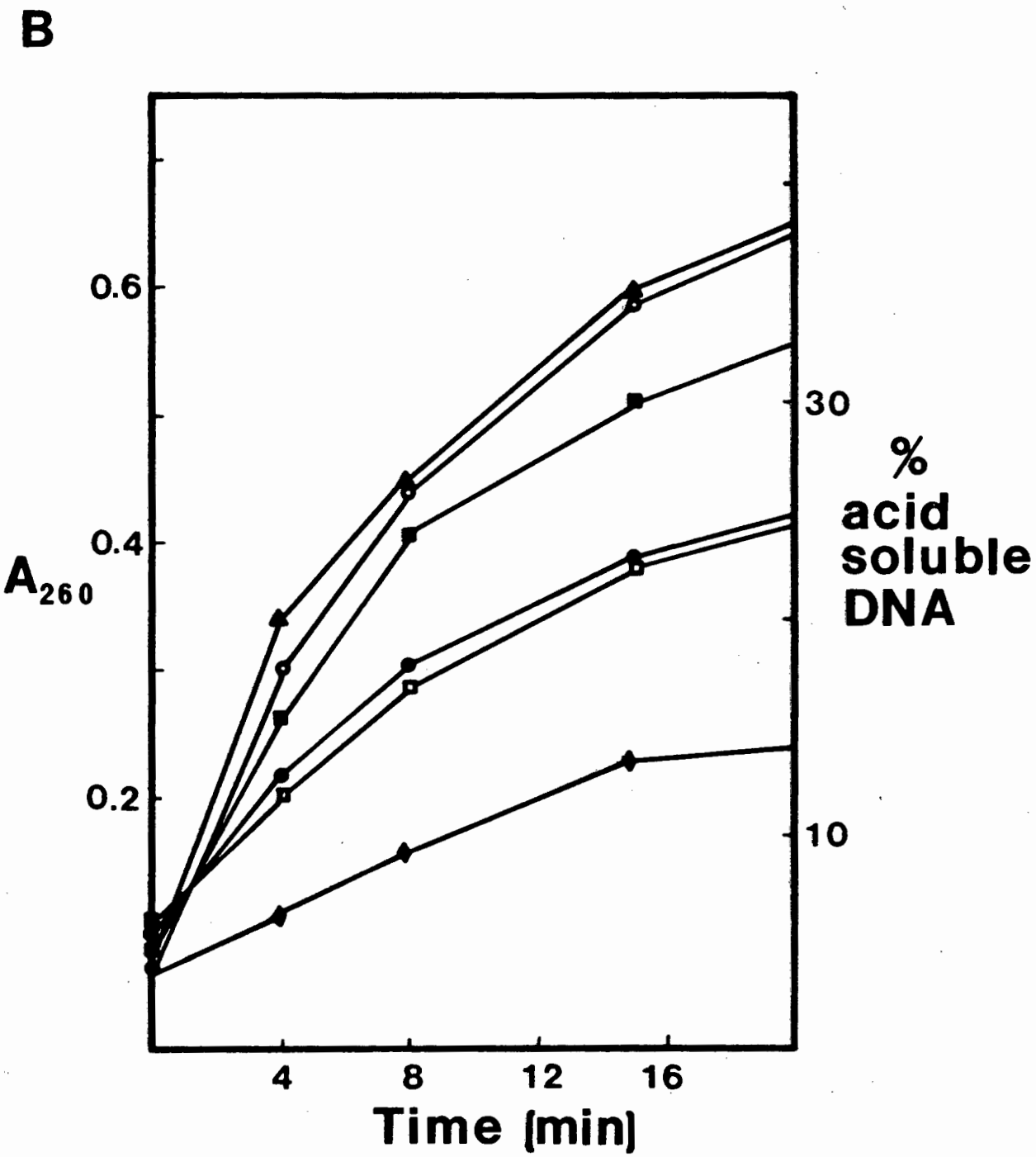
(A) Digestion kinetics over 8 hours

(B) Digestion kinetics over the first 15 minutes (i.e. initial rate)

▲ = 7 hour early blastula
○ = 8 hour early blastula
= 9 hour hatched blastula
= 12 hour mesenchyme blastula
□ = 21 hour gastrula
◆ = sperm

A


1.4
1.2
1.0
0.8
0.6
0.4
0.2
A260
80
60
40
20
% acid soluble DNA
1
2
3
4
5
6
7
8
digestion time (hours)


B
0.6
0.4
0.2
A_{260}
30
10
% acid soluble DNA
4
8
12
16
Time (min)

2.4 NUCLEOSOMAL DNA REPEAT LENGTHS OF SEA URCHIN EMBRYO AND SPERM CHROMATIN

2.4.1 INTRODUCTION

Nucleosomal DNA repeat length determinations demonstrate DNA packaging at the level of the nucleosome. The method determines the average length of DNA which is associated with the octamer of histones plus histone H1 over the whole chromatin. There are differences in these repeat lengths and these differences are probably due to the variable linker lengths of DNA connecting two core particles, which contain about 146 b.p. of DNA each. Repeat length differences have been observed between species and between various tissues from the same organism (for reviews see Kornberg, 1977 and Chambon, 1978). It also seems possible that the linker length can vary within a cell, as originally suggested by Lohr, et al (1977), and subsequently hypothesised by Prunell and Kornberg (1982). An improved method of analysis using exonuclease III has been employed to demonstrate that linker lengths could vary from one nucleosome to the next along a single chromatin fiber (Strauss and Prunell, 1982). Thus nucleosomal DNA repeat lengths are averages over the chromatin and therefore, do not reveal chromatin higher structure at a high level of resolution. It is nevertheless important to ascertain repeat lengths in order to relate histone content, especially histone H1 content, to DNA packaging in the nucleus.

It is conceivable that histone variants could play a role in the manifestation of different nucleosomal DNA repeat lengths. The programme of histone variant synthesis in the developing sea urchin embryo leads to the incorporation of these variants in the chromatin predominantly at specific

stages (see Section 1.2). For the purpose of determining the relationship between histone variants in chromatin and the nucleosomal DNA repeat lengths in that chromatin, repeat lengths have been determined at various stages of embryonic development.

2.4.2 RESULTS

Repeat length determinations were done according to Morris (1976). Photographic prints of DNA gels were scanned using a densitometer, the positions of the maxima of the peaks were measured from the origin and distances migrated were converted to base pairs by comparison to one of the standards, bacteriophages PM2 or ϕX 174 DNA digested with Hae III. The multimer sizes in base pairs were plotted against band number. The slope of the regression line of this plot gives a nucleosomal DNA repeat length which is independent of the effect of the exonuclease activity of micrococcal nuclease. Figures 2.4.1 and 2.4.2 give an example of a DNA gel and the densitometer tracings obtained in such a determination. In Figure 2.4.3 and Table 2.2 examples of the data for a plot of multimer size against band number are presented.

Average values for the repeat lengths of P. angulosus embryos and sperm are presented in Table 2.3. The sperm repeat length is longest at about 250 b.p. This is in agreement with previous work on other species of sea urchin. (Arceci and Gross, 1980; Keichline and Wassarman, 1977 and 1979; Spadafora, et al., 1976(b); Savic, et al., 1981).

The blastula and gastrula repeat lengths were analysed statistically using an analysis of variants. This showed that these values were not different at the 5% level of significance (6 values for each repeat length were used). Thus, the embryonic histone variants do not cause alterations in the chromatin structure at the level of repeat lengths, whereas sperm histones package the DNA into longer repeat lengths.

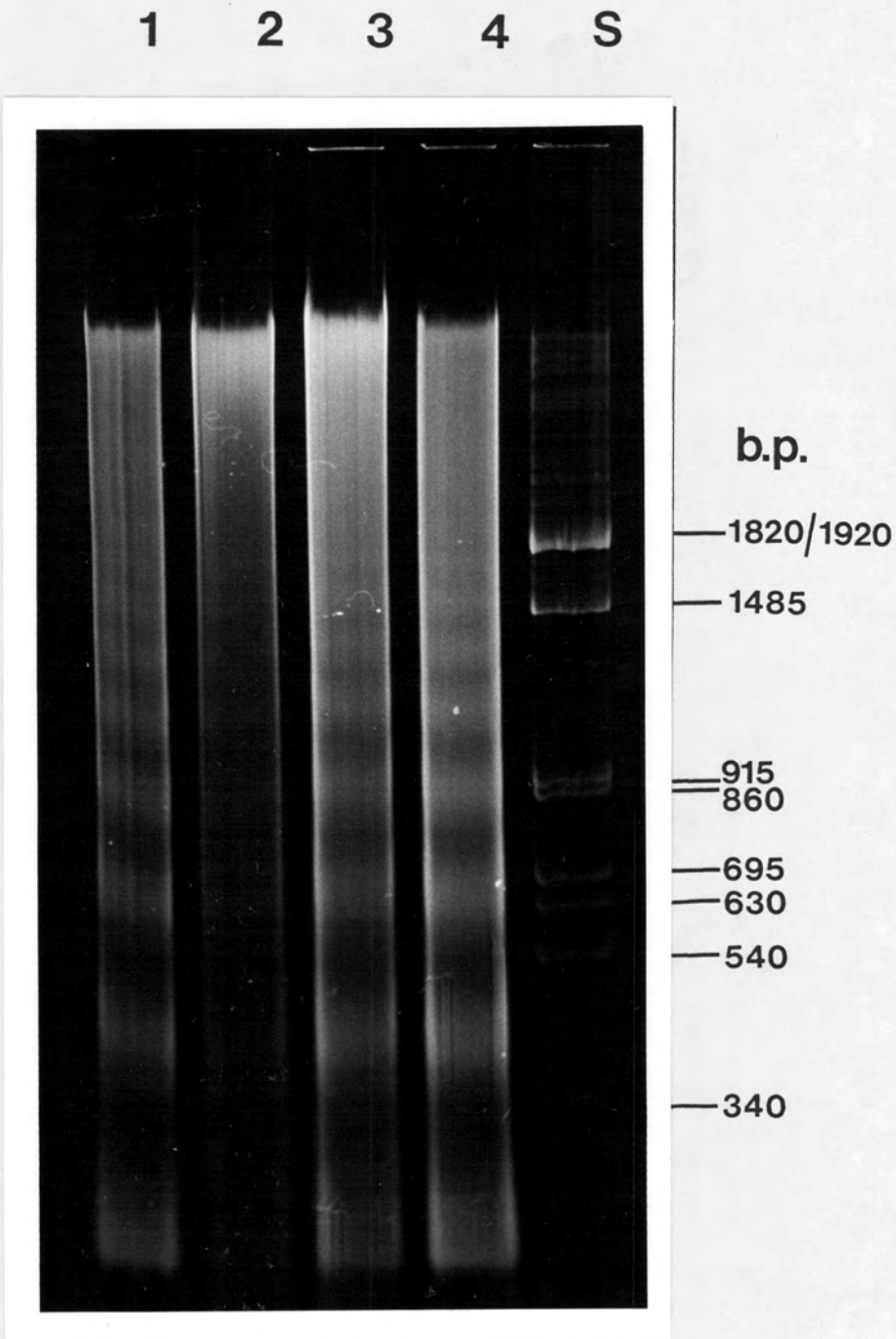


FIGURE 2.4.1: 2% agarose gel of DNA extracted from chromatin digested by micrococcal nuclease. Digestion was according to the legend of Figure 2.3.3. Chromatin was isolated from early blastula (7h) - lane 1; hatched blastula (9h) - lane 2; mesenchyme blastula (12h) - lane 3 and gastrula (21h) - lane 4. The lane marked S is the molecular weight standard, PM2 DNA digested with Hae III. In lanes 1 - 4, the chromatin preparations were digested to 12 - 16% DNA acid solubility.

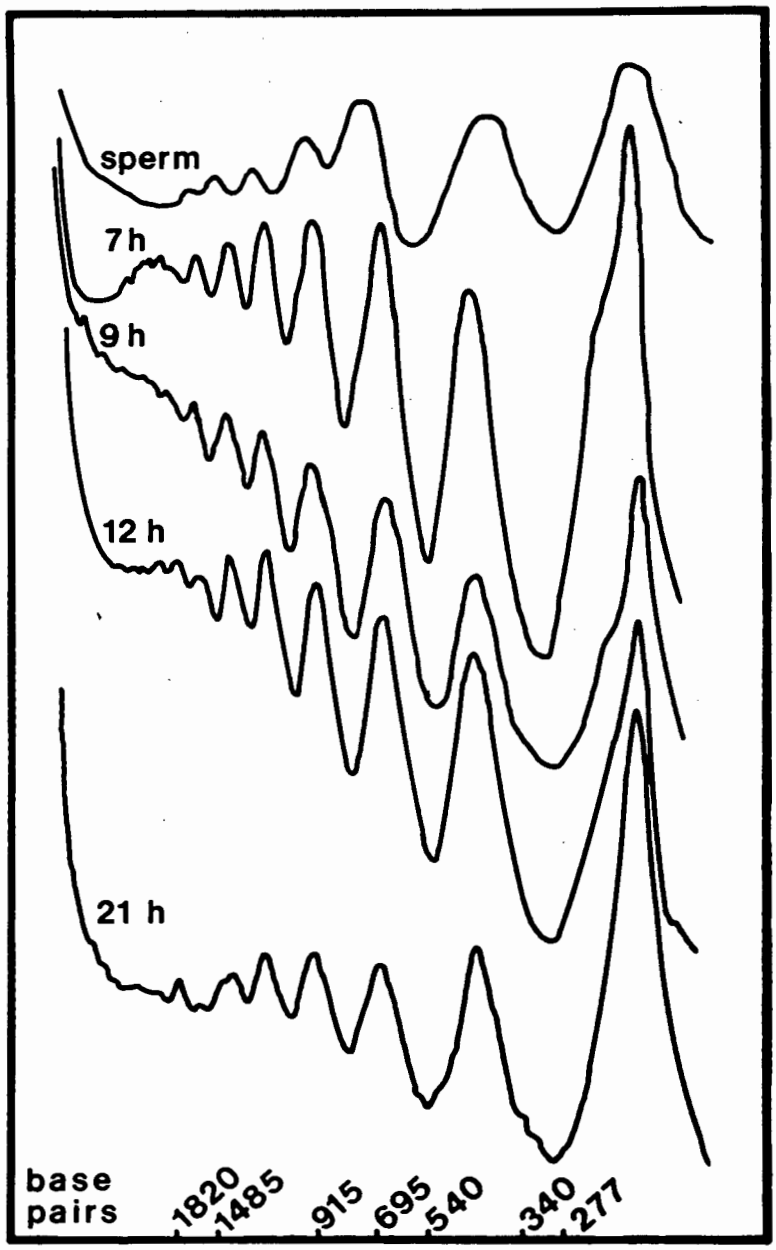


FIGURE 2.4.2 : Densitometer tracings of the agarose gel in Figure 2.4.1. The sperm tracing is from Figure 2.3.3 (D) lane 4.

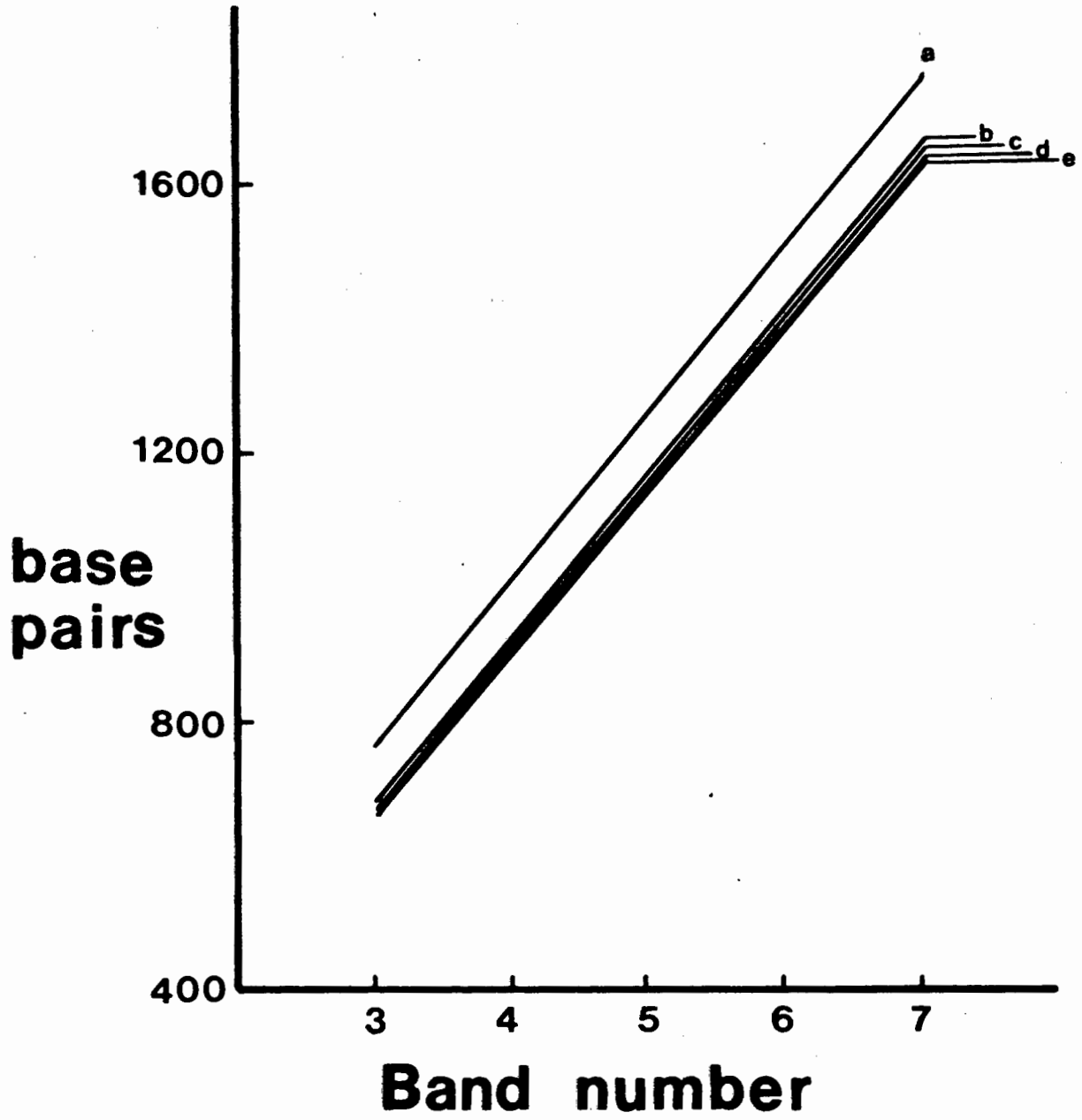


FIGURE 2.4.3: Determination of nucleosomal DNA repeat lengths from different developmental stages. Fragment sizes were estimated from the calibration curve which was a plot of log base pair length against distance migrated. The best straight line through the points was determined by linear regression analysis (see Table 2.3 for data).

a - sperm

b - mesenchyme blastula

c - hatched blastula

d - early blastula

e - gastrula

MULTIPLE	EARLY BLASTULA	HATCHED BLASTULA	MESENCHYME BLASTULA	GASTRULA	SPERM
1	188	186	190	186	173
2	417	412	426	421	443
3	643	650	670	648	777
4	906	916	934	910	1017
5	1155	1166	1190	1161	1268
6	1384	1398	1427	1392	1527
7	1610	1627	1660	1619	1771
REPEAT LENGTH	241	244	247	242	250
CORRELATION COEFFICIENT	0.998	0.999	0.999	0.998	0.999

TABLE 2.2: DNA fragment sizes (in b.p.) of micrococcal nuclease digested embryo and sperm chromatin. This is data from single lanes of 2% agarose gels. The correlation coefficients and repeat lengths are for the trimer to hexamer band sizes plotted against band number.

Embryonic stage	DNA repeat length (b.p.)
7 hour early blastula	239 ± 4
9 hour hatched bastula	242 ± 5
12 hour mesenchyme blastula	243 ± 4
21 hour gastrula	240 ± 5
sperm	250 ± 7

TABLE 2.3: NUCLEOSOMAL DNA REPEAT LENGTHS OF P. ANGULOSUS EMBRYO AND SPERM CHROMATINS

Repeat lengths were calculated as the slope of the linear regression plot of multimer size (b.p.) against band number. (3 lanes from at least 2 separate electrophoresis experiments were used to calculate means and standard deviations).

Arceci and Gross (1980) have observed nucleosomal DNA repeat length increases as development takes place. Their hypothesis states that when the "early" histones are predominant in the chromatin, a shorter repeat length is observed and when the "late" histones are predominant, a longer repeat length is observed. Intermediate repeat lengths are observed in chromatin

from intermediate embryonic stages. Savic, et al. (1981) have proposed a similar role for sea urchin embryo variants including the cleavage stage histones.

Previously, in Section 1.3, the significance of these early embryonic chromatin structural alterations has been questioned. It has been shown here that repeat lengths differences for blastula and gastrula chromatin (i.e. 4 base pairs) are not significantly different. Though the differences and standard deviations observed by Arceci and Gross (1980) and Savic, et al. (1981) are of a similar magnitude to those presented in Table 2.3 for the same embryonic stages, these authors have attached a different interpretation to the data.

Sperm repeat lengths were found to be different to blastula and gastrula repeat lengths at the 5% level of signficance. Others have determined similar longer repeat lengths for sperm chromatin of different species. (See Table 1.1).

Repeat lengths for the embryonic stages of Parechinus angulosus are 10-30 base pairs longer than those reported for other species of sea urchin. (Compare Table 2.3 with Table 1.1) This could be the result of either different preparation methods or a genuine difference in the chromatin structure. The components and the concentration of the various buffers used to isolate nuclei or chromatin could alter the chromatin structure such that the differences become apparent in repeat length determinations. The second reason for the longer repeat lengths in Parechinus angulosus is that there are genuine chromatin structural differences between this species and other species. For example, this species of sea urchin takes up to half

as long to reach blastula and gastrula stages. (Compare de Groot (1982) with Cohen, et al. (1975) and Newrock, et al. (1978)). However, a parallel experiment using as many species as possible would identify the reason for the differences mentioned above.

2.4.3 DISCUSSION

Nuclease digestion studies of chromatin have indicated that there is a regular arrangement of the DNA of approximately 34 to at least 75 kilobase pairs (Igo-Kemenes and Zachau, 1978) with a finer repeat of about 200 base pairs within the nucleus. These latter nucleosomal DNA repeat lengths have been determined for many types of cells and the observed differences have been interpreted to exist for many functional and structural reasons. The nucleosomal core particle invariably contains 146 b.p. of DNA and the connecting linker DNA is a likely reason for the variability of nucleosomal repeat lengths. The linker can vary from 20 b.p. in fungi and yeast to 100

b.p. in sea urchin sperm (Chambon, 1978). Morris (1976) and Noll (1976) have proposed that the difference in the structure of histone H1 is responsible for the changes in nucleosomal DNA repeat lengths. It has been suggested in the literature that there is a correlation between the linker length and the basicity and size of H1. However, it is probable that H1 basicity and size are not the sole determinants of the distances between nucleosomes.

Numerous structural models for protein-nucleic acid complexes have been proposed (for a review see Hèlène and Lancelot, 1982). By model building and other experimental data a nucleoprotamine model has been proposed (Warrant and Kim, 1978). Similarly, the mechanism of binding of the bacteriophage λ cro repressor to its operator site has been hypothesised (Ohlendorf, et al., 1982; Anderson, et al., 1981 and Anderson, et al., 1982). Also, a structure of the catabolite gene activator protein of Escherichia coli bound to DNA (McKay and Steitz, 1981 and Steitz, et al., 1982) and the interaction of various operon repressors and activators with their recognition sequences have been proposed (Gicquel-Sanzey and Cassart, 1982 and Matthews, et al., 1982). All of these models involve protein interactions which are specific for a particular sequence of DNA and it seems that the likely conformation of the protein sequences interacting directly with the DNA, is α-helical.

It has not been possible up to now to show a sequence specificity for histone interactions with DNA. However, it may be possible that the histone conformation at the regions of interaction with the DNA, could be α-helical. Present resolution of crystal structure analysis of core particles does not allow, however, to deduce the secondary structure of histones at such regions of interaction.

The conformation of histone H1 and its interactions with DNA could participate in determining the length of DNA between nucleosomes in a way similar to that suggested above. Thus, it may not purely be a function of the basicity and size of histone H1 which will determine the distances between nucleosomes but also the overall conformation of this histone and its interaction with DNA, with other chromosomal proteins and itself.

2.5 NUCLEOSOMAL CORE PARTICLE STRUCTURE IN CHROMATIN FROM SEA URCHIN EMBRYOS AND SPERM AS PROBED BY DEOXYRIBONUCLEASE I DIGESTIONS

2.5.1 PREPARATION OF CORE PARTICLES FROM SEA URCHIN EMBRYOS AND SPERM

Various methods have been devised to isolate nucleosomal core particles. (Simpson and Whitlock, 1976; Noll and Kornberg, 1977 and Lutter, 1978). In one group of these methods, the chromatin is extracted in buffers which dissociate histone H1, after mild micrococcal nuclease digestion, with the production of long chromatin. This then is isolated and redigested with micrococcal nuclease to core particles (Noll and Kornberg, 1977 and Lutter, 1978). In the second approach, after more extensive micrococcal nuclease digestion, chromatin is extracted in buffers which do not dissociate H1 and the 100mM NaCl soluble monomers which are core particles, are isolated. (Simpson and Whitlock, 1976). The first method has been shown to produce core particles which predominantly contain about 146 b.p. of DNA, which is only slightly nicked. (Lutter, 1978). Also, this method produces a high yield of core particles and the size of the DNA in the preparation can be more accurately controlled. Thus, the first method has been chosen to isolate core particles from sea urchin blastula, gastrula and sperm chromatin, although minor modifications have been made, especially in the case of sea urchin sperm.

2.5.1.1 Preparation of H1-Depleted Long Chromatin

For the isolation of core particles from sea urchin embryos the method used by Lutter (1978) has been modified. The major modification is in the initial micrococcal nuclease digestion of the chromatin to produce long chromatin strands free of histone H1. Endogenous nuclease activity is high in developing embryos (see Section 2.1.2). This causes digestion to take place more rapidly than in terminally differentiated cells. To overcome this problem a lower enzyme concentration is used for slightly longer times. Blastula core particles are nevertheless slightly nicked prior to DNase I digestion (see zero time lane in Figure 2.5.7). However, preparations generally had greater than 60% of the DNA as nominal 146 base pair DNA.

In order to determine the time required to digest the various embryo chromatin preparations with micrococcal nuclease to produce a high yield of polynucleosomes, the nuclease digestion pattern was followed by analysing the extracted products on linear sucrose gradients. Hatched blastula chromatin was prepared according to Keichline and Wassarman (1979) in the polyamine buffers of Hewish and Burgoyne (1973). Chromatin was digested, extracted and analysed according to the legend of Figure 2.5.1. After 30 seconds digestion, most of the chromatin pellets but as digestion continues, the yield of long chromatin increases as does the yield of mononucleosomes. Optimum digestion conditions are achieved when there is a low yield of mononucleosomes and a high yield of polynucleosomes. The digestion times chosen to prepare long chromatin from blastula and gastrula embryo, at the given enzyme:substrate ratio, were 4 minutes (see Figure 2.5.1 (e)). The optimum digestion time for sperm chromatin was determined to be 20 minutes by a similar analysis.

Sperm long chromatin was prepared differently. Due to the solubility properties of this chromatin, (see Section 2.1.3.3) long chromatin was extracted with 1.1 M NaCl. Also, sucrose gradients contained 1 M NaCl, as opposed to 500mM NaCl for blastula and gastrula chromatin. This high salt concentration dissociated H1 but did not dislodge the core histones, as can be seen in Figure 2.5.3.

Long chromatin depleted of H1, was isolated from the bottom half of these gradients. (the shaded area in Figure 2.5.2.) These chromatin preparations consisted of DNA which was predominantly larger than tetranucleosomes (see Figure 2.5.4, zero times).

FIGURE 2.5.1: Hatched blastula chromatin micrococcal nuclease digestion pattern as analysed by 5-20% linear sucrose gradients. Chromatin samples (5 mg DNA/ml) were digested for the times indicated in washing buffer at 1mM $CaCl_2$ with 20 units micrococcal nuclease/mg DNA. The reactions were terminated by adding EDTA to 5 mM and the chromatin was pelletted. Extraction of the pellets was with 500 mM NaCl, (1M NaCl for sperm chromatin) 10 mM TRIS-HCl (pH 7.5), 1 mM EDTA and 0.2 mM PMSF. This was clarified in a low speed spin and aliquots of the supernatants were applied to 5-20% linear sucrose gradients in 500 mM NaCl (1M NaCl for sperm chromatin), 10 mM TRIS-HCl (pH 7.5), 0.2 mM EDTA and 0.2 mM PMSF and centrifuged at 83000 x g for 16 hours at 5°C.

A - ½ minute digestion time

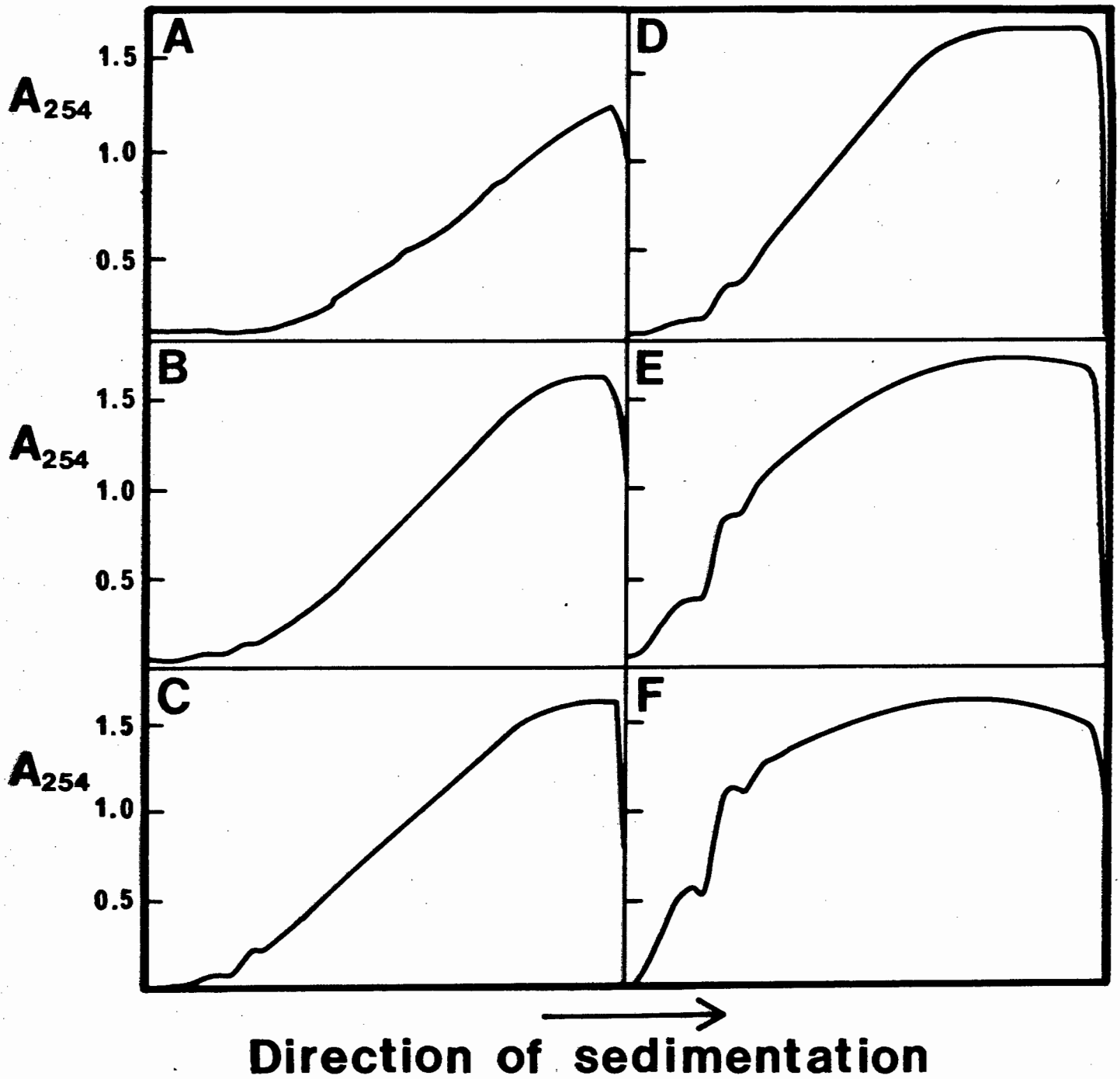
B - 1½ minutes digestion time

C - 2 minutes digestion time

D - 3 minutes digestion time

E - 4 minutes digestion time

F - 6 minutes digestion time


A
B
C
D
E
F
A_{254}
1.5
1.0
0.5
Direction of sedimentation

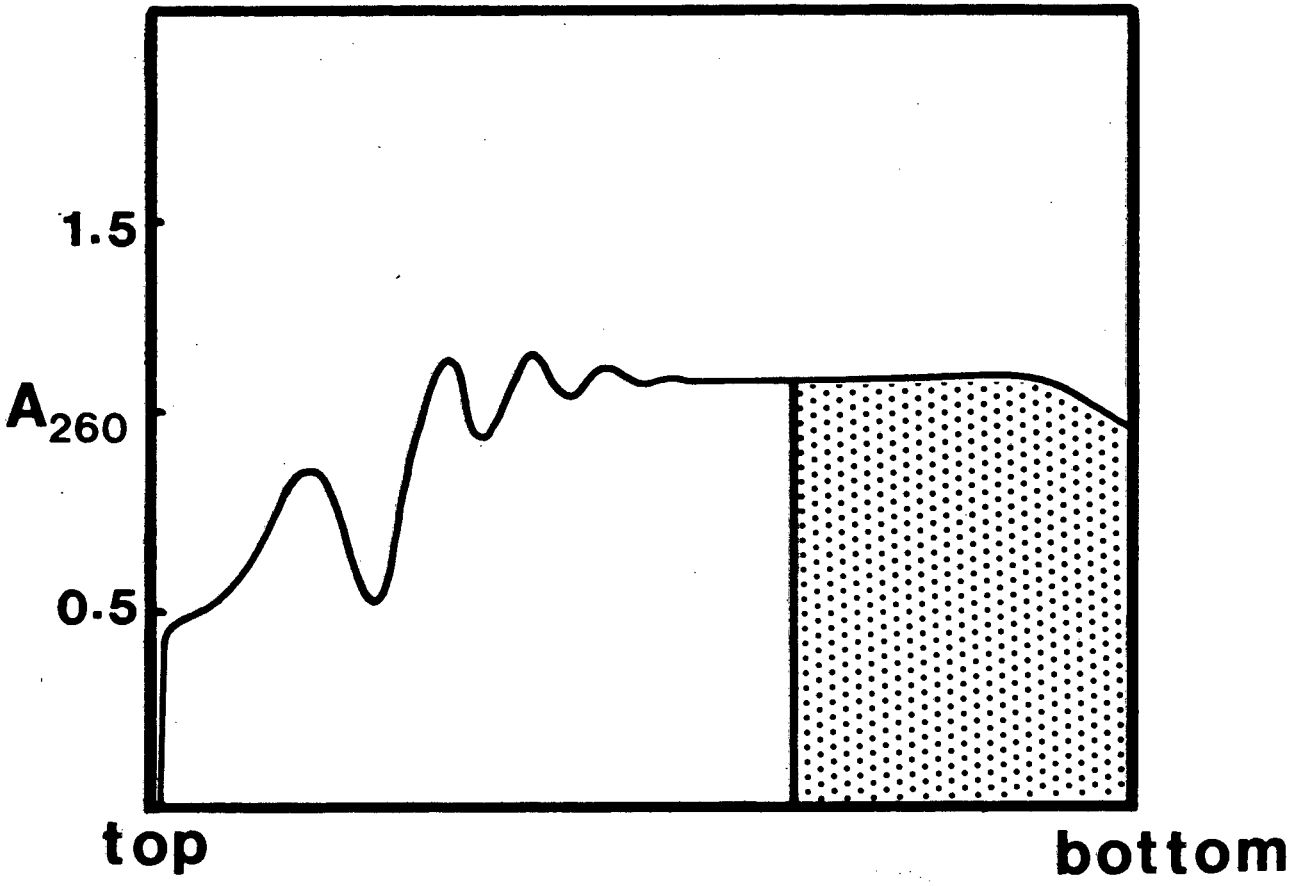


FIGURE 2.5.2: Chromatin from gastrula embryos digested for 4 minutes and analysed as in Figure 2.5.1. The shaded area was pooled, dialysed exhaustively against 50 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 0.2 mM EDTA and 0.2 mM PMSF and concentrated by pressure ultrafiltration to 0.5 - 1.0 mg DNA/ml.

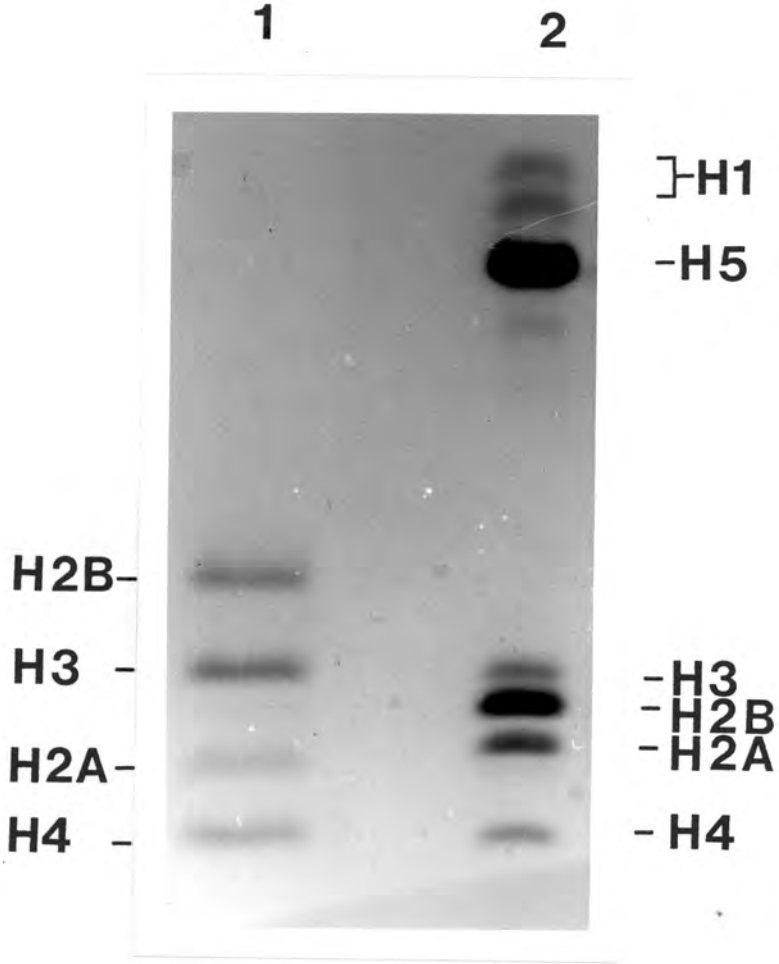


FIGURE 2.5.3: 15% polyacrylamide gel in SDS of histones extracted from core particles of sea urchin sperm.

lane 1 - sea urchin sperm core particle histones.

lane 2 - chicken erythrocyte histones (H5 and H2B enriched, for assignment purposes).

2.5.1.2 Redigestion of Long Chromatin to Core Particles

The long chromatin preparations were digested to core particles with micrococcal nuclease to a nominal DNA length of 146 b.p. Pilot digestion experiments of each preparation were done to determine the optimal redigestion times (Figure 2.5.4). An aliquot of each long chromatin preparation was digested as in the legend to Figure 2.5.4 and DNA was analysed on 8% denaturing polyacrylamide gels. The DNA was digested to a clearly visible single band in the 140-145 b.p. region at which stage the optimal time of redigestion was reached. It is important that longer DNA is not included in the core particle preparation because this would confuse the analysis of the subsequent DNase I digestion kinetics. The isolated fraction contains DNA which is only slightly nicked (see zero times of Figure 2.5.8).

Core particles were separated from micrococcal nuclease, oligonucleotides and oligonucleosomes on 5-20% linear sucrose gradients (Figure 2.5.5). The sedimentation coefficient was estimated by the method of Fritsch (1973) to be 11S. Thus nucleosomal core particles with the DNA digested flush, can be prepared from sea urchin blastula, gastrula and sperm chromatin.

DNase I digestion kinetics can be affected by single-stranded nicks in the core particle DNA which can be introduced at three stages during the isolation of core particles. Core particles prepared by the above method are free of endogenous nuclease nicking. Endogenous nuclease activity is inhibited when using the buffers developed by Hewish and Burgoyne (1973) to isolate nuclei or chromatin. Though mono-and oligonucleosomal fractions are extensively nicked these are separated from long chromatin to be used for core particle preparations during the first ultracentrifugation stage

on sucrose gradients. Nicks which are introduced during digestion of long chromatin to core particles using micrococcal nuclease (see zero time lanes in Figures 2.5.6, 2.5.11 and 2.5.12) are present but only as minor components. DNase I rate constant calculations do not involve the zero time lane as the slope of the initial digestion rate would be equal to that of a slope which passes through zero at zero time at which stage the substrate would have to be totally undegraded. Therefore, provided that the DNA of the core particle is predominantly undegraded and that the reaction kinetics are still linear, rate constants can be calculated.

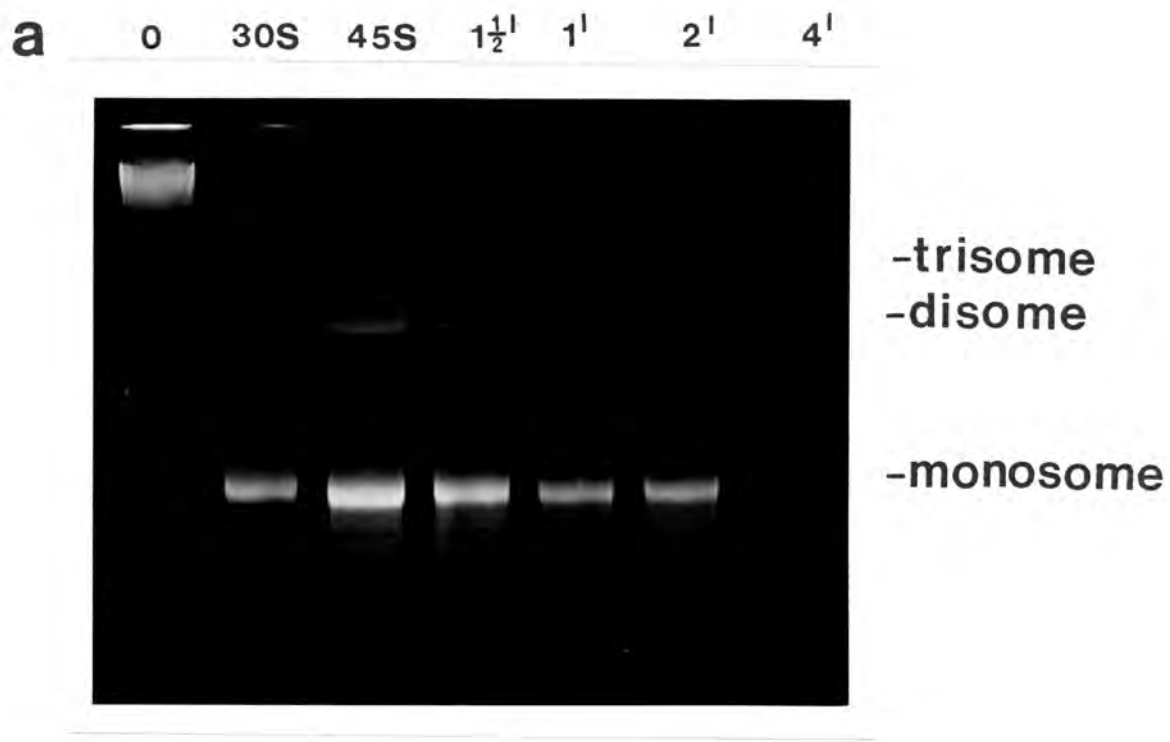


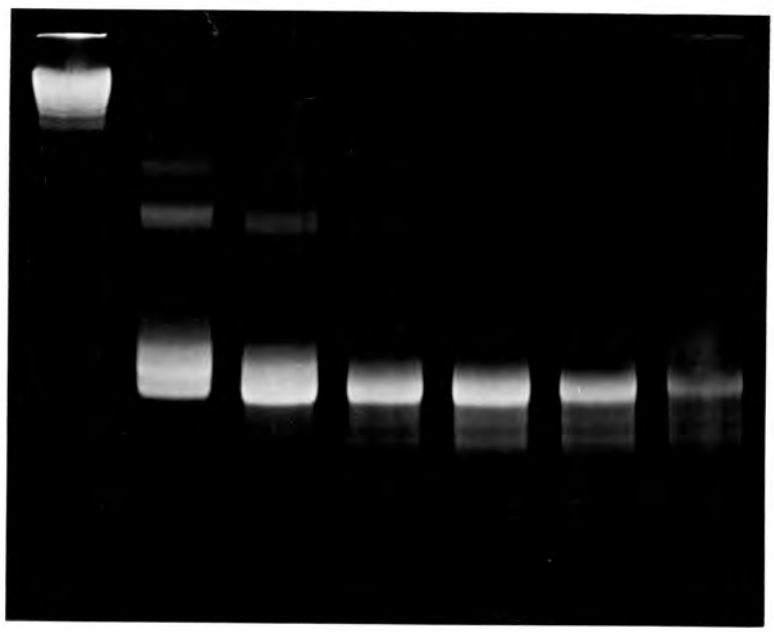
FIGURE 2.5.4: Redigestion of H1-depleted long chromatin with micrococcal nuclease. H1-depleted long chromatin from early blastula, gastrula and sperm cells was isolated as in Figures 2.5.1 and 2.5.2. Aliquots of these were redigested in the same buffer with 1 mM $CaCl_2$ and micrococcal nuclease concentration of 100 units/ml. The reaction was stopped at appropriate times, the DNA extracted and analysed on 8% polyacrylamide denaturing gels in TBE.

(A) - early blastula (optimal redigestion time = 4 minutes)

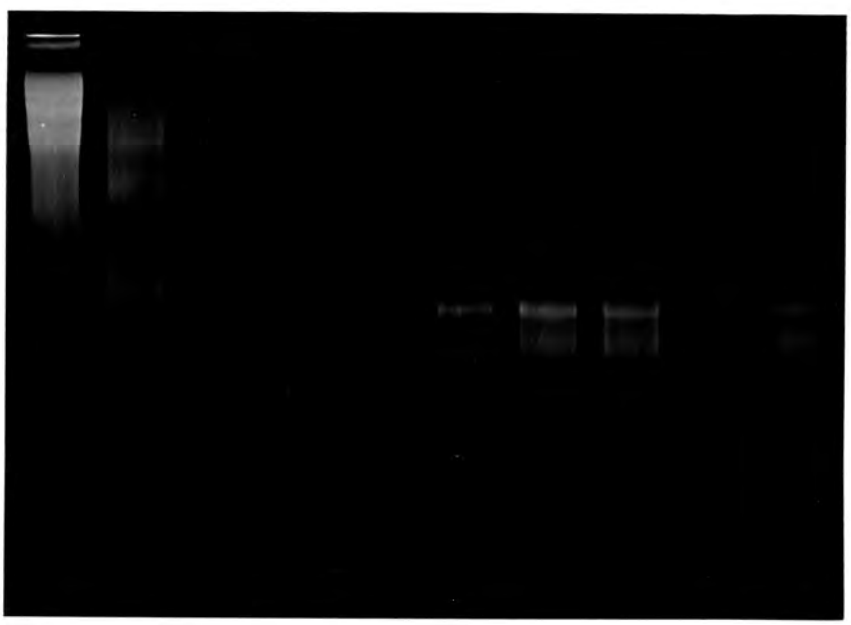
(B) - gastrula (optimal redigestion time = 2 minutes)

(C) - sperm (optimal redigestion time = 6 minutes)

b


0
15S
30S
45S
1½'
1'
2'
-trisome
-disome
-monosome

c


0
1'
2'
3'
4'
5'
6'
7'
8'
9'
-trisome
-disome
-mono-
some

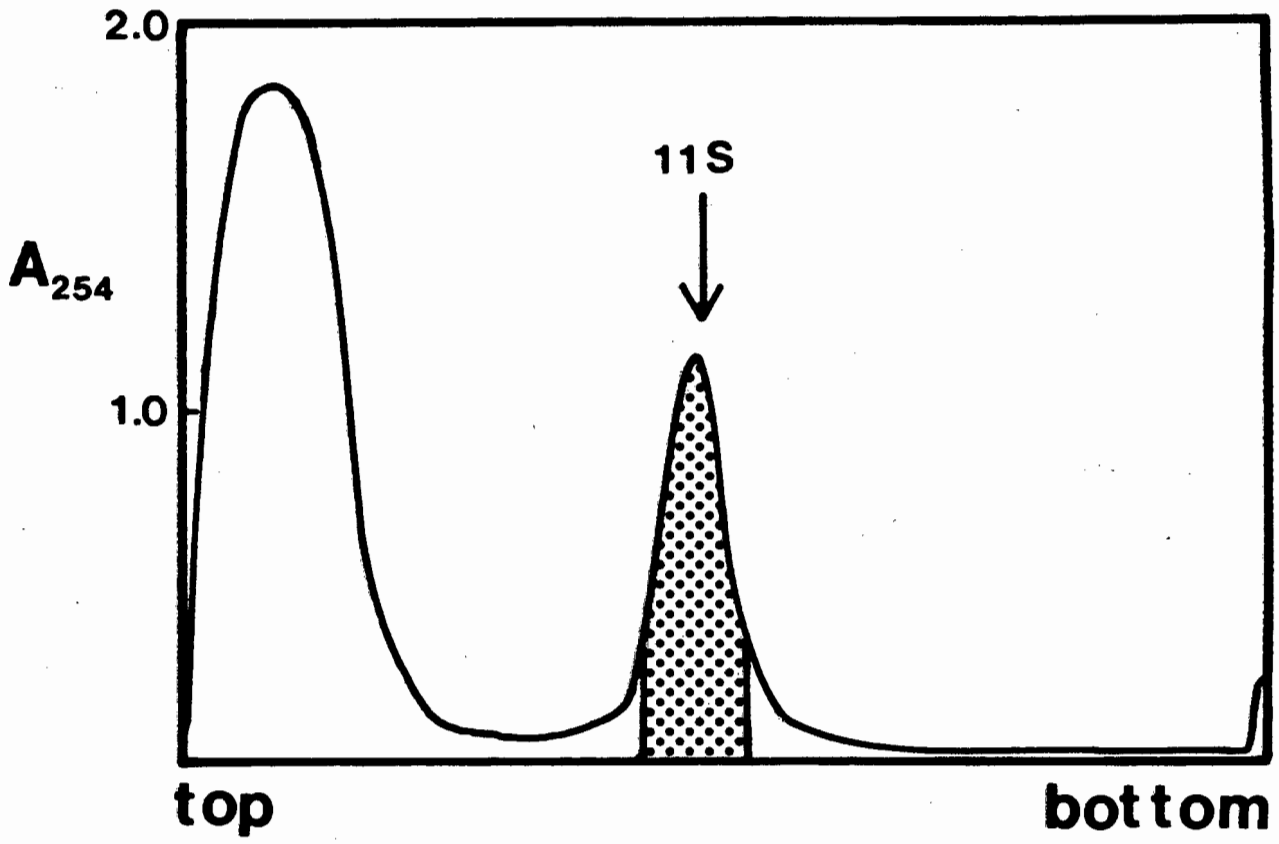


FIGURE 2.5.5: Isolation of core particles after redigestion for the optimal time. After redigestion, as in Figure 2.5.3, reactions were stopped by adding EDTA to 5 mM and samples were applied to 5-20% linear sucrose gradients in 50 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 0.2 mM EDTA and 0.2 mM PMSF and centrifuged at 182000 x g for 16 hours at 4°C. The monosome peak (shaded area) was dialysed exhaustively against the same buffer without NaCl.

2.5.2 DIGESTION OF SEA URCHIN EMBRYO AND SPERM CORE PARTICLES WITH DNase I

Various methods of analysis have been developed to determine the frequency of cutting of DNA by DNase I at specific sites on the nucleosomal core particle. (Simpson and Whitlock, 1976; Noll, 1977 and Lutter 1978). Each of these techniques involves a similar rationale in preparing, labelling and digesting 5'-labelled core particles with DNase I but the analysis of the digestion products differs in each case. Simpson and Whitlock (1976) qualitatively analysed their autoradiograms by inspection and demonstrated in this way the relative susceptibilities of the sites on the core particle to DNase I.

Noll (1977) has derived a quantitative analysis of cleavage of the DNA at each susceptible site based on the probability of cleavage at each site calculated from the radioactivity of the DNA fragments produced at these sites.

Lutter (1978) has refined the analysis by determining the fraction of unreacted substrate at each site, thus being able to calculate the apparent first-order rate constants for DNase I cleavage at each of these sites.

Analysis of the rate constants determined at particular sites, as developed by Lutter (1978), has here been used to characterise core particles, from sea urchin embryos and sperm, which contain different histone variants, in order to ascertain whether the presence of such variants in the core particle causes a change in susceptibility of the DNA to digestion.

2.5.2.1 DNase I Digestion Kinetics of Blastula and Gastrula Core Particles

Core particles were isolated from 7 hour blastula and 21 hour gastrula embryos as described in Section 2.5.1. 80% of the DNA in these core particles was about 146 b.p. in length as approximated from a gel similar to that in Figure 2.5.6 by counting the bases in the ladder up to 50 bases and then assuming the normal 10 base ladder for the bands greater than 50 bases. Core particles from 7 hour blastula chromatin contain predominantly embryo histone variants $H2B_{(2)}$ and $H2A_{(3)}$ while those from 21 hour gastrula chromatin contain predominantly embryo histone variants $H2B_{(1)}$, $H2B_{(3)}$, $H2A_{(1)}$ and $H2A_{(2)}$ (von Holt, et al., 1983).

These core particles were labelled with ^{32}P at the 5'-end using polynucleotide kinase and [γ-^{32}P] ATP. Greater than 70% of the label was incorporated into the DNA. The labelled core particles were than digested with DNase I, the DNA extracted and analysed on 8% denaturing polyacrylamide gels. The DNA was visualised by autoradiography (Figure 2.5.6).

Autoradiogram lanes were scanned using a densitometer and the tracings (Figure 2.5.7) were integrated to determine the relative amount of radioactivity in each band (see Section 4.2.5.1 for the method).

Rate constants of DNase I cutting at each site were determined according to Lutter (1978). The fraction of strands which are cut at a particular site was determined and the rate constant of cleavage at that site was calculated taking the necessary precaution to correct for the amount of additional cleavage which has occured between that site and the labelled 5'-end. (See Section 4.3.2.1 for the method of calculation). The results of these calculations are presented in Table 2.4 and Figure 2.5.8

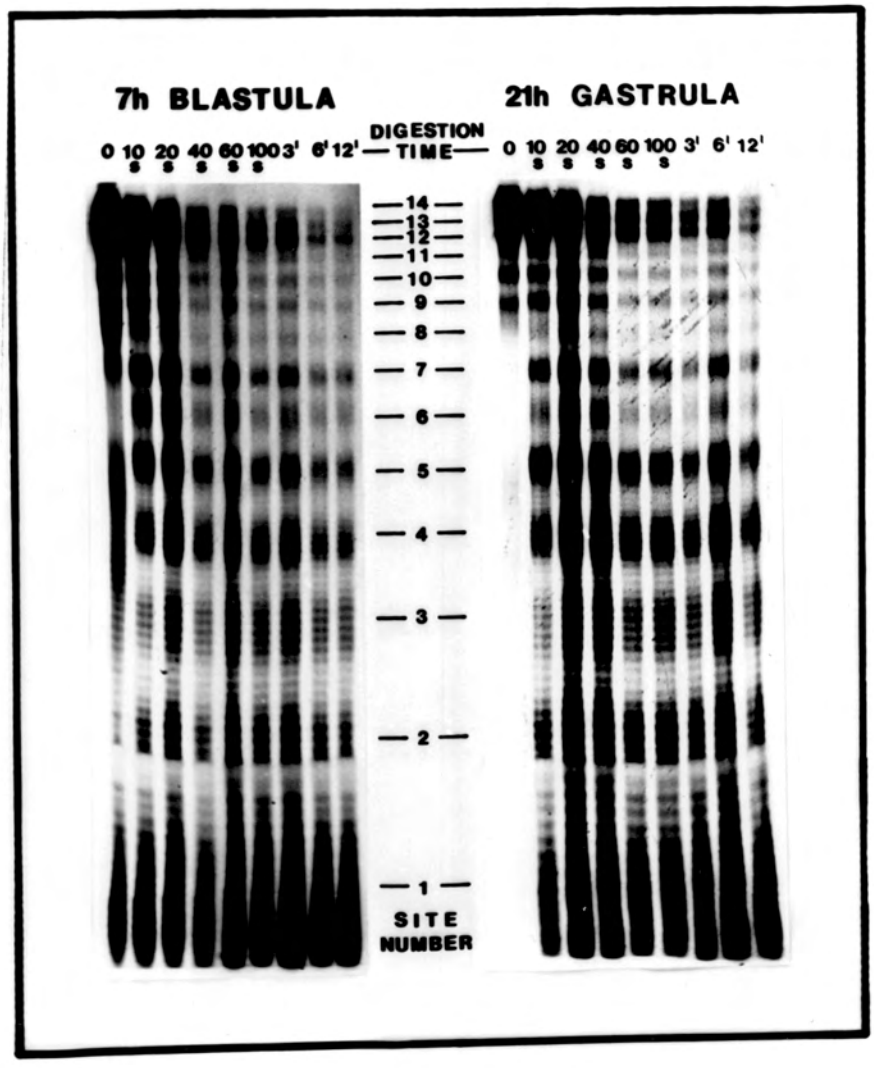


FIGURE 2.5.6 Autoradiogram of products of digestion by DNase I of blastula and gastrula core particles as analysed on an 8% denaturing polyacrylamide gel.

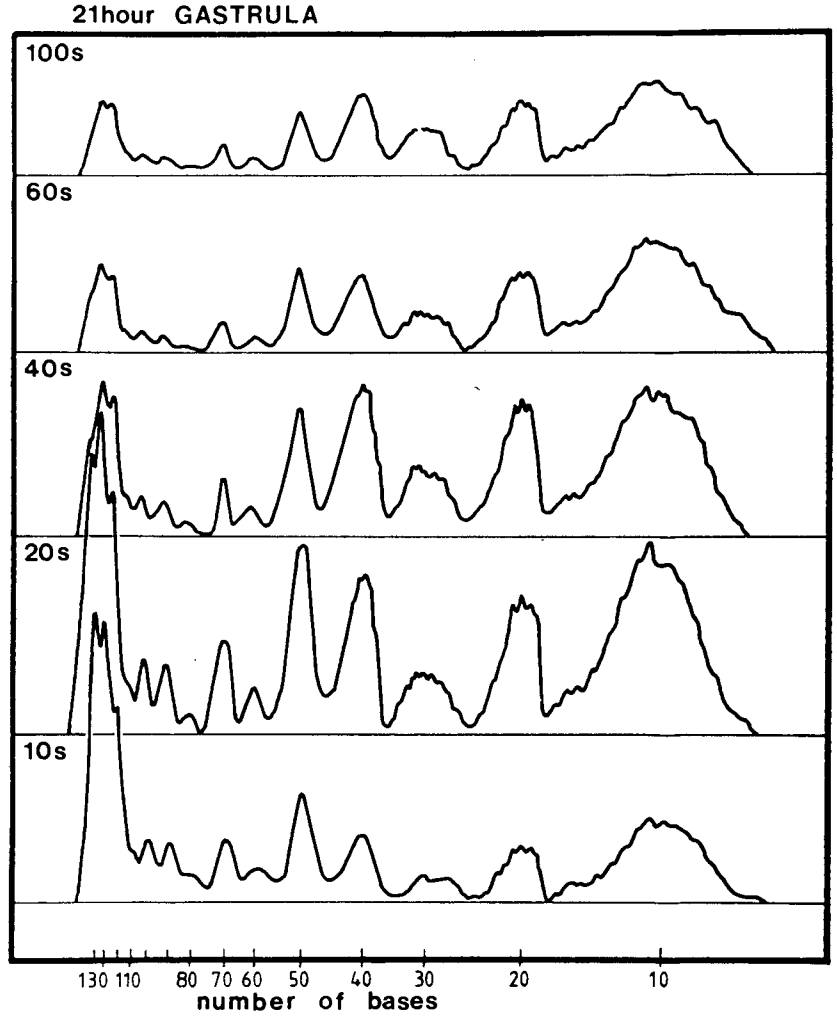


FIGURE 2.5.7 Densitometer tracings of the lanes indicated, from the autoradiogram in Figure 2.5.6.

SITE NUMBER	10 SECONDS		20 SECONDS		40 SECONDS		60 SECONDS	
	B	G	B	G	B	G	B	G
14	29.3	28.3	12.8	14.0	3.9	3.9	1.2	1.2
13	9.3	9.4	6.2	6.9	3.4	3.5	1.2	1.3
12	2.5	2.4	2.7	2.9	1.4	1.4	0.8	0.8
11	0.4	0.4	0.5	0.5	0.5	0.4	0.2	0.3
10	1.7	1.7	2.1	2.0	1.1	1.1	0.7	0.7
9	2.4	2.2	3.0	2.3	1.5	1.4	0.8	0.8
8	0.4	0.5	0.2	0.2	0.3	0.3	0.2	0.2
7	1.4	1.4	1.5	1.6	1.4	1.4	0.8	0.8
6	0.5	0.5	0.7	0.8	0.9	1.0	0.7	0.7
5	10.9	11.0	12.5	13.1	12.0	12.1	9.2	9.1
4	7.8	7.2	9.9	10.6	11.3	11.6	12.0	11.9
3	6.9	3.4	3.1	3.4	2.9	2.9	2.8	2.7
2	7.9	7.6	9.9	10.6	12.1	12.1	13.9	13.9
1	18.5	24.0	34.9	31.0	47.3	47.0	55.3	55.6

TABLE 2.4(a) Percentage of total radioactivity found in each band corresponding to each site of cleavage at each time of digestion. Column B is for the 7 hour blastula gel lanes and column G for 21 hour gastrula lanes.

SITE NUMBER	10 SECONDS		20 SECONDS		40 SECONDS		60 SECONDS	
	B	G	B	G	B	G	B	G
14	-	-	-	-	-	-	-	-
13	0.76	0.75	0.67	0.67	0.54	0.53	0.49	0.48
12	0.94	0.94	0.88	0.88	0.84	0.84	0.75	0.75
11	0.99	0.99	0.98	0.98	0.95	0.96	0.93	0.92
10	0.96	0.96	0.91	0.92	0.89	0.89	0.83	0.84
9	0.95	0.94	0.89	0.92	0.88	0.88	0.84	0.84
8	0.99	0.99	0.99	0.99	0.98	0.98	0.96	0.95
7	0.97	0.97	0.95	0.95	0.90	0.89	0.87	0.87
6	0.99	0.99	0.98	0.97	0.94	0.93	0.89	0.89
5	0.82	0.81	0.70	0.71	0.55	0.54	0.43	0.43
4	0.88	0.89	0.81	0.81	0.70	0.69	0.57	0.56
3	0.91	0.95	0.94	0.94	0.93	0.93	0.91	0.90
2	0.90	0.90	0.85	0.84	0.77	0.76	0.69	0.68
1	0.82	0.76	0.65	0.69	0.53	0.53	0.45	0.44
sum of P_{10n} values	2.13	2.15	2.79	2.73	3.62	3.64	4.40	4.40

TABLE 2.4(b) Fractions of each site which are not cleaved by DNase I after the times indicated ($1-P_{10n}$ in Lutter (1978)). Column B is for the 7 hour blastula gel lanes and column G for 21 hour gastrula lanes.

SITE NUMBER	7 HOUR BLASTULA		21 HOUR GASTRULA	
	RATE CONSTANT (s^{-1}) ($X10^3$)	CORRLEATION COEFFICIENT	RATE CONSTANT (s^{-1}) ($X10^3$)	CORRELATION COEFFICENT
13	8.68	0.977	9.18	0.983
12	4.26	0.980	4.29	0.980
11	1.25	0.991	1.29	0.999
10	2.67	0.976	2.63	0.990
9	2.16	0.945	2.43	0.998
8	0.74	0.954	0.72	0.952
7	2.28	0.994	2.27	0.985
6	2.08	0.997	2.09	0.999
5	12.86	0.999	12.59	0.999
4	8.55	0.997	8.60	0.999
3	0.18	0.921	0.80	0.995
2	5.34	0.999	5.26	0.999
1	11.51	0.980	10.99	0.996

TABLE 2.4(c) Rate constants of DNase I cleavage at the various sites. The natural logarithm of the uncleaved fraction (i.e. ln $(1-P_{10n})$) as a function of time is plotted and the slope of the regression line through these points is the rate constant. Rate constants were calculated from the $(1-P_{10n})$ values for time points 10, 20, 40 and 60 seconds as the digestion becomes non-linear at times greater than 60 seconds.

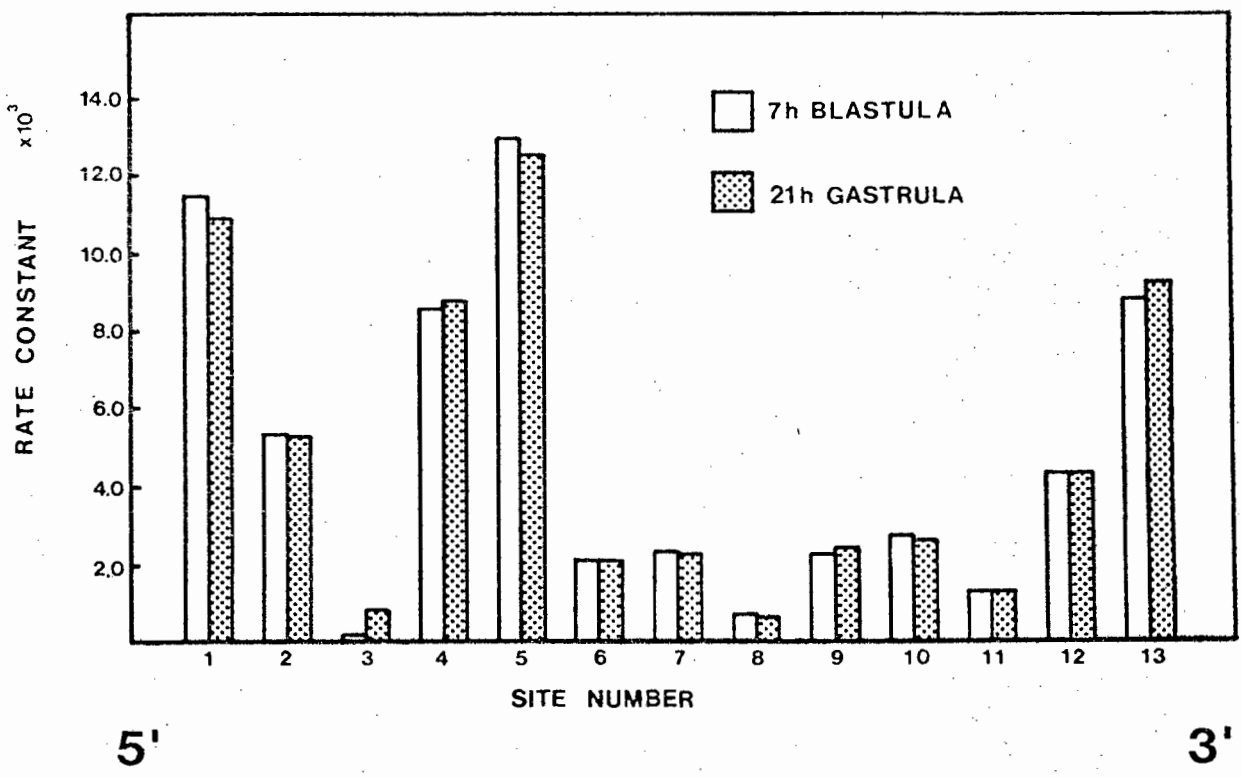


FIGURE 2.5.8 Bar chart of rate constants determined for each site of cleavage by DNase I on the core particles from 7 hour blastula and 21 hour gastrula.

The results presented above demonstrate that the DNase I digestion pattern of 5'-labelled core particles from 7 hour blastula and 21 hour gastrula chromatins is very similar. The rate constants at each cleavage site are not significantly different when comparing the digestion of blastula and gastrula core particles. Also, the trends in rate constants at the sites along the core particle DNA are similar. The digestion of both these core particles by DNase I corresponds to the digestion of rat liver core particles as calculated by Lutter (1978). As rate constants are here calculated from data for the first 60 seconds of digestion, where the reaction kinetics are linear, and as the substrate concentration becomes limiting after longer digestion, it is not possible to compare these results to the digestion pattern of S. purpuratus blastula and pluteus core particles (Simpson, 1981).

Thus, it has not been possible to show differences in the DNase I digestion kinetics of core particles which contain different histone H2A and H2B variants from sea urchin embryos.

Amino acid composition and partial sequence analysis of the sea urchin embryo histone H2A and H2B variants have demonstrated that the isotypes are distinctly different proteins (Brandt et al., 1979 and von Holt et al., 1983). The three H2B variants differ predominantly in the N-terminal 15 amino acids. The early $H2B_{(2)}$ has an N-terminal alanine residue whereas the late $H2B_{(1)}$ and $H2B_{(3)}$ N-terminals are both proline followed by alanine. There are 4 homologous amino acids in this region and two of the changes are glycine-alanine exchanges. However, the overall basicity of these peptide regions are similar and the basic amino acids are not clustered. Amino acid analyses of the purified H2A variants have shown their amino acid compositions to be similar.

The differences which are evident in the sequence of the variants of histones H2A and H2B from sea urchin embryo do not change the structure of the core particle as analysed by DNase I digestion kinetics. Either the histone variants do not display altered steric protection to the DNA or the technique is not sensitive enough to discriminate between small changes in particle shape. The first of these possibilities is discussed in greater detail below.

2.5.2.2 DNase I Digestion Kinetics of Sperm Core Particles

Core particles were prepared from sea urchin sperm chromatin as described in Section 2.5.1 using the necessary modifications to solubilise chromatin from this source. The histone content for these sperm core particles is shown in Figure 2.5.3. All four core histones H3, H4, H2A and H2B are present and there is no detectable trace of histone H1.

The length of the DNA in these core particles was approximated by a similar method to that used to approximate the DNA lengths in blastula and gastrula core particles. (Section 2.5.2.1). However, from Figure 2.5.11, it is observed that, superimposed on the 10-base ladder after DNase I digestion, there is a similar ladder which differs by 5 bases at the 5'-end. A plot of distance migrated, as determined from a scan of the 30 seconds digestion time lane (Figure 2.5.9), against the logarithm of the length of DNA (in bases) shows that the relationship is a cubic function and the correlation coefficient for the cubic regression line is 0.9998. (Figure 2.5.10). By extrapolation, the maximum DNA length has been approximated to be about 156 bases whereas the most abundant lengths of DNA at zero-time are about 145 bases. This will be discussed further below.

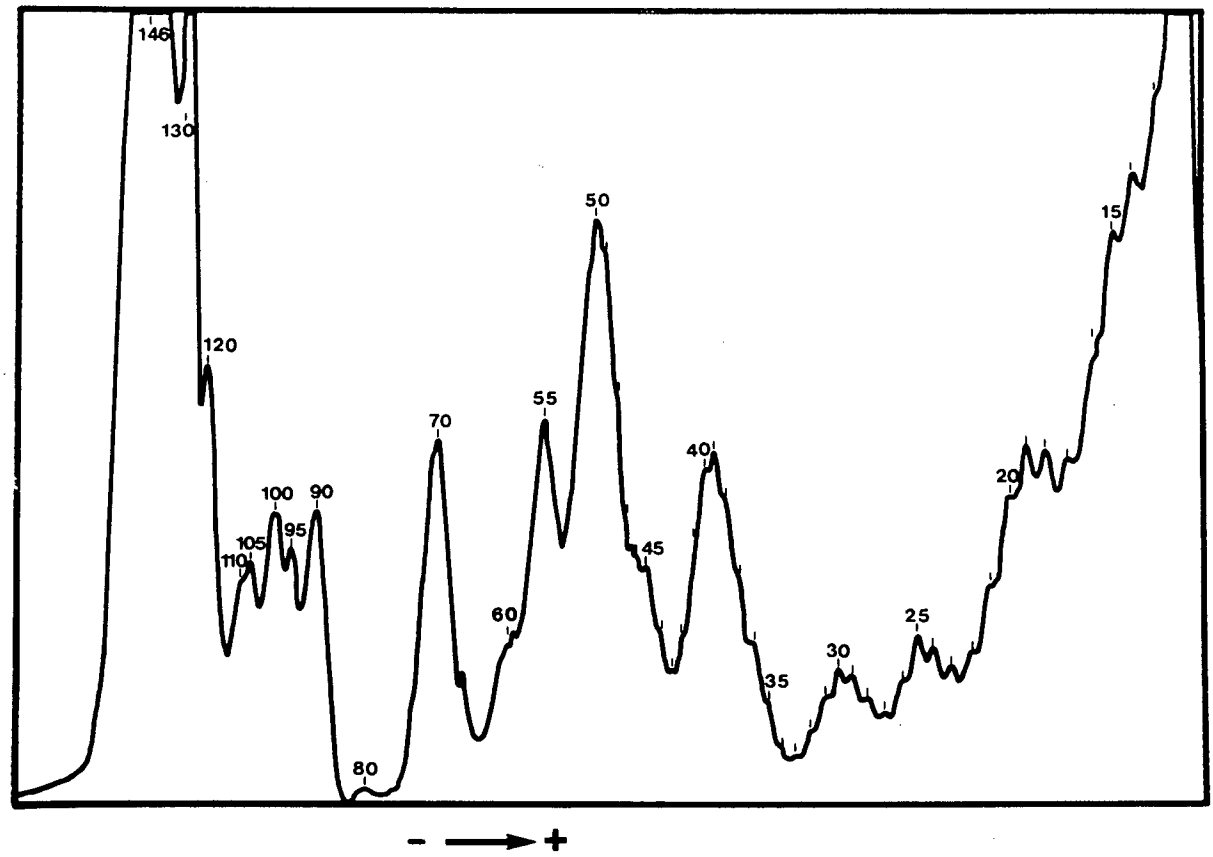


FIGURE 2.5.9 Densitometer scan of the autoradiagram of the 30 second digestion time lane of Figure 2.5.12(a)

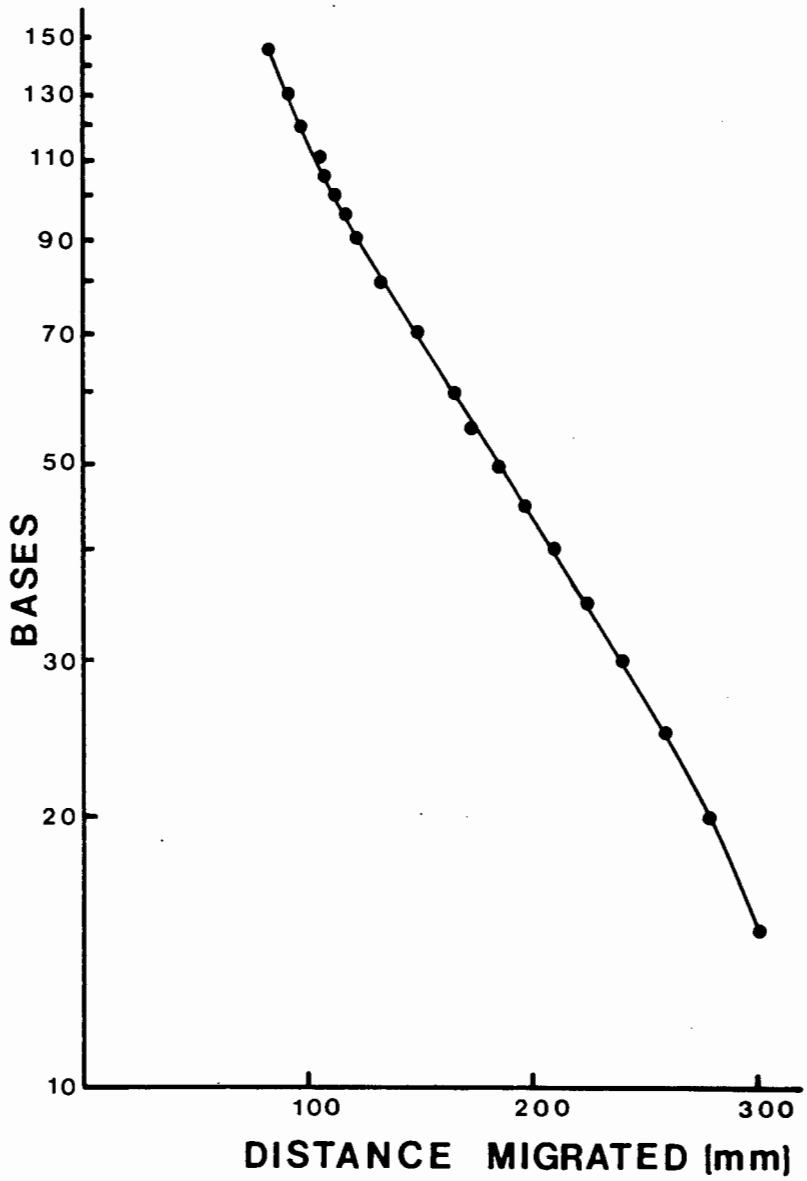


FIGURE 2.5.10 Logarithm of the DNA length in bases as a function of distance migrated in an 8% denaturing polyacrylamide gel.

These core particles were labelled with ^{32}P, digested with DNase I and the products were analysed on 8% denaturing polyacrylamide gels as in Section 2.5.2.1. Autoradiographs of these gels are presented in Figures 2.5.11 and 2.5.12.

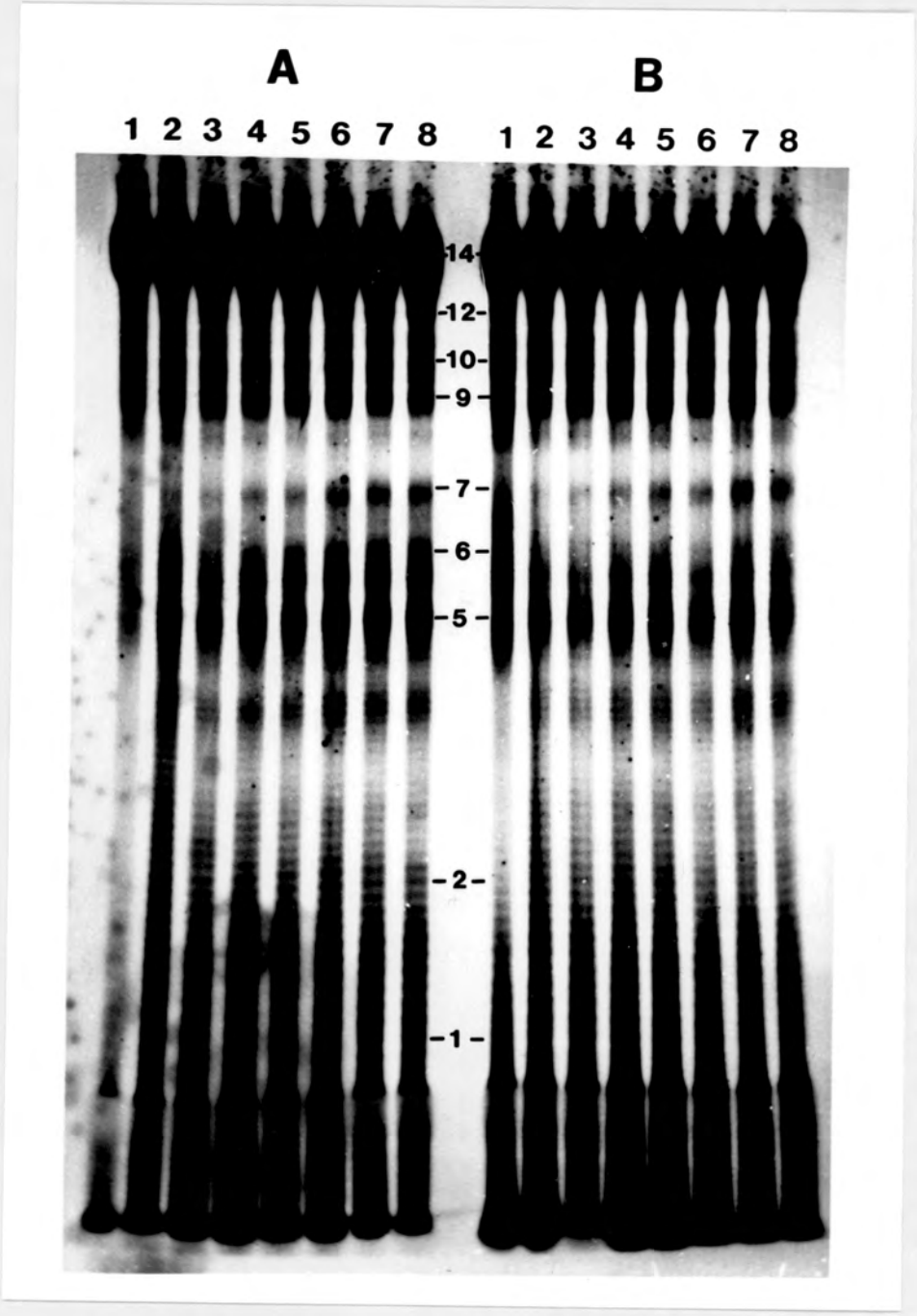


FIGURE 2.5.11 Autoradiogram of the products of DNase I (5 units/ml) digestion of sperm core particles as analysed on an 8% denaturing polyacrylamide gel

(A) = 6 minute redigested core particles (see Figure 2.5.4(c))

(B) = 7 minute redigested core particles (see Figure 2.5.4(c))

lane 1 = zero time

lane 2 = 15 seconds

lane 3 = 30 seconds

lane 4 = 45 seconds

lane 5 = 60 seconds

lane 6 = 75 seconds

lane 7 = 90 seconds

lane 8 = 105 seconds

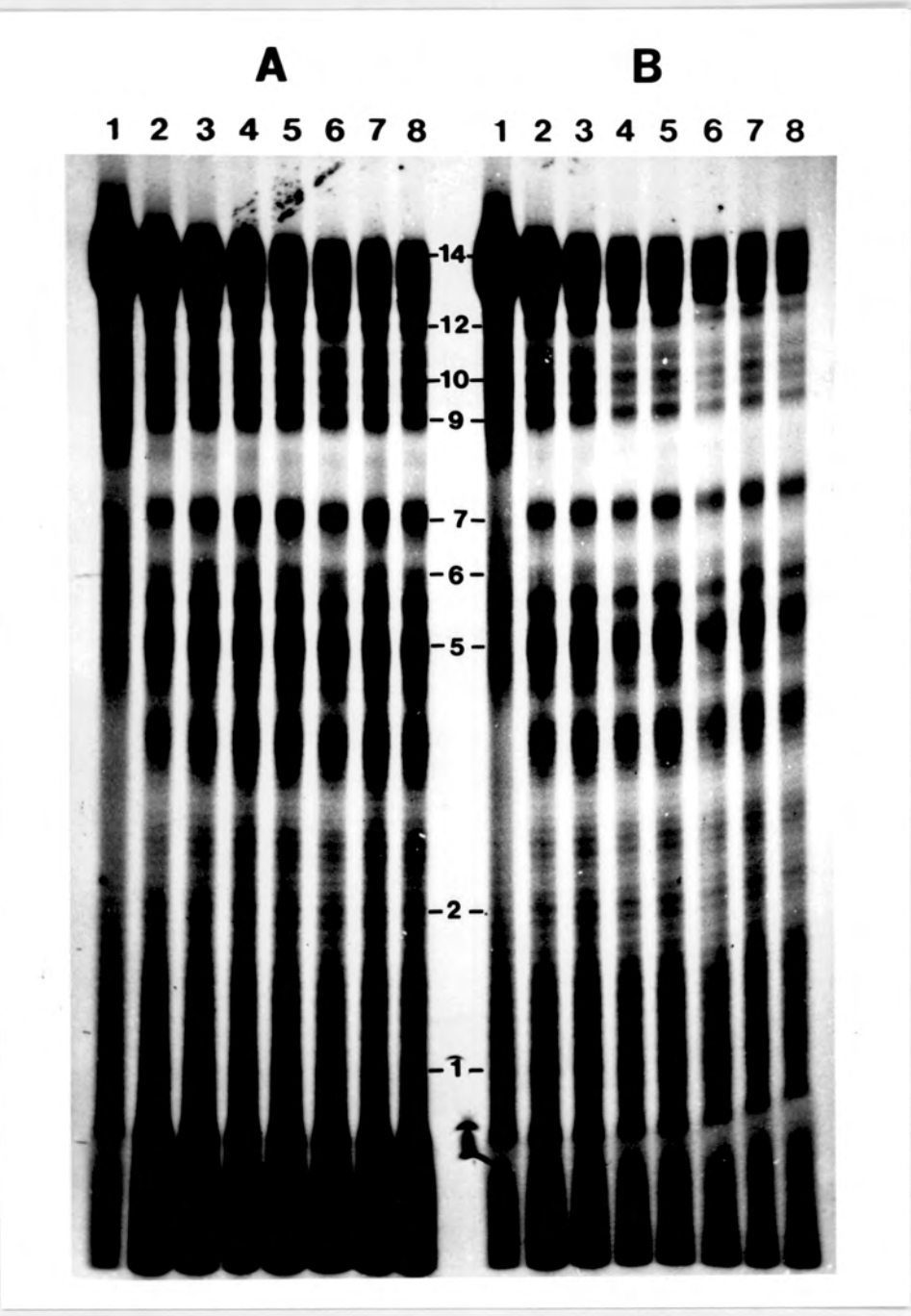


FIGURE 2.5.12 Autoradiogram of the products of DNase I digestion of sperm core particles (6 minute redigest) as analysed on an 8% denaturing polyacrylamide gel.

(A) = 500 units DNase I/ml

(B) = 5000 units DNase I/ml

lane 1 = zero time

lane 2 = 15 seconds

lane 3 = 30 seconds

lane 4 = 45 seconds

lane 5 = 60 seconds

lane 6 = 75 seconds

lane 7 = 90 seconds

lane 8 = 105 seconds

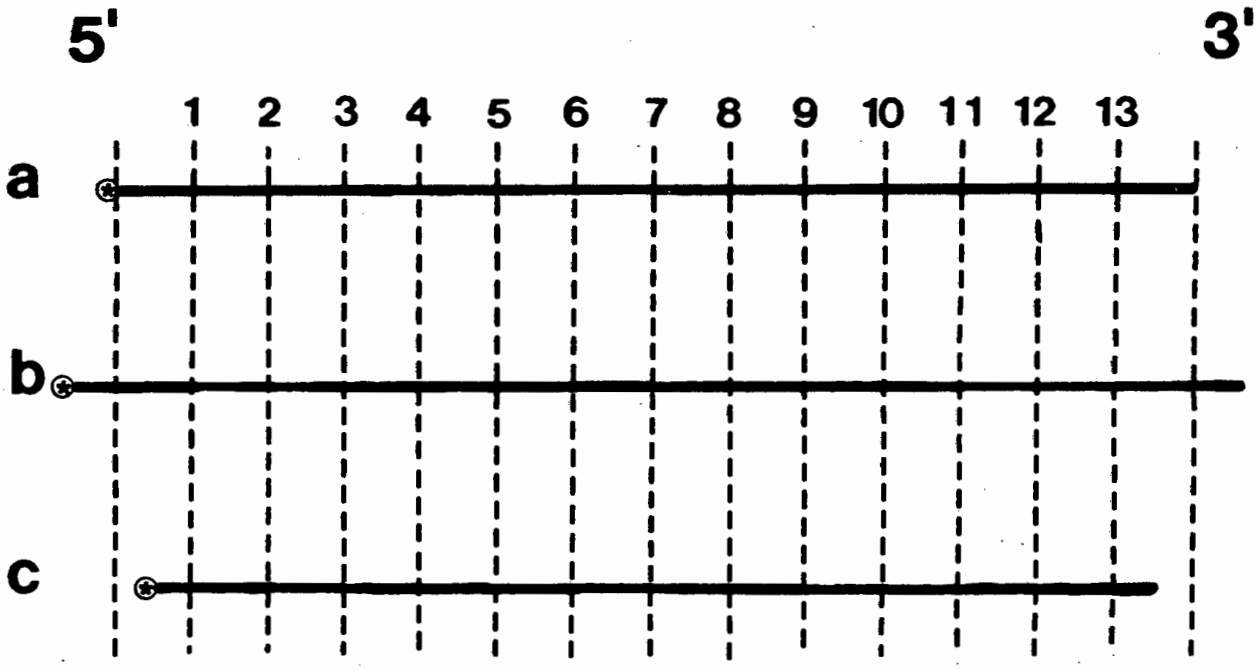


FIGURE 2.5.13 Possibilities for the superimposing of a 10 base ladder which is offset by 5 bases

(A) normal 10 base ladder on 140 bases of DNA

(B) 10 base ladder of DNA which is 5 or 10 bases longer

(C) 10 base ladder of DNA which is 5 or 10 bases shorter

Vertical broken lines are sites of cleavage, horizontal solid lines are single strands of DNA and asterisks are the ^{32}P labels.

It has been established here that there is a 10 base ladder of DNase I digestion products offset by 5 bases as well as the normal 10 base ladder. The possible structural alternatives considered in Figure 2.5.13 demonstrate that some of the core particle associated DNA strands are either longer or shorter by 5 bases at the 5'-end. If they were shorter, then the sites at 10 and 130 bases would show increased cleavage. This is not the case as will be demonstrated below. Also, the longest length of DNA in these core particles has been approximated to be about 156 bases.

Assuming that the ladder offset by 5 bases represents longer DNA strands the radioactivity at these sites (i.e. sites at 15, 25, 45, 55, 95 and 105 bases from the 5'-end) has been added to the radioactivity of the sites which are 5 bases shorter (i.e. sites at 10, 20, 40, 50, 90 and 100 bases from the 5'-end) in order to calculate rate constants. Thus, the following summations were performed:

$$R_{10} + R_{15}$$
$$R_{20} + R_{25}$$
$$R_{40} + R_{45}$$
$$R_{50} + R_{55}$$
$$R_{90} + R_{95}$$
$$R_{100} + R_{105}$$

Where R_n is the radioactivity at a site n bases from the 5'-end.

Also, a similar relationship can be seen between the heights of the peaks at 15, 25, 45, 55, 95 and 105 bases from the 5'-end and the heights of the peaks at 10, 20, 50, 50, 90 and 100 bases from the 5'-end.

The rate constants of DNase I cleavage were determined for each site by the method of Lutter (1978). The amount of radioactivity in each gel band was determined by slicing each band out and counting by liquid scintillation counting. The results of these calculations are presented in Table 2.5 and Figure 2.5.14.

SITE NUMBER	15 SECONDS		30 SECONDS		45 SECONDS		60 SECONDS	
	500	5000	500	5000	500	5000	500	5000
14	48.6	40.5	34.9	25.7	26.1	19.9	21.2	17.1
13	4.2	4.8	5.3	4.6	5.0	4.3	4.9	4.0
12	2.0	2.0	2.3	2.2	2.6	2.3	2.6	2.4
11	0.7	1.0	0.9	1.1	1.0	1.1	1.0	1.1
10	1.9	2.6	2.5	2.9	2.7	2.9	2.5	2.8
9	3.1	3.5	3.5	3.7	3.9	3.9	4.0	3.8
8	1.6	1.8	1.9	2.0	2.0	2.2	2.0	2.2
7	1.8	2.7	2.5	3.3	3.2	3.7	3.3	4.3
6	1.9	2.5	2.4	3.0	2.9	3.3	3.0	3.5
5	5.4	7.3	7.1	9.4	8.6	10.4	9.2	10.9
4	5.2	8.1	7.5	11.3	9.3	12.5	10.2	12.8
3	1.2	1.5	1.8	2.2	2.3	2.5	2.4	2.7
2	3.9	5.9	5.6	8.1	6.6	9.1	7.1	9.5
1	18.5	15.8	21.8	20.5	23.7	22.0	26.6	22.9

TABLE 2.5(a) Percentage of total radioactivity found in each band correspon ding to each site of cleavage as described above, at each time of digestion. Column 500 is for sperm core particles digested with 500 units DNase I/ml an column 5000 is for 5000 units DNase I/ml.

SITE NUMBER	15 SECONDS		30 SECONDS		45 SECONDS		60 SECONDS	
	500	5000	500	5000	500	5000	500	5000
14	-	-	-	-	-	-	-	-
13	0.92	0.89	0.87	0.85	0.84	0.82	0.81	0.81
12	0.96	0.96	0.95	0.93	0.92	0.91	0.91	0.90
11	0.99	0.98	0.98	0.97	0.97	0.96	0.97	0.96
10	0.97	0.95	0.94	0.92	0.93	0.91	0.92	0.90
9	0.95	0.94	0.93	0.91	0.91	0.89	0.89	0.88
8	0.97	0.97	0.96	0.95	0.95	0.94	0.95	0.93
7	0.97	0.95	0.95	0.93	0.93	0.91	0.92	0.88
6	0.97	0.96	0.96	0.94	0.94	0.93	0.93	0.92
5	0.92	0.89	0.89	0.84	0.85	0.81	0.83	0.79
4	0.93	0.89	0.89	0.84	0.86	0.81	0.84	0.80
3	0.98	0.98	0.98	0.97	0.97	0.96	0.96	0.96
2	0.95	0.93	0.93	0.90	0.91	0.88	0.90	0.88
1	0.81	0.84	0.78	0.80	0.76	0.78	0.73	0.77
sum of P_{10n} values	1.69	1.86	1.99	2.27	2.25	2.49	2.43	2.62

TABLE 2.5(b) Fractions of each site which are not cleaved by DNase I after the times indicated ($1-P_{10n}$ in Lutter (1978)). Columns are designated as the number of units DNase I per ml of incubation mixture.

SITE NUMBER	500 UNITS DNase I/ml		5000 UNITS DNase I/ml	
	RATE CONSTANT (s^{-1}) ($X10^3$)	CORRELATION COEFFICIENT	RATE CONSTANT (s^{-1}) ($X10^3$)	CORRELATION COEFFICENT
13	2.73	0.988	2.12	0.965
12	1.31	0.995	1.44	0.994
11	0.49	0.994	0.53	0.968
10	1.07	0.973	1.25	0.967
9	1.45	0.998	1.44	0.981
8	0.64	0.992	0.81	0.985
7	1.24	0.994	1.59	0.998
6	0.93	0.994	1.02	0.986
5	2.46	0.997	2.68	0.971
4	2.32	0.994	2.37	0.942
3	0.47	0.980	0.47	0.977
2	1.16	0.985	1.30	0.955
1	2.26	0.996	1.90	0.935

TABLE 2.5(c) Rate constants of DNase I cleavage at the various sites on sperm core particle DNA. The natural logarithm of the uncleaved fraction (i.e. ln $(1-P_{10n})$) as a function of time is plotted and the slope of the regression line through these points is the rate constant. Rate constants were calculated from the $(1-P_{10n})$ values for time points 15, 30, 45 and 60 seconds as digestion becomes non-linear at times greater than 60 seconds.

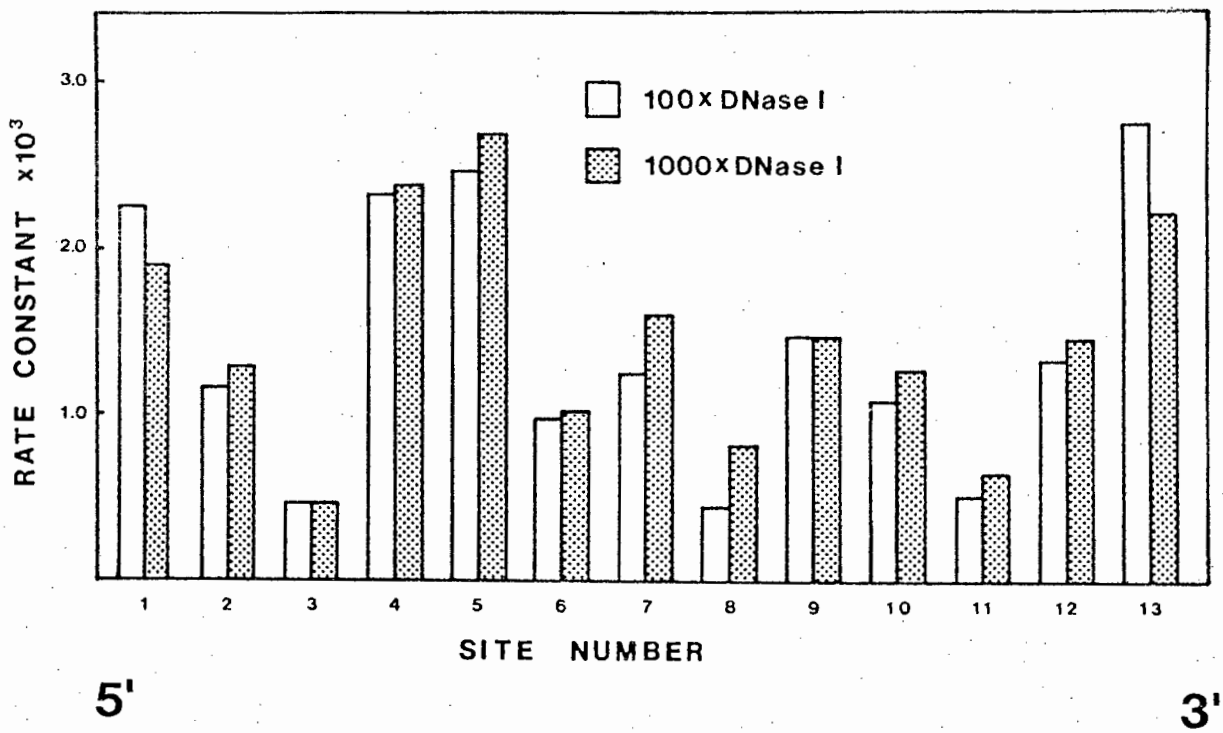


FIGURE 2.5.14 Bar chart of rate constants determined for each site of cleavage by DNase I on sperm core particles at 500 units DNase I/ml (100X) and 5000 units DNase I/ml (1000X)

The results presented above show that the rate of digestion of sperm core particles by DNase I is different to that in blastula and gastrula core particles. Sperm core particle digestion rates at sites 1, 2, 4, 5, 12 and 13 are 3-5 times smaller than for the corresponding sites in blastula and gastrula core particles whereas at sites 3, 6, 7, 8, 9, 10 and 11 the rate constants are 1-2 times smaller. Although the digestion rates are different, the most protected sites are the same, namely 3, 8 and 11.

In order to determine the rate constants of cleavage by DNase I at each site, it was necessary to increase the enzyme concentration 100- and 1000-fold. This increase did not alter the digestion kinetics (Figure 2.5.14). This is typical of a first-order reaction where the substrate concentration is the limiting factor and the enzyme concentration is very much smaller than the substrate concentration. It is concluded that the affinity of the enzyme, DNase I, for the substrate, sperm core particles, is reduced. This could be due to stronger binding of the core histone proteins to the DNA due to the increased arginine content of the sperm histones especially the histone H2B's (see Section 2.1.3.3). Alternatively, the longer histone H2B's in sea urchin sperm core particles could sterically protect the cleavage sites and in this way deny the enzyme access to the site. This will be discussed further in Section 3.2.

Sea urchin sperm core particles, as prepared in Section 2.5.1, show a micrococcal nuclease digestion pause at a site which is 5 bases from the site which , in other core particles, is the entry of the DNA into the histone-DNA complex. (Figures 2.5.11 and 2.5.12). The digestion pause becomes less evident after longer redigestion times when preparing core particles from H1-stripped long chromatin. This suggests that in sperm core particles there is a more protected site on the outside of the core par-

ticle near the entrance and exit points of the DNA (Figure 2.5.11). As approximated above, the sperm core particle DNA maximum size ranges between about 146 and 156 bases, thus, it is possible that the digestion pause sites are on both ends of the core particle DNA although it is not conclusively established here as labelling was at the 5'-end only.

It has previously been suggested, based on the qualitative analysis of DNase I digestion products of 5'-labelled S. purpuratus sperm core particles, that sperm core particle DNA is less susceptible to DNase I if compared to chicken erythrocyte core particles (Simpson and Bergman, 1980). The quantitative analysis of P. angulosus sperm core particle digestion by DNase I presented here, has now established that such a decrease exists. In addition, for specific sites the extent of the decrease varies.

These investigations have demonstrated that the kinetic analysis of DNase I digestion reveals peculiarities in the structure of the core particle isolated from sperm due to the presence of isohistones. However, the same technique is insensitive to the presence of isohistones with less pronounced changes in their structure in core particles from blastula and gastrula.

2.5.3 NUCLEOSOMAL CORE PARTICLE STRUCTURE AS PROBED BY DNase I

It has been proposed that the highly conserved histones H3 and H4 form a tetramer, $(H3)_2(H4)_2$, which is critical in nucleosome formation because it is capable of forming nucleosome-like particles with DNA while the less conserved histones H2A and H2B are proposed to form heterotypic dimers which associate with opposite faces of the H3-H4 tetramer and with DNA (Klug, et al., 1980). This octamer interacts with about 146 b.p. of DNA presumably by ionic interactions between the basic amino acid side chains

and the phosphates of the DNA. (Burton, et al.,1978)

The establishment in this laboratory of the existence of a large variety of isohistones (for reviews see von Holt, et al., 1979 and 1983) has prompted investigation as to the structural consequences and possible functions of these protein molecules. The high degree of conservation of primary structure of histone H3 and H4 (for reviews see von Holt, et al., 1979 and Isenberg, 1979) indicates that there is a selection pressure to maintain a unique H3 and H4 interaction between the proteins themselves and the DNA. However, H2A and H2B show a greater range of isotypes. Both of these proteins have a conserved hydrophobic core with N-terminal domains which are highly variable. The highly conserved hydrophobic cores are probably involved in protein-protein interactions between the various histone molecules in the core particle. Also, tryptic digestion of chromatin and H1-depleted chromatin has yielded peptides predominantly from the N-terminal domains of these histones (Kato and Iwai, 1977; Böhm et al., 1980, 1981 and 1982 and Grigor'ev and Kresheninnikov, 1982).

DNase I digestion of 5'-labelled core particles has shown that the sites of digestion are on average 10.4 base pairs apart (Lutter, 1979 and Prunell, et al., 1979) and that these sites are not cleaved at an equal rate of digestion (Simpson and Whitlock, 1976; Noll, 1977 and Lutter, 1978). It has been postulated that the larger differences in digestion rates between the various sites results from some sites being exposed to and other sites being protected from the enzyme, presumably by steric hindrance by the histones (Lutter, 1978 and Finch, et al., 1977). This then would apply to the sites 3, 6, 8 and 11. The smaller differences in digestion rates (i.e. at sites 1, 2, 4, 5, 7, 9, 10 and 12) have been postulated to result from the

angular dispositions of these sites on the surface of the core particle. (Klug and Lutter, 1981).

It could be argued that the regions responsible for protection of the DNase I insensitive sites are the N-terminal sequences of the core histones which can be cleaved off by trypsin. Changes within these regions could alter the DNase I protection pattern both qualitatively and quantitatively. However, the experiments presented show that the DNase I digestion rates of early blastula and gastrula core particles which contain different H2A and H2B histone variants are very similar. The latter representing various subtypes of the short H2B histones (von Holt, et al., 1983). Thus, variation in the N-terminal primary structure of the short H2B does not manifest itself in protection sites on the core particle. Protection is conferred either by the more conserved regions of these histones or largely by the histones H3 and H4. This latter would imply that the conformation of the core particle itself is a highly conserved structure.

However, the results of DNase I digestion kinetics of sea urchin sperm core particles show that the N-terminal regions of the long histones H2B plays a role in the protection. It has been demonstrated that there is an overall decrease in susceptibility of DNase I cleavage on the DNA of these core particles when compared to blastula and gastrula core particle digestion kinetics. (see Section 2.5.2.2). The observed differences are of two types. Firstly, the rate constants at frequently cut sites are 3-5 times smaller in sperm core particles than in blastula and gastrula core particles and secondly, the rate constants at the infrequently cut sites only differ by a factor between 1 and 2.5. Thus, the decreased susceptibility results predominantly from a decrease in cutting frequency at the sites which are more frequently cut. These sites are situated mainly near the entry and exit sites of the DNA on the core particle.

That histone H2B is positioned near the ends of the DNA of the core particle has been shown by Mirzabekov, et al. (1978) and Shick, et al. (1980). These authors have shown that histones bind discrete DNA segments of about six nucleotides with 4 nucleotide-long histone-free gaps separating areas of binding. The unbound nucleotide stretches correspond to the DNase I cutting sites. The sites of most interest are the sites of infrequent cutting as these are presumed to be protected by protein (i.e. sites 3, 8 and 11). (Lutter, 1978 and Finch, et al., 1977). Histones H2B and H2A seem to bind almost exclusively at or near these sites (Shick, et al., 1980 and Klug, et al., 1980) and histone contact sites were not detected within 20 nucleotides of the 5'-end of the core particle DNA. (Shick, et al., 1980). Also, the histone which seems to be located closest to the 5'-ends of the single strands of the core particle DNA, is H2B.

It is significant that the histones H2B are probably located near the 5'-ends of one strand of the nucleosomal core particle DNA because it has been demonstrated here (Section 2.5.2.2.) that there is a micrococcal nuclease and probably also a DNase I, digestion pause 5 bases on the outside of the core particle DNA. P. angulosus sperm core particles contain long H2B variants which have 4-5 N-terminal, repeating penta- and hexapeptides. (Strickland, et al., 1977(a), 1977(b) and 1978). By the criteria on which a nucleoprotamine model has been hypothesised (Warrant and Kim, 1978), it is possible to envisage that these repeating penta- and hexapeptides could form α-helical domains which would be stabilised by the lysine and arginine interactions with the DNA phosphates, and which would be separated into domains by the proline residues. These penta- and hexapeptide domains would presumably extend along the DNA at the end(s) of the core particle and result in 1 or 2 sites which would be, at least partially, protected from nuclease digestion and which seems to be about 5 bases from the entry

of the DNA into the core particle. This would explain the nuclease digestion pause which is shown to exist in Section 2.5.2.2.

However, it is also possible that these penta- and hexapeptides could form similar α-helical conformations in, probably, the major groove of the DNA, by extending along the DNA but towards the centre of the length of DNA associated with the histone octamer. Interactions of this type could reduce the affinity of DNase I for the DNA and therefore, reduce the digestion rates at the sites which are closest to the positions of the histones H2B on the DNA. (Shick, et al., 1980). It has been observed that the digestion rates of these sites (i.e. sites 1, 2, 4, 5, 12 and 13) are diminished 3-5 times when compared to blastula and gastrula core particle digestion rates.

Sea urchin sperm core particles seem to have evolved to perform a specific function which could involve the compaction of DNA in the nucleus into a higher order structure which is different from that in somatic cells. The longer H2B's in sea urchin sperm could assist the H1's in wrapping the sperm DNA into ordered, coiled higher structures which are designed to be extremely compact while in the nucleus, but which can be efficiently transferred to and associated with the chromatin in the sea urchin egg, upon fertilization.

Decreased DNase I digestion rates have also been demonstrated in core particles, isolated from mouse myeloma cells, which bind RNA polymerase II. (Baer and Rhodes, 1983). RNA polymerase II-core particle complexes are protected from DNase I cleavage at sites 1, 2, 4, 5 and 13, while sites 3, 6, 7 and 8 are exposed. On release of RNA polymerase II, by treatment with 0.3M KCl, digestion rates at each site were restored although increased

rates of digestion were observed at sites 1, 2 and 13. These increases have been attributed to a partial loss of histones H2A and H2B.

3. CONCLUSIONS

1. In this thesis it is shown that nucleosomal DNA repeat lengths are not significantly different in Parechinus angulosus embryos at different stages of development although other workers (Savic, et al., 1981) have suggested that early embryonic isohistones package chromatin into shorter, and late embryonic variants into longer, repeating units. Thus nucleosomal DNA repeat length determinations do not reveal differences in chromatin structure which result from the altered interactions of isohistones in the chromatin.

2. Analysis of DNase I kinetics of 5'-labelled nucelosomal core particles containing various isohistones, has shown that the longer sperm H2B variants not only partially protect a site on the DNA which is 5 bases on the outside of the core particle but also decrease the DNase I digestion rates at the sites closest to the ends of the core particle DNA. It is concluded that the repeating penta- and hexapeptides of the sperm H2B's are probably the peptide regions which interact with the DNA at or near these sites as the shorter embryonic H2B's in blastula and gastrula core particles do not show these altered protection sites.

4. MATERIALS AND METHODS

4.1.1 GROWTH OF SEA URCHIN EMBRYOS

Mature, Parechinus angulosus, ready to spawn, were collected from rock pools at low tide from the local coastline. Spawning, fertilisation and growth was done according to Hinegardner (1967 and 1975). Spawning was induced by intracoelomic injection of 0.5 M KCl. Sperm was collected by inverting a male animal on a petri dish. Eggs were collected by inverting the females and placing them on raised wire mesh supports about 2 cm above the bottom of plastic trays. Sea water was poured into the trays so that the gonophores were completely covered thus allowing the eggs to drip and settle on the bottom. Animals were removed after about 1 hour and the egg suspension was filtered through a nylon mesh (pore size = 250 µm). Eggs were washed by settling 3 times in filtered sea water (no. 41 Whatman filter paper) by gravity and resuspended at a final concentration of about 4 x 10^6 eggs per 100 ml filtered sea water containing 100 mg Penicillin G and 50 mg Streptomycin sulphate per liter. 5ml of a 1/1000 dilution of fresh sperm was used to fertilise such a culture. Fertilisation was monitored by phase contrast microscopy and only cultures which showed greater than 95% fertilisation were used. Growth was at 20°C with aeration using a rotating platform shaker.

4.1.2 ISOLATION OF NUCLEI OR CHROMATIN

4.1.2.1 Rat Liver Nuclei

4.1.2.1.1 In $MgCl_2$ Buffers

Nuclei were prepared from rat livers according to the method of Blobel and

Potter (1966). Rats were killed by cervical dislocation and the livers were excised. All subsequent procedures were performed at 4°C. The liver lobes were trimmed of connective tissue, diced and suspended in 3 volumes of 0.25M sucrose 50 mM TRIS-HCl (pH 7.5), 25 mM KCl and 1.5 mM $MgCl_2$. PMSF in DMSO was added to all solutions to a final concentration of 0.2 mM just prior to use. The diced livers were then homogenised in a glass-teflon Potter homogeniser. (3-4 passes) The homogenate was then filtered through first 1 layer and then 4 layers of cheesecloth. The filtrate was centrifuged at 500 xg for 20 minutes. The pellet was resuspended in at least 9 volumes of 2.3 M sucrose in 50 mM TRIS-HCl (pH 7.5), 25 mM KCl and 1.5 mM $MgCl_2$. In order to obtain a proper nuclear pellet, it is important to resuspend the pellet evenly using a glass rod. The suspension was centrifuged at 45000 xg for 60 minutes to obtain a white pellet. If necessary, the nuclear pellet was washed a second time in 2.3 M sucrose in the same buffer. The supernatant was carefully decanted and the centrifuge tube walls were wiped clean with a tissue wrapped around a glass rod. The nuclear pellet was resuspended in 0.25 M sucrose in the same buffer by vortex mixing.

4.1.2.1.2 In Polyamine Buffers

Nuclei were prepared according to the technique of Burgoyne, et al. (1970). Excision and trimming of livers was as in 4.1.2.1.1 above. Livers were diced in 0.34M sucrose in buffer B (60 mM KCl, 65 mM NaCl, 0.15mM spermine, 0.5 mM spermidine, 5 mM 2-mercaptoethanol and 15mM TRIS-HCl (pH 7.4) containing 2 mM EDTA and 0.5 mM EGTA (7 ml/g of liver). PMSF in DMSO was added to all solutions to a final concentration of 0.2 mM just prior to use. Honogenisation was in a glass-teflon Potter homogeniser (3-4 passes). The homogenate was filtered as in 4.1.2.1.1 and the filtrate was lay-

ered on 0.3 volumes of 1.37 M sucrose in buffer B containing 1mM EDTA and 0.25 mM EGTA and then centrifuged at 16 000 x g for 15 minutes. The pellet was thoroughly mixed in at least 7 volumes 2.3M sucrose in buffer B containing 0.1 mM EDTA and 0.1mM EGTA and then centrifused at 45 000 x g for 60 minutes. The clean white pellet was resuspended in 0.34M sucrose in buffer B containing 0.1mM EDTA and 0.1mM EGTA.

4.1.2.2 Chicken Erythrocyte Chromatin

Chicken erythrocyte chromatin was isolated according to the method of Ruiz-Carrillo, et al. (1980). Chicken blood was collected from freshly slaughtered chickens in 0.25M trisodium citrate. This was immediately filtered through 1 layer of cheesecloth and transported to the laboratory on ice. All subsequent operations were at 4°C. Erythrocytes were collected by centrifugation at 2 000 x g for 10 minutes. The pellets were suspended in washing buffer A (4.1.2.1.3) and pelleted as above. This washing cycle was repeated until the supernatant was colourless. Erythrocytes were resuspended in washing buffer A containing 0.5% Nonidet LE. This caused cell lysis and chromatin was pelleted at 600 x g for 10 minutes. The pellet was washed at least 3 times in washing buffer A or until the supernatent was clear. The yield was about 1.2 g of DNA per litre of blood processed.

4.1.2.3 Sea Urchin Embryo Chromatin

4.1.2.3.1 In Polyamine Buffers

All operations were done at 4°C. Chromatin was isolated essentially according to Keichline and Wassarman (1979) except that the Mg-buffers were replaced by a buffer containing the polyamines, spermine and spermidine,

(buffer A) (Hewish and Burgoyne, 1973). Embryos were washed 4 times in calcium-magnesium-free-sea water (0.48 M NaCl, 11 mM KCl, 2.4 mM $NaHCO_3$, 0.5 mM Na_2EDTA) by centrifuging at 600 x g for 10 minutes. Embryos were then resuspended in 0.5 M sucrose, 10 mM TRIS-HCl (pH 7.4) and 0.4 mM PMSF. The cells were dissociated by using a loose fitting glass Dounce homogeniser (5 passes used for gastrula embryos and 15 passes for blastula embryos). This was then adjusted to a concentration of 1:10 relative to packed cell volume and an equal volume of 120 mM KCl, 30 mM NaCl, 0.3 mM spermine, 1.0mM spermidine, 10 mM 2-mercaptoethanol, 30 mM TRIS-HCl (pH 7.4), 10 mM EDTA, 2.5 mM EGTA and 0.5% Nonidet LE was added slowly. The chromatin was pelleted gently and washed 3 times in 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 spermidine, 5 mM 2-mercaptoethanol, 15 mM TRIS-HCl (pH 7.4), 0.2 mM EDTA, 0.2 mM EGTA and 0.2 mM PMSF (washing buffer A) by centrifuging at 600 x g for 10 minutes. The chromatin pellet was resuspended in washing buffer at the desired concentration.

4.1.2.3.2 In $MgCl_2$ Buffers

This preparative technique was identical to the isolation of chromatin in polyamine buffers (4.1.2.3.1) except that the polyamine buffers were replaced by 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM TRIS-HCl (pH 7.5). (Keichline and Wassarman, 1979). Embryos were washed 4 times in calcium-magnesium-free sea water (4.1.2.3.1). The embryo pellet was resuspended in 0.5 M sucrose, 10 mM TRIS-HCl (pH 7.5) and 0.4 mM PMSF. The cells were dissociated using a loose fitting glass Dounce homogeniser (5 passes used for gastrula embryos and 15 passes for blastula embryos). This was adjusted to a concentration of 1:10 relative to packed cell volume and an equal volume of 20 mM NaCl, 10 mM $MgCl_2$, 20 mM TRIS-HCl (pH 7.5) and 0.5% Nonidet LE was added slowly. The chromatin was pelleted at 600 x g for 10 minutes and was

washed 3 times in 0.25 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM TRIS-HCl (pH 7.5) and 0.2 mM PMSF. The chromatin pellet was resuspended in this final washing buffer at the desired concentration.

4.1.2.4 Sea Urchin Sperm Chromatin

Sperm was collected from male sea urchins after intracoelomic injection of 0.5 M KCl by inverting individuals on 100 ml beakers filled to the brim with sea water. The sea water was gently decanted and the sperm slurry was filtered through a plastic mesh (pore size = 250 μm) and pelleted at 2000 x g for 5 minutes. The sperm pellet was washed 4 times in washing buffer A (4.1.2.3.1) by repeated pelleting and resuspension in the same buffer. The pellet was then resuspended in 10 volumes 0.5 M sucrose, 10 mM TRIS-HCl (pH 7.4) and 0.4 mM PMSF using 3-4 passes of a loose fitting glass Dounce homogeniser. To this an equal volume of 120 mM KCl, 30 mM NaCl, 0.3 mM spermine, 1.0 mM spermidine, 10 mM 2-mercaptoethanol, 30 mM TRIS-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA and 0.5% Nonidet LE was added slowly. Chromatin was pelleted at 600 x g for 10 minutes. The chromatin pellet was washed by resuspending in washing buffer A (4.1.2.3.1) using a loose fitting glass Dounce homogeniser and subsequent centrifugation at 600 x g for 10 minutes. Chromatin was resuspended in washing buffer A at the desired concentration.

4.1.3 PREPARATION OF SEA URCHIN EMBRYO AND SPERM NUCLEOSOMAL CORE PARTICLES

All solutions used in the preparation of core particles were autoclaved. Nucleosomal core particles were isolated according to Lutter (1978) with modifications. Chromatin was isolated from embryos (4.1.2.3.1) and sperm

(4.1.2.4) in polyamine buffers. Chromatin pellets were resuspended in washing buffer A at DNA concentration of 5 mg/ml. $CaCl_2$ was added to a final free concentration of 1 mM (taking into account the EDTA and EGTA in washing buffer A) from a 100 mM stock solution. Micrococcal nuclease was added to 20 units/mg DNA and digestion was at 37°C for 3½ minutes in the case of blastula and gastrula chromatin and 20 minutes in the case of sperm chromatin. The reaction was stopped by adding EDTA to a final concentration of 5 mM and cooling on ice. The chromatin was pelleted at top speed in a benchtop centrifuge for 3 minutes. Digested chromatin pellets were extracted with 600 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF in the case of blastula and gastrula chromatin and 1.1 M NaCl in the same buffer in the case of sperm chromatin for 3-4 hours at 4°C. The insoluble material was pelleted at 4000 x g for 5 minutes. The supernatant contains soluble digested chromatin. (85-95% of the DNA was solubilised as measured by the absorbance at 260 nm).

Aliquots of these supernatants containing 4-6 mg DNA in 2-3 ml were applied to linear 5-20% (w/w) sucrose gradients in 500 mM NaCl (1 M NaCl for sperm chromatin), 10 mM TRIS-HCl (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF and centrifuged at 83 000 x g for 16 hours at 4°C. The gradients were analysed on an Isco gradient fractionator by displacement from the bottom using 40% sucrose. Chromatin fractions larger than tetranucleosomes (see Figure 2.5.2) were pooled, dialysed overnight against 10 mM TRIS-HCl (pH 7.5), 50 mM NaCl, 0.2 mM EDTA, 2 mM 2-mercaptoethanol and 0.2 mM PMSF and then concentrated by pressure ultrafiltration to 0.5 -1.0 mg DNA/ml. This fractionation separates stripped H1 and non-histone proteins from long chromatin.

Pilot experiments were performed on each H1-stripped long chromatin prepar-

ation in order to determine the optimum micrococcal nuclease redigestion time to produce core particles, the DNA of which was flush. A 100-200 µl aliquot of H1-stripped long chromatin (0.5-1.0 mg DNA/ml) was made 1mM $CaCl_2$ and digested with micrococcal nuclease at a concentration of 100 units/ml. Samples (about 10 µg DNA) were removed at regular time intervals, the DNA was extracted (Section 4.2.2.1), and then analysed on an 8% denaturing polyacrylamide gel (220 mm x 230 mm x 1.8 mm) (Section 4.2.3.2) which was photographed. (Section 4.2.3.3). The optimum time for redigestion was determined and a bulk digestion was then performed under identical conditions except that volumes were proportionally increased. The reaction was stopped by adding EDTA to 5 mM and cooling to 4°C. In order to separate core particles from oligonucleotides and undigested chromatin, aliquots were applied to linear 5-20% (w/w) sucrose gradients in 50 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF and then centrifuged at 182 000 x g for 16 hours at 4°C. Fractionation was as above and the monosome peak (see Figure 2.5.5) was pooled, dialysed exhaustively against 10 mM TRIS-HCl (pH 7.5) and 1.0 mM EDTA and then concentrated by pressure ultra-filtration to a concentration of 0.5 - 1.0 mg DNA/ml.

4.1.4 ISOLATION OF SEA URCHIN SPERM DNA

Sperm cells were obtained from male sea urchins as in Section 4.1.1 and 4.1.2.4. DNA was extracted according to the method of Kedes, et al. (1975). Sperm cells were washed 5 times in 0.54 M KCl by repeated centrifugation, at 1000 x g for 10 minutes, and resuspension. 4 ml of packed sperm was diluted to 12 ml with 0.54 M KCl and resuspended and then 200 ml 0.1 M TRIS-HCl (pH 8), 10 mM EDTA, 10 mM NaCl, 0.5% SDS and 100 µg/ml proteinase K at 55°C was added. This was incubated at 55°C for 5 hours after which 20 mg proteinase K was added and the incubation was continued for 16

hours at 50°C. This resulted in a clear, viscous solution which was phenol extracted 6 times (freshly distilled water-saturated phenol made 0.1% 8-hydroxyquinoline was used) and then chloroform-isoamyl alcohol (24:1) extracted twice. This solution was then dialysed exhaustively against 15 mM TRIS-HCl (pH 7.4) and 1 mM EDTA. The DNA was then precipitated by adding sodium acetate to 1 M and EDTA to 0.33 mM and 2 volumes of 96% ethanol. Precipitation was carried out at -30°C overnight. The DNA was pelleted at 10000 x g for 10 minutes and then dried under vacuum. The yield was about 32 mg DNA/ml packed sperm as measured by the absorbance at 260 mn.

4.2 ANALYTICAL TECHNIQUES

4.2.1 DNA DETERMINATION

DNA concentrations were determined spectrophotometrically using a PYE-Unicam SP-1800 spectrophotometer. Concentrations of nuclear suspensions were estimated by diluting aliquots 10 - 50 fold in 150 mM NaCl or an equivalent ionic strength buffer and a further 10 fold dilution in 10% SDS. The absorbance at 260 nm was measured in the range 0.2 - 1.8 and related to the DNA concentration by the following relationship.

DNA concentration (mg/ml) = 0.05 x absorbance at 260 nm x dilution factor

The absorbance was determined in cuvettes with a 1 cm pathlength and the spectrophotometer was zeroed at 320 nm (i.e. where DNA has a very low absorbance relative to that at 260 nm). DNA concentrations of chromatin and nucleosome solutions were determined by the same method.

4.2.2 APPROXIMATION OF SEDIMENTATION COEFFICIENTS ON LINEAR SUCROSE GRADIENTS

Isokinetic sucrose gradients were designed to allow particles of specific density and constant hydrodynamic properties to move at a uniform rate in a centrifugal field. This rate is proportional to the sedimentation coefficient which may be determined mathematically or by the use of an internal standard. (Noll, 1967). Such gradients have an exponentially convex form and are produced using a constant volume mixing chamber. However, Fritsch (1973) has shown that sedimentation velocity of all kinds of macromolecules is constant in a linear 5-20% sucrose gradient at temperatures 5-20°C. The distance sedimented ($r - r_m$) is related to sedimentation coefficient by the equation,

$$r - r_m = \alpha S_{20,w} \omega^2 t$$

where r and r_m are the distances from the rotor axis to the macromolecular zone and to the meniscus, respectively, ω is the rotor speed, t is the time and α is a proportionality constant which depends on the kind of macromolecule.

Thus, mononucleosomes (11S) centrifuged through a linear 5-20% gradient in a Beckman SW40 rotor would migrate about half way down the tube if centrifuged at 18200 x g for 16 hours at 5°C (see Figure 2.5.5).

4.2.3 DNA ELECTROPHORESIS

4.2.3.1 Non-denaturing Gels

1.8, 2.0 or 2.5% agarose (Sigma-type V (high gelling temperature)) slab

gels were run either vertically (220 mm x 230 mm x 1.8 mm) or horizontally (150 mm x 100 mm x 4-6 mm) until the bromophenol blue dye front had travelled 100-150 mm at a voltage of 10 volts/cm. The gel and tray buffer was 90 mM TRIS-borate (pH 8.3) and 2.5 mM EDTA. (TBE buffer) (Peacock and Dingman, 1967) From 5 to 10 µg of DNA were applied to a single lane, except in the case of restriction fragments where 1-2 µg were applied into 10 mm slots. Samples for electrophoresis were dissolved in 9 mM TRIS-borate (pH 8.3), 0.25 mM EDTA (i.e. to 1/10 TBE), 5% glycerol, 0.01% bromophenol blue and 0.4% Sarkosyl NL 35 (Ciba-Geigy).

4.2.3.2 Denaturing Gels

Polyacrylamide gel electrophoresis under denaturing conditions was done according to Maxam and Gilbert (1977). 8% acrylamide, 0.4% methylene bisacrylamide gels were run in 90 mM TRIS-borate (pH 8.3), 2.5 mM EDTA (TBE buffer) and 8M urea. The reservoir buffer was TBE buffer. Gels for pilot redigestions were 200 mm x 230 mm x 1.8 mm and gels for the 5'-labelled DNAse I digestion products were 400 mm x 400 mm x 0.8 mm. Electrophoresis was at 1200 volts for the larger gel and 400 volts for the smaller gel. Both gels were run at 50-60°C. Samples for electrophoresis were dissolved in 8 M urea, 1/10 diluted TBE buffer and 0.01% bromophenol blue, heated to 100°C for 1 minute and cooled rapidly on ice.

To make 150 ml of gel solution, the following components were mixed:

75 g	Urea
15 ml	900 mM TRIS-Borate (pH 8.3), 25 mM EDTA
30 ml	40% acrylamide -2% methylene bisacrylamide
46 ml	water
1 ml	10% ammonium persulfate (freshly made up)

When the urea was dissolved the solution was deaerated, using a Venturi pump. A 10 ml plug of acrylamide was poured into the gel mould first to seal the spacers by adding 10 µl TEMED (N,N,N',N'-tetramethylethylene diamine) and quickly pouring the gel solution down the inside edges of the gel mould along the spacers. After this had set, the gel solution was poured, after adding 80 µl TEMED, as a slow continuous stream down the one edge of the mould. Care was taken not to introduce bubbles into the solution during puring. The slot (10 mm wide) former was then inserted and the gel was allowed to set at an angle (10° above horizontal) for 2-3 hours.

4.2.3.3 DNA Preparation for Electrophoresis

Nucleosomal DNA which was to be analysed by electrophoresis was stripped of its protein contaminants by adding 0.6 M Na-acetate, 1% SDS and 5 mM EDTA (final concentrations). Proteins were removed by phenol (water saturated) extraction 3 times and chloroform-isoamyl alcohol (24:1) extraction twice.

The following technique was used to perform these extractions on small volumes (i.e. 20 - 100 µl). 1.5 ml Eppendorf vials were used. To the DNA solution was added an equal volume of phenol or chloroform-isoamyl alcohol. This was shaken on a Vortex mixer and then centrifuged at 12000 x g for 30 seconds. The bottom, organic phase was removed by inserting a micropipette to the bottom of the tube and then sucking up only the bottom phase. This minimises DNA losses. DNA was then precipitated by added 2-3 volumes of ice-cold 96% ethanol, mixing by inverting the tube and then cooling at -30°C for about 16 hours or in liquid nitrogen vapour for 1 hour. The precipitated DNA was pelleted at 12000 x g for 10 minutes and the pellet was washed with ice-cold 70% ethanol. DNA pellets were dried under vacuum for about 30 minutes and then dissolved in the appropriate sample application buffer for gel electrophoresis.

4.2.3.4 DNA Gel Staining, Visualization and Photography

DNA gels were stained in a solution which contained 0.5 µg ethidium bromide/ml for about 30 minutes. The DNA was visualized as fluorescence of the DNA-ethidium bromide complex upon irradiation with ultraviolet light with a wavelength of 254 nm using a UV-transluminator. Gels were photographed with a standard single lens reflex camera fitted with a 50mm macro lens. Kodak Panatomic X or Ilford Pan F 35 mm film was used and developed according to the instructions for each type of film. A yellow filter (Wratten No. 4) was fitted to the lens so that only the fluorescence would expose the film. From Figure 4.1, it can be observed that a red filter (Wratten No. 24) decreases the amount of light which reaches the film by about 12% whereas a yellow filter (Wratten No. 4) does not absorb light above 460 nm but transmits no light below 440 nm.

4.2.4 Determination of Acid Soluble DNA products after Micrococcal Nuclease Digestion

The digestion kinetics of micrococcal nuclease on nuclei or chromatin can be followed by monitoring the production of acid - soluble deoxyribonucleotides. (Clark and Felsenfeld, 1971). The nuclease digests both DNA and RNA to acid-soluble products, thus an RNA contaminant or large amounts of nuclear RNA would make it difficult to interpret the results with much certainty if the nucleic acid determination was performed by measuring the absorbance at 260 nm. Because yeast cells are rich in RNA it was necessary to measure acid soluble DNA products after micrococcal nuclease digestion of yeast chromatin by an alternative method. (Lohr, et al., 1977(a)). However, in the case of sea urchin embryo cells, cytoplasmic RNA would not contaminate the chromatin preparations unduly as isolation techniques using

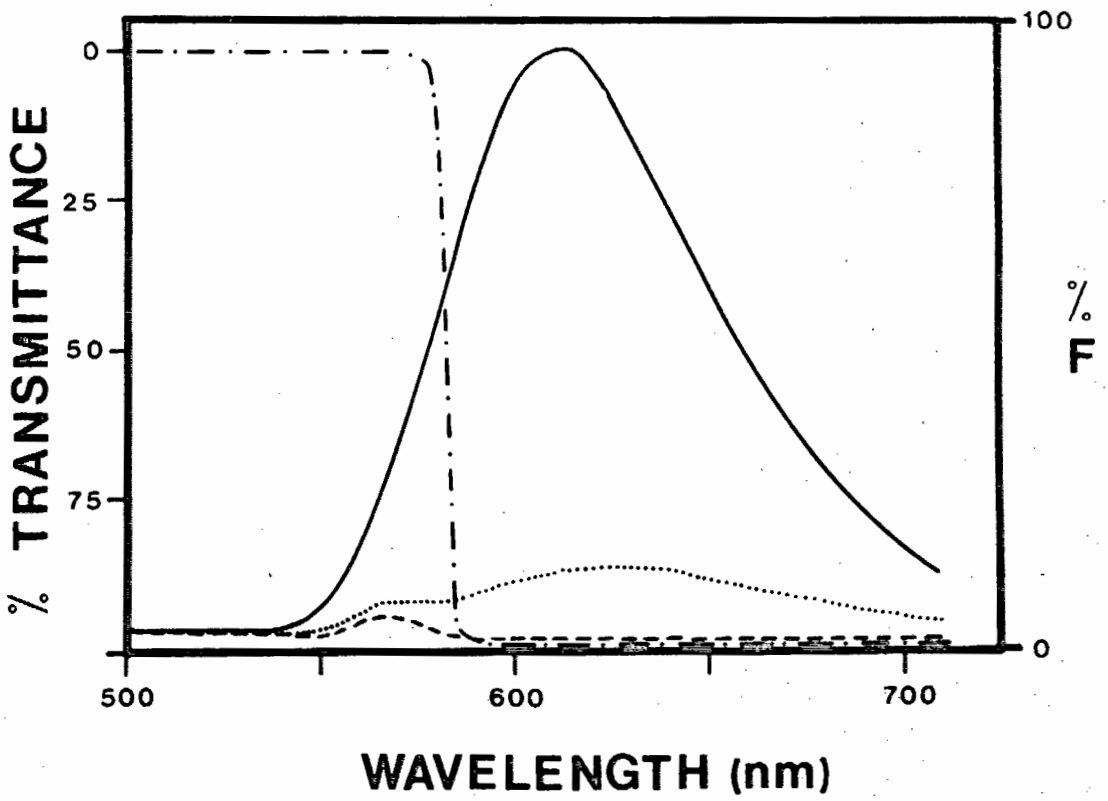


FIGURE 4.1 Comparison of the light transmitted by a red filter (Wratten No. 24) with the fluorescence of ethidium bromide-DNA complexes irradiated with 254 nm UV-light.

-·—·—·- transmission of red filter (Wratten No. 24)

——————— - fluorescence spectrum of a solution of DNA (1 µg/ml) and ethidium bromide (1 µg/ml) in TBE

. . . . . - fluorescence spectrum of ethidium bromide (1 µg/ml) in TBE

– – – – – – - fluorescence spectrum of TBE

detergents, will remove cytoplasmic contaminants. (Keichline and Wassarman, 1979). The amount of RNA per nucleus of the sea urchin, Strongylocentrotus purpuratus, mesenchyme blastula amounts to 0.17 pg RNA (Davidson, 1976; pages 157 - 163) which is 10% of the mass of DNA per nucleus. If the half-life of gastrula nuclear RNA is similar to that in blastula nuclei, then the proportion of RNA relative to DNA in this chromatin would be similar because the rate of synthesis of total heterogeneous nuclear RNA is comparable. Thus, it is possible to monitor micrococcal nuclease activity on sea urchin blastula and gastrula chromatin with a reasonable degree of reliability by measuring the absorbance at 260 nm.

Chromatin at a DNA concentration of 1 mg/ml was digested with micrococcal nuclease (60 units/mg DNA) in Buffer A with 1.0 mM $CaCl_2$ at 37°C. Aliquots containing 100 - 200 µg DNA were removed at zero time and at various times during digestion and the reaction was stopped by adding EDTA to 5 mM from a 250 mM stock solution. 20 µg bovine serum albumin, from a 1 mg/ml stock solution was added and the volume was brought to 0.6 ml by adding water. 0.4 ml 2 M NaCl and 2 M PCA was added (i.e. final concentrations of NaCl and PCA were 0.8 M). This was then cooled at 4°C for 30 minutes after which the insoluble material was pelleted at 10000 x g for 10 minutes. The supernatant was removed with a Pasteur pipette and the absorbance at 260 nm was measured. The percentage acid-soluble DNA was determined by the equation below taking into account the hyperchromic effect.

$$\% \text{ acid soluble DNA} = \frac{A_{260} \text{ of acid soluble DNA} \times 0.6}{A_{260} \text{ of undigested sample in 10\% SDS}} \times 100$$

4.2.5 Chromatin Solubility Assay

Sea urchin gastrula and sperm and chicken erthrocyte chromatin were digested with micrococcal nuclease (60 units/mg DNA) at a DNA concentration of 1 mg/ml in buffer A with 1.0 mM $CaCl_2$ at 37°C for 4 minutes. The reaction was stopped by adding EDTA to 5 mM and the chromatin suspension was dialysed exhaustively against 0.2 mM EDTA brought to pH 7.5 with TRIS base, at 4°C. The insoluble material was pelleted and to aliquots of the soluble chromatin supernatant, containing about 50 µg DNA/ml, equal volumes of twice the required concentrations of NaCl in 0.2 mM EDTA-TRIS (pH 7.5) were added. Precipitation was for 3-4 hours at 4°C. Insoluble material was removed by centrifugation at 12000 x g for 10 minutes. The supernatants were removed with a Pasteur pipette and the absorbances at 260 nm of the chromatin which remained soluble at the particular ionic strengths were measured.

4.2.6 AUTORADIOGRAPHY OF RADIOACTIVE DNA GELS

Wet 8% polyacrylamide gels for autoradiography were transferred to sturdy, thin sheets of plastic. The gel and its support were covered with sandwich wrap and exposure to Du Pont Cronex 4 X-ray film was accomplished by placing the film against the radioactive gel and inserting these in an X-ray film cassette. Exposure was at -30°C and for from 16 hours to 7 days. If necessary, a Du Pont Cronex lightning plus intensifying screen was placed against the X-ray film surface which was not in contact with the radioactive gel, in order to decrease exposure times.

Du Pont Cronex 4 X-ray film was developed in Agfa developer G-127 (1+5 dilution) for 4 minutes, stopped in running water for 30 seconds and fixed in Kodafix fixer for 2-3 minutes. The film was washed in running water for at least 30 minutes after fixing.

4.2.7 QUANTITATION OF RADIOACTIVITY IN DNA GELS

^{32}P-labelled DNA fragments were separated in 8% polyacrylamide gels containing 8 M urea. The fragments were visualised by autoradiography and the amount of radioactivity in each band was determined by either cutting the fragments from the gel and counting the radioactivity by liquid scintillation counting or scanning the gel lanes with a Vitatron TLD 100 densitometer and then, by integration, calculating the areas under the peaks.

Autoradiogram lanes were scanned on the densitometer at a slow speed so that bands which were close together could be best resolved. Densitometer tracings were digitized using a Summagraphics Bit Pad and a Tektronix 4051 computer programmed to estimate the areas under the peaks. The programme fits a series of Gaussian curves to the densitometer tracing. The fitting involves the estimation of 3 parameters, the height, the width and the position of each peak. The best height is determined by least squares and the best position and width parameters are determined by the user on the basis of the summed Gaussian curves to the original densitometer tracing. The programme was designed and written specially for the purpose of estimating the areas under the peaks of electrophoretogram and chromatagram tracings by Dr B T Sewell.

The range of intensities of the bands on the X-ray film was greater than the linear range of the X-ray film. 3-4 exposures of different times were therefore made of each gel. Lanes were scanned on the densitometer and the band intensities were scaled on the basis of the areas under the fitted Gaussian curves.

4.3 ENZYMATIC DIGESTIONS AND MODIFICATIONS

4.3.1 Micrococcal Nuclease Digestions

Micrococcal nuclease (3.1.31.1) (Sigma) was dissolved in 10 mM TRIS-HCl (pH 7.5) and 10 mM $CaCl_2$ at a concentration of 20 000 Kunitz units/ml and stored frozen. Repeated freezing and thawing did not reduce the enzyme activity.

Micrococcal nuclease digestion of chromatin was performed at a DNA concentration of either 1 or 5 mg/ml in buffer A (see Section 4.1.2.3.1) to which $CaCl_2$ was added to a final concentration of 1 mM from a 100 mM $CaCl_2$ stock solution. EDTA and EGTA in buffer A was titrated out by adding an equivalent molar excess of $CaCl_2$. $CaCl_2$ was added just prior to enzymatic digestion. The nuclease concentration was 20 or 60 units enzyme/mg DNA and digestions were at 37°C.

Micrococcal nuclease redigestions of H1-depleted long chromatin was performed at a DNA concentration of 0.5 - 1.0 mg/ml in 50 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 0.2 mM EDTA and 0.2 mM PMSF to which EDTA was added to a final concentration of 1.2 mM. Micrococcal nuclease concentration was 100 Kunitz units/ml and digestion was at 37°C. Reactions were stopped by adding a 5-fold molar excess of EDTA and cooling on ice.

4.3.2 Pancreatic Deoxyribonuclease (DNase I) Digestion of Nucleosomal Core Particles

DNase I (3.1.21.1) (Sigma) was dissolved in 50% glycerol and 1 mM $CaCl_2$ at

a concentration of 10000 units/ml and stored at -30°C. Over a period of 18 months the enzyme activity decreased by less than 20% of its original activity when stored in this manner.

Samples of ^{32}P-labelled core particles (25-50 µg DNA/ml) were in 70 mM TRIS-HCl (pH 7.6), 5 mM dithiothreitol and 10 mM $MgCl_2$. The enzyme concentration was 5 units/ml and digestion was at 37°C. Aliquots (0.5 - 1.0 g labelled DNA) were removed at specific times and the DNA was prepared for electrophoresis.

Rate constants of DNase I cleavage at each site on the nucleosomal core particle were determined according to Lutter (1978). ^{32}P-labelled DNase I digestion products were analysed on 8% denaturing polyacrylamide gels. After autoradiography, the amount of radioactivity in each band was determined (Section 4.2.7). Thus, a value R_{10n} is measured which is the amount of radioactivity in a band 10n bases from the 5'-end where n is an integer from 1 to 14. Using the equation

$$P_{10n} = \frac{R_{10n}}{\sum_{m=n}^{N} R_{10m}}$$

where N = 14, P_{10n}, which is the fraction of strands cut at a site 10n bases from the 5'-end after time t, is calculated. 1 -P_{10n} is therefore the fraction of strands which are not cleaved by the enzyme at the site 10n bases from the 5'-end and this is related to the digestion rate by the

equation,

$$1 - P_{10n} = e^{-kt}$$

where k is the apparent first-order rate constant. The slope of the linear regression line of the plot of ln $(1 - P_{10n})$ upon t is the apparent first-order rate constant in seconds $^{-1}$.

Rate constant calculations were performed on a Tektronix 4051 computer programmed by Mr J.D.Retief.

4.3.3 Removal of 5'-end Phosphate Groups from DNA by Treatment of the DNA with Bacterial Alkaline Phosphatase

To an aliquot of oligonucleosomes (see Figure 2.1.4) (15 - 30 µg DNA) in 2 mM EDTA-TRIS (pH 7.5) $MgCl_2$ was added to a final concentration of 2 mM from a 100 mM stock solution. Bacterial alkaline phosphatase (3.1.3.1) (Bethesda Research Laboratories) was added to a final concentration of 80 units /ml and this was incubated at 37°C for 2 hours. Oligonucleosomes were then pelleted at 100 000 rpm for 35 minutes in a Beckman Airfuge and then dispersed in 70 mM TRIS-HCl (pH 7.6), 10 $MgCl_2$ and 5 mM dithiothreitol.

4.3.4 5'-end Labelling of DNA with T4 Polynucleotide Kinase

Oligonucleosome fractions, treated with bacterial alkaline phosphatase or nucleosomal core particles prepared by digestion of chromatin with micro-

coccal nuclease, were labelled at the 5'-end with ^{32}P according to Lutter (1978). The DNA concentration of the nucleosome preparations were 25-50 µg/ml (i.e. 30 - 60 pmol of 5'-ends) in 70 mM TRIS-HCl (pH 7.6), 5 mM dithiothreitol and 10 mM $MgCl_2$. 10 - 20 pmol [γ - ^{32}P] - ATP (Amersham) (5000 or greater Ci/mmol) were added and incubated with 3-4 units T4 polynuceotide kinase (2.7.1.78) (Amersham) for 1 hour at 37°C. Greater than 70% incorporation of label (as measured on a Sephadex G-50 column eluted with 0.2 mM EDTA-TRIS (pH 7.5)) shows that at least 25-35% of the core particles were labelled.

4.3.5 Restriction Endonuclease Digestion of Plasmid, Bacteriophage and Chromatin DNA

Restriction endonucleases, from Bethesda Research Laboratories, were stored as prescribed by the manufacturers. Digestion of phage PM2 (Boehringer), or plasmid pBR322 (Sigma) DNA was with 2 units of enzyme per µg of DNA at 37°C for 1 hour in the buffer indicated below for each enzyme. After digestion, the DNA was extracted and prepared for electrophoresis (see Section 4.2.3.4).

Digestion of chromatin fractions (see Figure 2.1.4 and Section 2.2) was in the buffers prescribed by the manufacturers to optimise enzyme activity. Enzyme concentrations were increased to 1.5 units/ µg DNA and digestion was for 7 hours at 37°C after which DNA was extracted and prepared for electrophoresis (see Section 4.2.3.4).

Sea urchin sperm DNA was digested with restriction endonucleases in the buffers mentioned above. To 8 µg DNA in 50 µl buffer 25 units of enzyme was added and incubation was at 37°C for 16 hours. The DNA was then ex-

tracted and prepared for electrophoresis (see Section 4.2.3.4).

Restriction endonuclease digestion buffers:

Bam H1	20 mM TRIS-HCl (pH 8.0)
	7 mM $MgCl_2$
	100 mM NaCl
Bgl II	20 mM TRIS-HCl (pH 7.4)
	7 mM $MgCl_2$
	7 mM 2-mercaptoethanol
Eco R1	100 mM TRIS-HCl (pH 7.2)
	5 mM $MgCl_2$
	50 mM NaCl
	2 mM 2-mercaptoethanol
Hae III	50 mM TRIS-HCl (pH 7.5)
	5 mM $MgCl_2$
	0.5mM dithiothreitol
Hind II	10 mM TRIS-HCl (pH 7.6)
	10 mM $MgCl_2$
	50 mM NaCl
	15 mM dithiothreitol
Hind III	20 mM TRIS-HCl (pH 7.4)
	7 mM $MgCl_2$
	60 mM NaCl

Sal I	8 mM TRIS-HCl (pH 7.6)
	6 mM $MgCl_2$
	150 mM NaCl
Xba I	6 mM TRIS-HCl (pH 7.4)
	100 mM NaCl
	6 mM $MgCl_2$

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