POLY(GLUTAMIC ACID) PROMOTED ASSEMBLY OF NUCLEOSOME CORES ON THE HISTONE GENE QUINTET OF PSAMMECHINUS MILIARIS.

JACQUES D. RETIEF

1986

Submitted in fulfilment of the requirements for the degree of Docter of Philosophy in the Faculty of Science at the University of Cape Town, South Africa.

CERTIFICATE OF SUPERVISOR

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

Professor C. von Holt
Head, Department of Biochemistry
University of Cape Town

ABSTRACT

This thesis investigates whether DNA and histones contain sufficient information to direct nucleosome cores into specific positions.

The "in vitro" assembly of nucleosome cores promoted by poly(glutamic acid) has been optimized with respect to rate and yield. This was achieved by paying attention to the purity of the core constituents and in particular by the use of histones in their octameric form. The suitability of a number of octamer purification protocols, to produce pure undenatured histone octamers, has been investigated and the methodology improved.

The particles assembled on random DNA have been found to be indistinguishable from native nucleosome cores by the following criteria: Their S value on sucrose gradient centrifugation, resistance to Micrococcal nuclease digestion, DNase I digestion patterns, DNase I digestion kinetics at the susceptible sites, electronmicroscopic appearance, histone content and electrophoretic mobility.

Cores were also assembled on unique DNA, namely the intact h22 histone quintet of Psammechinus miliaris. Low resolution mapping, by indirect endlabelling of polycores assembled on the quintet, did not reveal any preferred sites of assembly. To investigate the core associated DNA at single base pair

resolution, a series of fragments, excised from the H2A-H1 and the H1-H4 spacer areas, were inserted into pGV403 plasmids. These plasmids can be strand specifically end-labelled with the Klenow fragment at the two different Tth 111 I excision sites utilised to isolate the propagated insert. On the free linearised DNA a complex digestion pattern is produced due to the sequence specificities of Micrococcal nuclease and DNase I. When cores are assembled on this DNA the digestion pattern is changed. This pattern reveals two preferential frames of assembly and indicates that in the remainder of the fragments cores are assembled, randomly, or in a number of overlapping frames.

It is concluded that the DNA fragments investigated and the histone octamer contain enough structural information to influence the positions occupied by some nucleosome cores. The implications of these findings are discussed.

ACKNOWLEGEMENTS

I would like to thank the following persons who have assisted in the completion of this work:

Prof. C. von Holt for encouragement, guidance and constructive critisism during this project.

Prof. M.L. Birnstiel for the pBRh22 clone and its complete sequence.

Dr. Trevor Sewell for electron microscopy and many hours spent on computer programs, especially those used to manipulate the densitometer data.

Johan Greyling and Sylva Schwager for generous gifts of histone octamers.

The past and present members of the workshop team, Claus Achleitner, Hermann Alk and Robb Senekal for designing and constructing all the electrophoresis equipment, especially power supplies, electrophoresis chambers and the "Monster" densitometer.

Winston Davids for reproduction most of the photographs in this thesis.

All my friends and co-workers for discussions and support.

Jenny Rees for proof-reading and enthusiastic encouragement.

Council for Scientific and Industrial Research and the University of Cape Town Research Committee for financial assistance.

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

Abbreviation	Description
AMPS	Ammonium peroxodisulfate
Bis-acrylamide	N,N',-Methylene-bis-acrylamide
bp	Base pairs
BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
DNase	Deoxyribonuclease
DTT	Dithiotreitol
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis-(2-amino-ethyl ether) N,N'-tetra-acetic acid
IPTG	Isopropyl- β-D-thiogalactopyranoside
L broth	Luria broth
p	Plasmid
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
Sarcosyl	Sarcosyl NL35 (T.M. Ciba-Geigy)
SDS	Sodium dodecyl sulphate (Dodecyl hydrogen sulfate sodium salt)
TEMED	N,N,N',N',-Tetramethylethylendiamine
Tris	Tris(hydroxymethyl)-aminomethane
Xgal	5-Bromo-4-chloro-indoyl-β-D-galactoside

1. SCOPE OF THIS INVESTIGATION

The object of this investigation is to determine whether histone octamers and DNA contain enough structural information to specifically position the nucleosome core on a defined DNA sequence.

To answer this question, nucleosome cores must be assembled "in vitro" on DNA with a known sequence, initially in the absence of any tissue factors that can influence the positioning of the cores.

I will first review the evidence for the existence or otherwise of specific positioning of nucleosomes "in vivo" and "in vitro". I will then consider the suitability of "in vitro" assembly protocols.

In the experimental section I describe the assembly protocol developed and the results obtained in applying the protocol to the assembly of cores on random and unique DNA.

The implications of these results are discussed.

2. LITERATURE REVIEW

The fundamental repeating unit of eukaryotic chromatin is the nucleosome (Kornberg 1974). The nucleosome consists of two each of the four core histones H3,H4,H2A,H2B, called the octamer and one histone H1, associated with approximately 200 base pairs of DNA. Removal of the H1 histone and further digestion of the DNA with nucleases gives rise to a nucleosome core particle, consisting of the histone octamer associated with 145 base pairs of double stranded DNA. For reviews see Kornberg (1977), Felsenfeld (1978), Rint and Nover (1980), Mirzabekov 1980, Kornberg and Klug (1981), Cartwright et al. (1982), Igo-Kemenes et al. 1982. The core particle is ubiquitous in all eukaryotic chromatin. The crystal structure of this canonical core has been studied using neutron diffraction to 16 Å resolution (Bently et al. 1984), and by X-ray diffraction to 5 Å resolution (Finch et al. 1981, Richmond et al. 1984). The structure of the histone octamer has been resolved at 3.3 Å resolution (Burlingame et al. 1985). In the models derived from the core and octamer crystals there are considerable discrepancies with regard to the shape of the core, the path of the DNA and the position of the subunits. (Klug et al. 1985, Moudrianakis et al. 1985.

Nucleosome cores play an obvious structural role in the packing of DNA into higher order chromatin structures. In addition the large number of histone variants expressed at various stages of the life cycle (Cohen et al. 1975, Brandt et al. 1979) suggest that, beyond that role, they may be important in functional

aspects of chromatin. For review see Von Holt (1985).

Such a possibility is suggested by the fact that "in vitro" transcription, of the chicken tRNA gene, is dependant on the position of the functional promoter sequence (TTGA) on the assembled nucleosome core. Maximal transcription occurs when the promoter is positioned on the middle axis of the nucleosome core (Wittig and Wittig 1981, 1982a and b).

If the position of nucleosomes on a gene can influence the activity of that gene, it is important to understand the forces involved in the sequence specific assembly of nucleosomes since these forces would ultimately play a role in determining the gene activation.

In this context the term positioning should be defined. Positioning can mean that every single nucleosome core in the genome is attached to a specific DNA sequence (Prunell and Kornberg 1977). A more general definition of positioning is any defined relationship between cores and a DNA sequence (Zachau and Igo-Kemenes 1981). This relationship may be simple, with each core occupying an unique position, or complex, with nucleosome cores occupying several defined frames within a specific sequence. The concept of positioning could be expanded to include inhibition of core assembly relative to a defined DNA sequence. Such a sequence is found around the initiator site in the SV40 virus (Varshafsky et al. 1977, 1979, Saragosti et al. 1980, Hiwasa et al. 1981, Jacobovits et al. 1982). The antithesis to positioning is the random association of octamers

and DNA, in which the nucleosome cores are placed with equal affinity on any part of a DNA sequence.

2.1 "IN VIVO" DISTRIBUTION OF NUCLEOSOMES:

2.1.1 Random association:

To answer the question whether nucleosome cores assembled "in vivo" are associated randomly or specifically with the DNA sequence, Prunell and Kornberg (1977) produced 145 base pair DNA fragments by extensive micrococcal nuclease digestion of rat liver chromatin. These DNA fragments were limit digested using Exo III to produce two single stranded fragments 70 bases long. If the original cores each occupied an unique position on the DNA then the single stranded fragments should not cross hybridise. In the experiment, however, random hybridisation was observed. From this the authors concluded that nucleosome cores are randomly associated on most single copy DNA sequences. In the light of the sequence specificity of micrococcal nuclease and Exo III (Dingwall et al. 1981, Hörz and Altenburger 1981) these results may have to be reinterpreted. In addition, the presence of complex frames of positioning, or of populations of positioned cores cannot be excluded using this experimental protocol.

One advantage of random association could be the simplification of DNA packaging, to accommodate a large number of differently structured genes (Kornberg 1981). It is has also been argued that, because of tissue specific variation of nucleosome spacing, (Compton et al. 1976, Thomas and Thompson 1977) the DNA sequence cannot be responsible for a high degree of

positioning. This argument rests on the fact that the DNA sequences in the genome are identical in all tissues. Kornberg (1981) also makes the point that the DNA affinity of the histone octamer to any sequence investigated is orders of magnitude less than that of proteins, such as repressors, that are known to bind DNA sequences specifically.

2.1.2 Specific positioning:

Nucleases are commonly used in experiments to determine specific positioning. These experiments vary considerably in complexity but can nevertheless be divided into two categories according to the level of nuclease digestion.

In the experiments of the first category only partial digestion with micrococcal nuclease is employed (Wittig and Wittig 1979, Brown et al. 1979, Gottesfeld and Bloomer 1980, Gottesfeld 1980). The sequence composition of a partial digest is largely determined by the sequence specificity of micrococcal nuclease, since digestion takes place preferentially at AT rich sequences. This has been well established by the investigations of Nedospasov and Georgiev (1980), Dingwall et al. (1981), Hörz and Altenburger (1981), Pauli et al. (1982). A similar sequence specificity has also been reported for DNase I (Fedor and Daniell 1983, Drew and Travers 1984). Many of the experiments on positioning have been carried out before the sequence specificity of the nucleases were known or fully appreciated and may thus require reinterpretation.

In the second category more extensive digestions with micrococcal nuclease have been used to produce 145 base pair cores or dimers with subsequent identification of the protected DNA. Specific positioning has been found on Drosophila genes (Levy and Noll 1980), as well as on SV40 DNA (Ponder and Crawford 1977). A combination of Exo III and S1 after micrococcal nuclease treatment showed specific positioning in three frames on rat satellite I DNA (Igo-Kemenes et al. 1980). McGhee and Felsenfeld (1983) have drawn attention to a number of limitations of such experiments. The main problem lies in the production of GC rich core populations due to the cleavage preference of micrococcal and other nucleases for AT rich sequences. This type of "in vivo" probing for specific positioning inherently lacks controls as to the nuclease susceptibility of naked DNA. Indirect end-labelling (Wu 1980), on the other hand, makes such controls easy. Precisely spaced nucleosome cores were found on the non-transcribed spacer areas histone gene repeat of Drosophila melanogaster. The 5' ends of the histone genes remain in an exposed conformation throughout the development of the Drosophila embryo (Samal and Worcel 1981). Using the same technique, the promoter areas of the Psammechinus miliaris histone gene repeat (h22) were found to be sensitive to DNase I digestion during the expression of the early histone genes (Bryan et al. 1983). This sensitivity disappeared abruptly at the blastula stage. Some sequences near the 3' end of the genes were very resistant to micrococcal nuclease throughout development. These findings have however not been interpretted by the author in terms of nucleosome positioning.

The probing for positioning is complicated by findings which indicate that, for the same gene, specific positioning and random association may be present in different tissues. This was found for the tRNA gene cluster in various tissues of Xenopus laevis (Gottesfeld 1980). A sequence-specific arrangement of nucleosome cores were also found on the "oocyte-type" tRNA genes of Xenopus laevis (Gottesfeld and Bloomer 1980). For Xenopus laevis tRNA genes in active liver and kidney cells, there is no apparent positioning of the nucleosomes. However, in transcriptionally inactive erythrocytes nucleosomes were positioned in a single predominant frame (Bryan et al. 1981). Whereas these experiments point to the possibility that it may be transcriptional activity which can be correlated with the absence or presence of positioning, the $tRNA^{Lys}$, $tRNA^{Phe}$ and $tRNA^{Met}$ genes have been found to be specifically associated with nucleosomes in chicken embryo cells, (Wittig and Wittig 1979) presumably very active cells.

In the experiments considered up to now the sequence specific positioning or the random association had to be probed on the background of virtually all or a large fraction of the genomic DNA. Intranuclear "in vitro" assembly of unique pieces of DNA can take place in the frog oocyte system. End-labelled circularised somatic 5S RNA gene of Xenopus borealis and the histone gene repeat of Drosophila melanogaster, have been injected into the germinal vesicles of Xenopus laevis oocytes. The assembled nucleosome cores have been found to be

regularly spaced, with a 180 base pair periodicity, but randomly positioned (Gargiulo and Worcel 1983). These results differ from those obtained by indirect end-labelling of Drosophila embryo chromatin. In these experiments specific positioning within the histone gene repeat had been found (Samal and Worcel 1981). The discrepancies between the two experiments have not been satisfactorily explained.

2.1.3 Positioning in multiple frames:

The two alternatives, specific positioning, or random association, may be an over simplification of possible DNA-histone interactions. Under "in vivo" conditions nucleosome cores may occupy a number of frames, on genomic as well as satellite DNA (Fittler and Zachau 1979 and Zhang et al. 1983, 1984). The indirect end-labelling method used does not allow resolution better than 10-15 bases. Therefore nucleosome cores occupying multiple frames displaced by 10 base pairs or less may well be interpreted as being randomly associated.

An example of the confusion arising from multiple frame positioning is found in the much investigated satellite DNA chromatin from various sources. Whereas Musich et al. (1977, 1982) demonstrated the specific positioning of nucleosome cores on α-satellite DNA, Singer (1979) found no difference in spacing between bulk chromatin and satellite DNA, namely 185 base pairs for both. This would indicate random association between cores and DNA, since the DNA sequence repeat of the satellite is 9 base pairs. However, the same digestion patterns obtained by Musich et al. (1977) are present on free DNA due to

the sequence specificity of the nucleases employed (Fittler and Zachau 1979).

These discrepancies were finally resolved when techniques were developed to enrich the African Green Monkey α-satellite DNA, allowing the use of direct end-labelling techniques (Zang et al. 1983). It was established that the nucleosome cores are positioned on the 172 base pair repeat sequence of α-satellite DNA in eight strictly defined positions. 35% of the nucleosomes occupy one of these frames while each of the other registers occupy approximately 10% of the nucleosomes. Similar results were obtained on mouse satellite DNA (Zhang and Hörz 1984). The mapping experiments on satellite DNA were carried further by cloning the DNA fragments, obtained from core preparations, into M13 with subsequent sequencing. Of 50 clones completely sequenced 50% are derived from two major sites, the remainder come from 16 less preferred sites (Böck et al. 1984). These results have been subsequently confirmed by "in vitro" reconstitution experiments (Linxweiler and Hörz 1985).

2.2 "IN VITRO" ASSEMBLY SYSTEMS:

An "in vitro" assembly system should allow the investigation of the structural features governing the interaction of both the DNA and octamer. It would also be desirable if putative factors, which control the positioning of the nucleosome cores, could also be investigated. Though regular spacing of nucleosomes has been reported "in vitro", using the frog oocyte assembly factor, no specific positioning in such a system has been observed yet (Laskey and Earnshaw 1980). Such positioning has been reported for other types of "in vitro" assembly systems (Simpson and Stafford 1983 and Simpson et al. 1985).

Assembly forced through dialysis of a histone-DNA mixture from 2 M NaCl down to physiological ionic strength results in the association of histone octamers with SV40 viral DNA (Wasylyk et al. 1979). Minichromosomes assembled in this way have been mapped electron microscopically. The results indicate that there are preferred positions for assembly. In a poly(glutamic acid) assembly system, Simpson and Stafford (1983) have demonstrated the specific positioning of a single core on a 260 base pair fragment of a 5S RNA gene of Lytechinus varigatus, using direct end-labelling and limited DNase I digestion. This positioning agrees with one of two possible phasing schemes proposed for the 5S RNA genes from Drosophila (Louis and Schedl 1980) but does not agree with any of four possible phases in the Xenopus 5S RNA gene (Gottesfeld and Bloomer 1980).

Experiments demonstrating the specific positioning of nucleosome cores on E coli DNA have been carried out by Chao et al. (1979) and Ramsay et al. (1984). Since prokaryotic DNA is not covered by nucleosome cores "in vivo", the specificity may not be biologically significant.

2.2.1 Desirable features of an assembly system:

The assembly system of choice should not contain any denaturants such as urea which disrupts the histone octamer as this may seriously interfere with the fidelity of assembly. It is concievable that a mixture of partially denatured histones and properly associated octamers may eventually interact nonspecifically with DNA. Also, the presence of urea will lower the melting temperature of the DNA. This is of great concern as the regions in the DNA serving as a substrate for assembly may well be AT rich. Such low melting regions can form loops, stem loops or cruciform structures (Lilley 1980 and Panayotatos and Wells 1981) and thus influence the interaction between the octamers and DNA (Nickol and Martin 1983). In addition, urea may affect the structure of putative assembly factors to be assessed at a later stage.

The ionic strength during the assembly reaction should be between 50 and 300 mM NaCl since the properties of nucleosome cores have been reported to be essentially stable within that salt concentration range. Lower and higher salt concentrations have been shown to influence the structure of the core particle (McGhee et al. 1980, Wilhelm and Wilhelm 1980, Greulich et al.

1985). Increasing the ionic strength, from 0.1 M to 0.6 M NaCl, increases the size and the anisotropy of the core particle (Ausio et al. 1984).

During assembly by dialysis from high salt concentrations the ionic strength of the solution is altered according to a fairly rigid protocol. This makes it difficult to investigate the influence of ionic strength itself on the assembly and positioning of the cores.

The high ionic strength solutions used in many protocols during the assembly process can induce conformational changes, such as Z-DNA formation, in areas of the DNA. Such induced Z-conformation of poly[d(G-C).d(C-G)] can be stabilised by individual core histones at 150 mM NaCl (Russell et al. 1983). Also, core-like particles can be assembled on poly[d(Gm^5-C).d(C-Gm^5)] in the Z-form by a trout testis assembly factor. Though these particles have characteristics similar to natural nucleosome cores, namely an $s_{20,w}$ of 10.6 S, they contain 170-250 base pairs of DNA (Miller et al. 1982).

Specific tissue factors can play a role in the assembly and positioning of nucleosome cores (Gottesfeld and Bloomer 1982). The assembly reaction should therefore take place under conditions which will allow, at a later stage, investigation of the effects of these factors. High NaCl concentrations may well interfere with their efficiency and thus make it impossible to ultimately assemble a functional chromatin fiber associated

with enzymatically active proteins.

It is essential that the assembly system should produce a high yield of cores, free from aggregates, core-like particles or sub-core particles. This is of great importance since various assembly protocols have been reported to result in products that are very hard to distinguish from the natural cores. Core-like particles containing an octamer of histones H3 and H4 have been assembled (Simon et al. 1978, Stockley and Thomas 1979). Particles containing a hexamer of histones, consisting of $(H2A)_2$, $(H2B)_2$, $(H4)_1$ and $(H3)_1$ protecting 102 base pairs of DNA have also been assembled at physiological ionic strength (Ellison and Pulleyblank 1983a, b, c).

It would be desirable for the assembly protocol to be equally suitable for the assembly of cores in milli-, or nanogram amounts in order to allow the initial physio-chemical characterisation of the cores, and to analyse at high resolution the positioning of cores on unique DNA.

To find an assembly system that would lend itself to the further investigation of "in vitro" nucleosome core positioning, the literature was reviewed.

2.2.2 Core particle assembly at high ionic strength:

These assembly protocols involve extensive dialysis from high salt concentrations down to lower ionic strength in the presence or absence of urea. For review see McGhee and Felsenfeld (1980). Histones are reconstituted with DNA to form core particles with a high degree of fidelity (Axel et al. 1974; Camerini-Otero et al. 1976; Sollner-Webb et al. 1976; Tatchell and Van Holde 1977). Core particles can also be assembled on circular DNA with good yields (Camerini-Otero and Felsenfeld 1977). Dilution of a high ionic strength solution, containing the assembly components, to low ionic strength also leads to core assembly (Germond 1976). Rapid dilution followed by extended incubation, leads initially to low yields of cores which act as transient nucleation centres for supernumerous histones which in turn interact with DNA to form further cores. (Stein 1979). This latter method appears to produce initially a small number of nucleosome cores. Step dilution has resulted in good yields of cores (Simpson and Kunzler 1979).

In these assembly protocols the reactants are all well characterised, since only histones and the DNA participate in the reaction. However, high urea and salt concentrations may make it difficult to develop such assembly protocols further to ultimately assemble, for example, active positioning factors. In addition, long dialysis steps may lead to degradation of DNA and proteins.

In a variation of the salt assembly procedure, histone migration from a large excess of natural cores or polycores to free DNA is promoted by first increasing the ionic strength of the solution, followed by a decrease (Wilhelm et al. 1978) Though in this way the purification of histones is avoided, the necessary prior removal of H1 may disturb the histone stoichiometry since the conditions of H1 removal also leads to a partial dissociation of the histones H2A and H2B. This may lead to formation of particles containing non-stoichiometric ratios of core histones. The presence of non-histone proteins in the donor core population may also pose a problem.

2.2.3 Core particle assembly in crude and partially purified nuclear lysates:

Nucleosomes have been assembled using crude nuclear lysates such as those obtained from Xenopus oocytes (Laskey et al. 1977 and Gilkin et al. 1984) or Drosophila embryos (Nelson et al. 1979). In these protocols very rapid assembly occurs and the spacing of the nucleosomes resemble closely that of the natural chromatin fibre. For review see Laskey and Earnshaw (1980).

From such lysates presumptive assembly factors have been partially purified. An acidic, thermostable protein has been obtained from Xenopus eggs (Laskey et al. 1977) which acts as an assembly factor (Laskey et al. 1978, Mills et al. 1980). This protein appears to be similar or identical to the ubiquitous nucleoplasmin as judged by its immunological cross

reactivity (Krohne and Franke 1980a and 1980b). The crude assembly extract obtained from Drosophila embryos is rich in topoisomerase activity. The assembly activity was initially ascribed to the topoisomerase (Germond et al. 1979). However, further fractionation of the crude extracts revealed that the activity was due to the presence of high molecular weight RNA (Nelson et al. 1981). HMG proteins 1 and 2 exhibit assembly activity, though considerably less than other tissue factors (Bonne-Andrea et al. 1984). Extracts from trout testis also contain core assembly activity (Miller et al. 1985). The better characterised assembly factors appear to be polyanionic in nature. These factors could be polyanionic proteins such as nucleoplasmin (Laskey et al. 1978, Krone and Franke 1980a and 1980b) and HMG proteins with their polyanionic clusters (Bonne-Andrea et al. 1984).

2.2.4 Core particle assembly with non protein polyanions:

It appears that the octamer can be sufficiently stabilised "in vitro" by proteins with polyanion clusters, or RNA, to allow nucleosome core formation (Nelson et al. 1981). A simple synthetic polyanion with this potential is poly(glutamic acid) (Stein et al. 1979). The use of a synthetic polyanion has the advantages of homogeneity and purity over natural partially purified assembly factors. The assembly reaction promoted by poly(glutamic acid) has been reported to assemble 80% of the DNA in 1 hour, considerably faster than that in the salt assembly systems, although not as rapid as the assembly promoted by some crude nuclear lysates. Poly(glutamic acid) assisted assembly takes place at low ionic strength, preventing DNA conformational changes (Peck et al. 1982, Russel et al. 1983).

of the nucleases during subsequent core particle characterisations. The scale of preparation made it possible to monitor the DNA electrophoresis by ethidium bromide staining, and to use gradient centrifugation in long centrifuge tubes that allow high resolution sedimentation of the nucleosome cores and polycores. Milligram amounts of assembled core particles could be prepared with ease.

DNA was treated with proteinase K (Muller 1983), followed by repeated phenol and chloroform extractions and ethanol precipitation (Maniatis et al. 1982). The purified DNA gives the typical UV absorption spectrum (Fig. 1) and is free from proteinase K as no degradation of histones assembled on such DNA was observed (Fig. 7 Gel D Lane 2).

When plasmid DNA was used in protocols with unique DNA the preparation involved additionally two RNase A digestions and two caesium chloride centrifugation steps. The resulting plasmids were homogeneous on gel electrophoresis (Fig. 31).

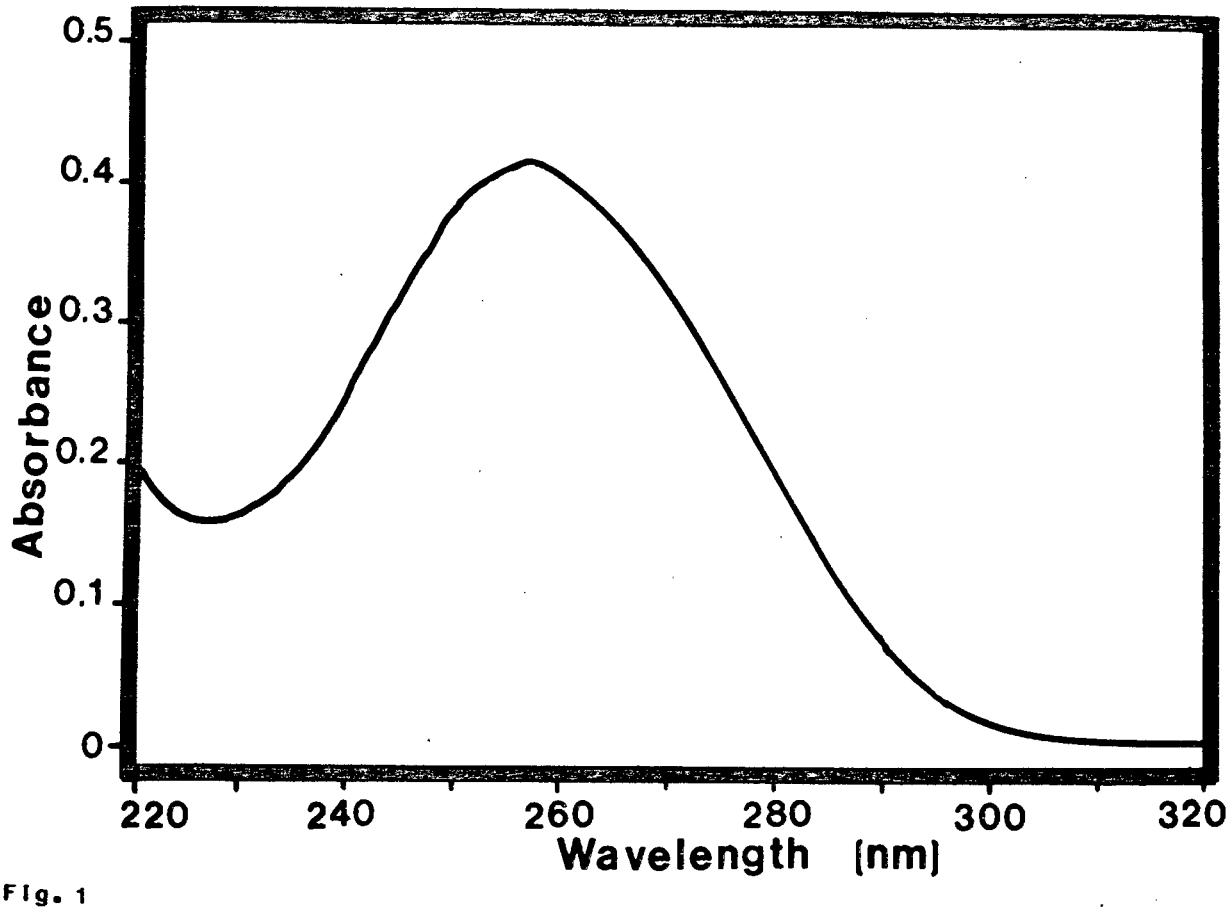


Fig. 1
Absorbance spectrum of purified DNA.
A 2 mg/ml solution of random DNA was diluted 1/100 with 2 M NaCl, 10 mM phosphate pH 7.6.

3.2.2 The histone octamers:

I consider it essential that the input histones should be in their natural octameric conformation. This form guarantees that the core histones are present in stoichiometric amounts. This is important because an excess of histones H3 and H4 can lead to the formation of a subnucleosomal particle [$(H3\text{-}H4)_2$, 64 bp DNA] (Oudet et al. 1977). Such a particle can also supercoil closed circular DNA (Camerini-Otero and Felsenfeld 1977). In addition particles of the composition [$(H3\text{-}H4)_4$,

145 bp DNA] with an S value of 10.4 can also form (Simon et al. 1978, Klevan et al. 1978). This particle is similar in appearance to the natural core particle (Thomas and Oudet 1979 and Stockley and Thomas 1979).

Therefore the efficacy of histone octamer production is important.

3.2.3 Salt extracted histone octamers:

The method of Ruiz-Carillo and Jorcano (1979) involves the sequential dissociation of chromosomal proteins from DNA resulting ultimately in the dissociation of the histone octamer after 10 hours exposure to 2 M NaCl. The highly viscous DNA is then removed from the histones by ultracentrifugation for 18 hours. I found that octamers prepared by this method contain a considerable amount of OD_{260} absorbing material (Fig. 2) and the core histones are not present in stoichiometric amounts (Fig. 7 A. Lane 4). In view of these findings this method of octamer isolation was not further pursued.

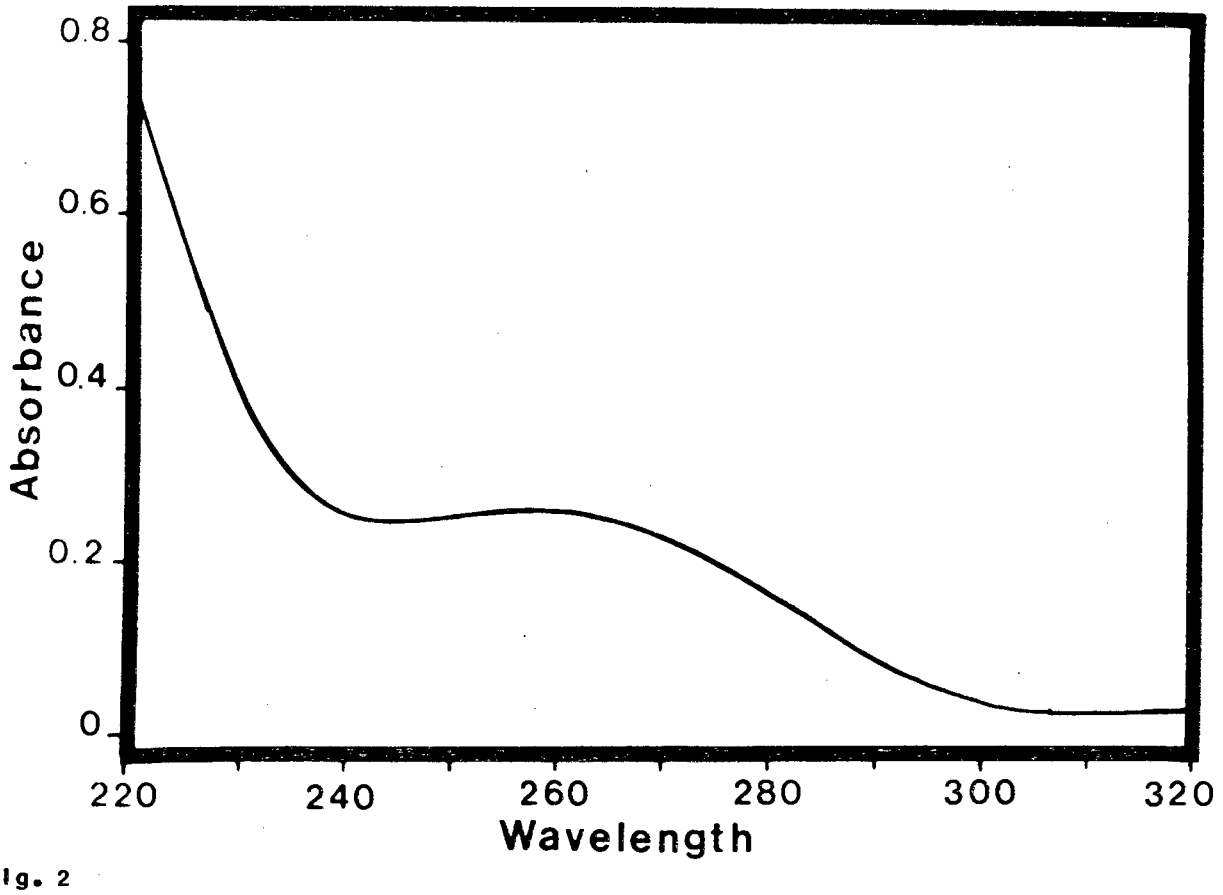


Fig. 2
Absorbance spectrum histone octamers purified by salt extraction.
Histone octamers purified by salt extraction were diluted in 2 M NaCl, 10 mM phosphate pH 7.6 and measured against the diluent.

3.2.4 Histone octamers extracted from partially digested chromatin immobilised on hydroxyapatite:

For assembly reactions histones are mostly isolated after dissociation from partially digested chromatin bound to hydroxyapatite (Faulhaber and Bernardi 1967 and Simon and Felsenfeld 1979). The chromosomal proteins are successively dissociated at typical NaCl concentrations.

To remove H1 and H5 the chromatin digest has been treated with an ion exchange resin prior to hydroxyapatite adsorption. (Oohara et al. 1983) or spun through sucrose gradients containing 0.6 M NaCl (Stein 1979). I found that histones H1 and H5 are completely removed by eluting the hydroxyapatite

column first with 0.6M NaCl (Fig. 7 B. Lane 1 and 2).

During the subsequent elution of the hydroxyapatite column with 3 M NaCl, the increase in the salt concentration, at the leading edge of the eluant, does not occur instantaneously. This causes the histone octamers to partially dissociate (Bloom and Anderson 1978 and Simon and Felsenfeld 1979) (Fig. 3). The leading fractions are therefore enriched with histones H2A and H2B (Fig. 3 a and Fig. 3 B. Lane 1). The trailing edge of the peak is enriched with H3 and H4 (Fig. 3 b and Fig. 7 B. Lane 2). The stoichiometry of the core histones produced by this method therefore depends on how the fractions are pooled. It also follows that the octameric complex isolated from a hydroxyapatite column is an octamer reconstituted from its component H2A-H2B dimers and H3-H4 tetramer.

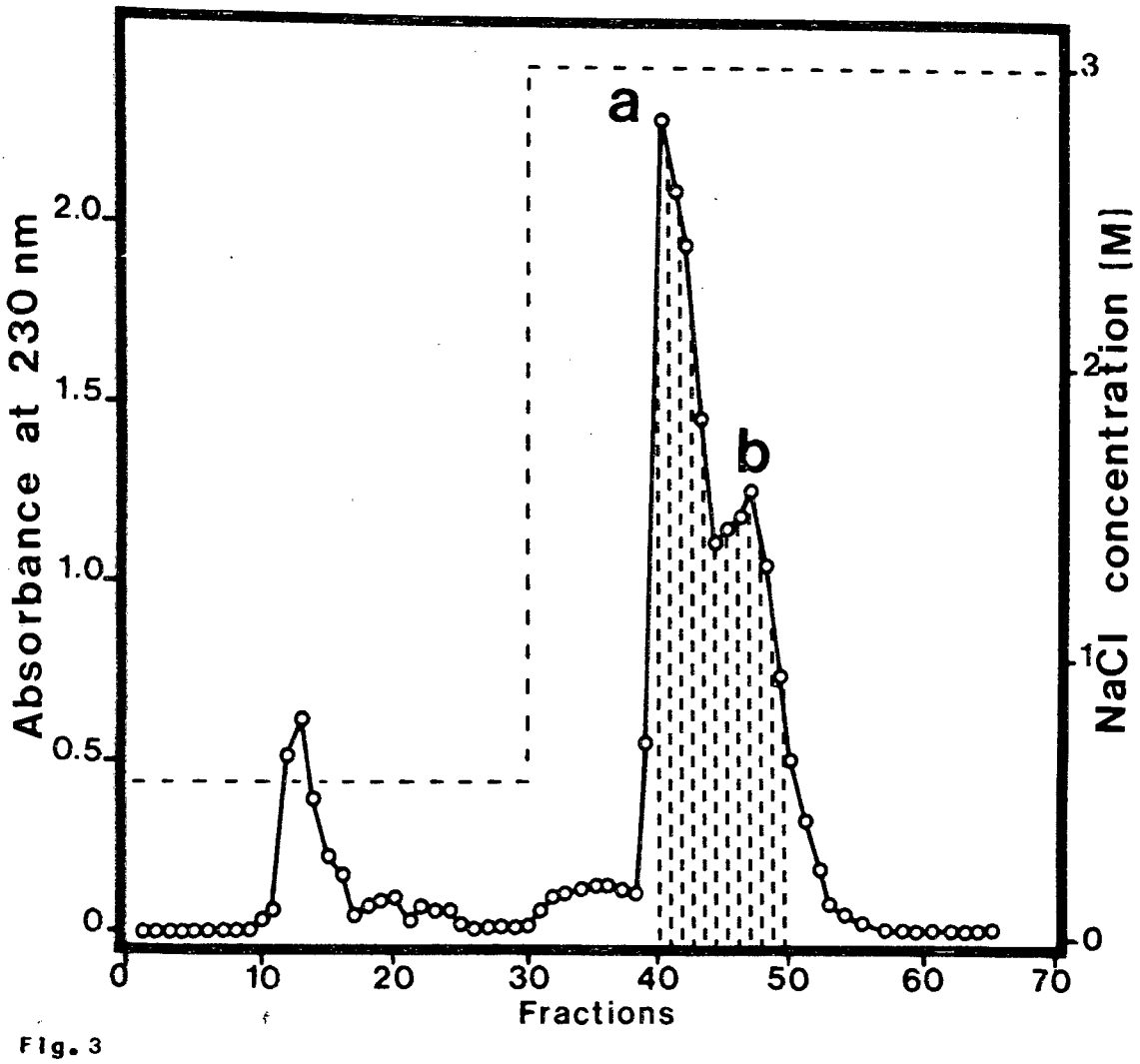


Fig. 3
Elution profile of chromatin immobilized on hydroxyapatite:
Column: 2.5 cm diameter, 20 cm long.
Matrix: hydroxyapatite.
Sample: A partial digest of chicken erythrocyte chromatin.
Eluant: NaCl step gradient in 5 mM Na_2HPO_4 and 5 mM NaH_2PO_4
The shaded area indicates the fractions pooled for subsequent experiments.

3.2.5 Further purification of octamers:

None of the purification protocols investigated produced histones of adequate purity. Methods to further purify the octamers were therefore investigated. It is known that the histone octamer is stable in solution under appropriate salt and pH conditions (Thomas and Kornberg 1975). In order to restore stoichiometry of the core histones and separate the octamer from excess dimers and tetramers the pooled fractions

from the hydroxyapatite column were subjected to exclusion chromatography on a Sepharose 6B or an Ultragel ACA 54 in 2 M NaCl, 20 mM Tris/HCl pH 8.0. (Greyling et al. 1983, Retief et al. 1984) (Fig. 7 C. Lane 1). The resulting octamers are free from oligo- or polynucleotides and exhibit the low absorbance at 280 nm typical for the histones.

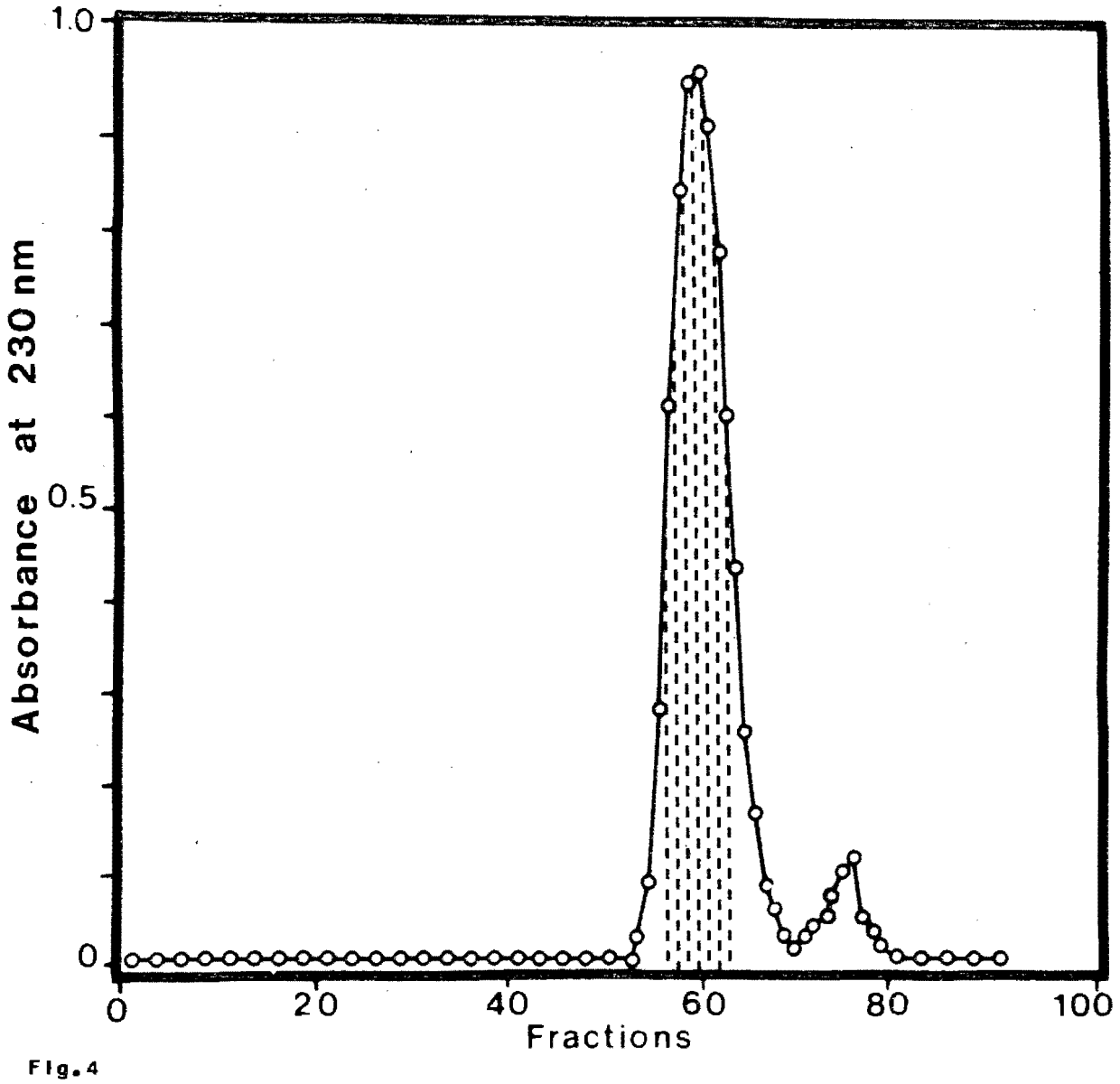


Fig.4
Exclusion chromatography of partially pure histone octamers.
Column: 2.5 cm diameter, 100 cm long.
Matrix: Sepharose CL 6B.
Sample: Histone octamers purified by hydroxyapatite chromatography.
Eluant: 2M NaCl, 0.1 mM PMSF and 10 mM Tris/HCl pH7.6.
The shaded area indicates the fractions pooled for subsequent experiments.

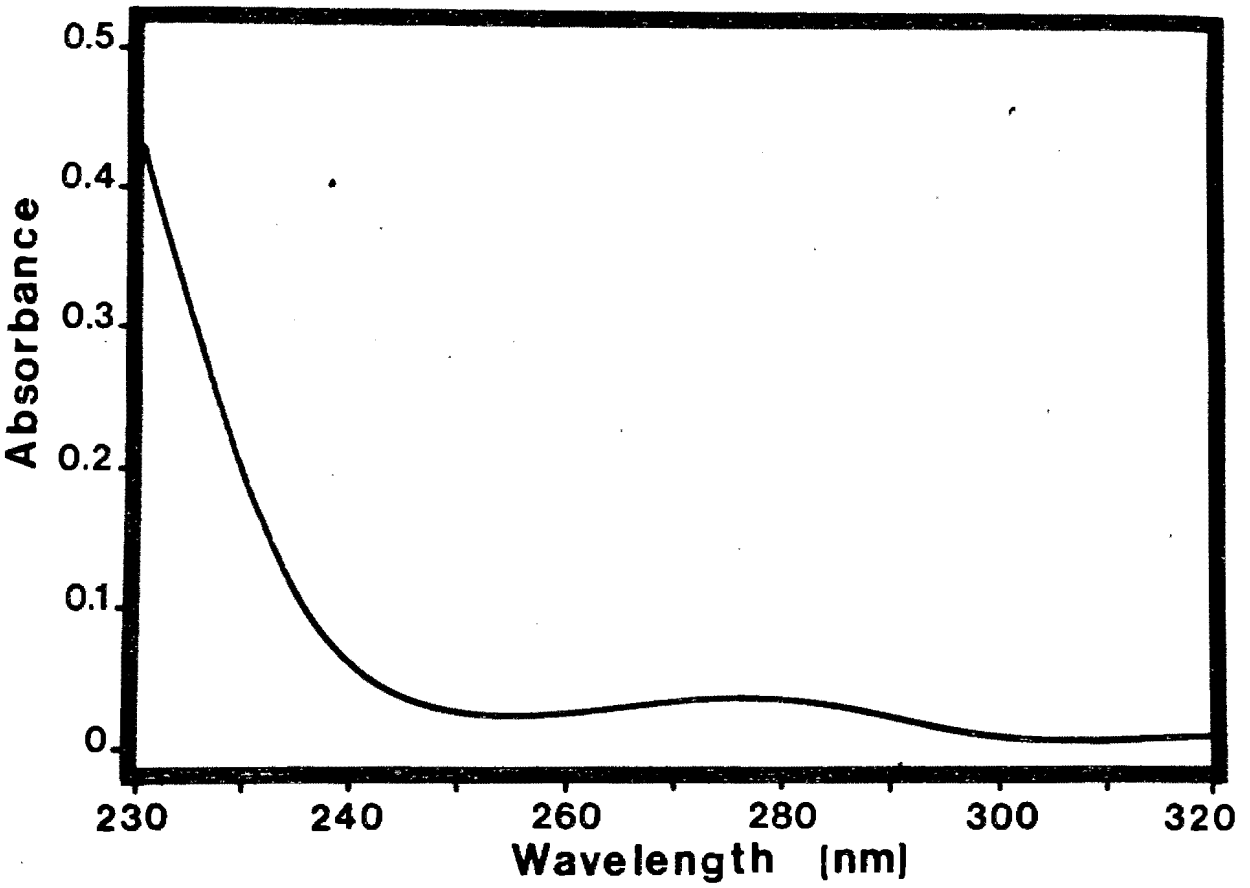


Fig. 5
Absorbance spectrum of histone octamers purified by exclusion chromatography. Histone octamers purified first by hydroxyapatite, and then exclusion chromatography were diluted in 2 M NaCl, 10 mM phosphate buffer pH 7.6 and measured against the diluent.

3.2.6 Octamer storage:

The histone octamer is stabilized by 50% glycerol at low salt concentrations (Beaudette et al. 1982). We found that histone octamers can be stored in 50% glycerol and 1 M NaCl at -20°C for several years without any degradation (Fig. 7 C Lane 2.), or dissociation of the octamer (Fig. 6).

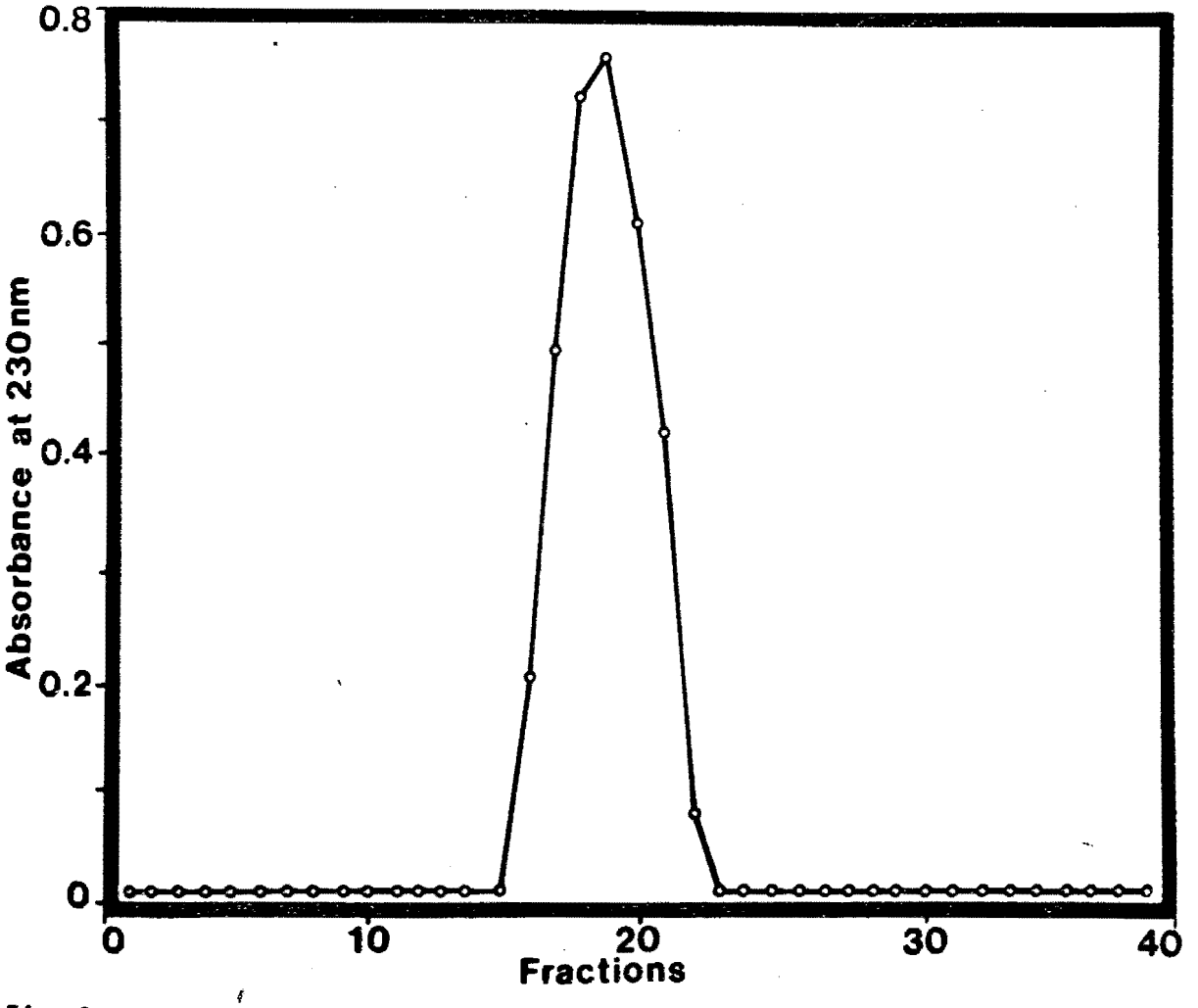


Fig. 6
Exclusion chromatography of purified histone octamers stored in glycerol.
Column: 1 cm diameter, 45 cm long.
Matrix: Ultra gel ACA 44.
Sample: Histone octamers purified by hydroxyapatite chromatography followed by molecular sieve chromatography and storage in glycerol.
Eluant: 2 M NaCl, 0.1 mM PMSF and 10 mM Tris/HCl pH 7.6.

The analysis of histones on SDS gels is commonly used to assess the quality of the histone preparations. The metachromatic staining of the Coomassie Brilliant Blue (Duhamel et al. 1980 Neuhoff et al. 1985) may mask small deviations in the histone stoichiometry.

Within these limitations, the core histones produced by elution from hydroxyapatite and exclusion chromatography are present in approximately stoichiometric ratios and do not contain histone H1, HMG proteins, membrane proteins, or small molecular weight breakdown products. (Fig. 7 C, Lane 1) The histone octamers freshly prepared or after storage in glycerol crystallize into the typical tubes Greyling et al. (1983).

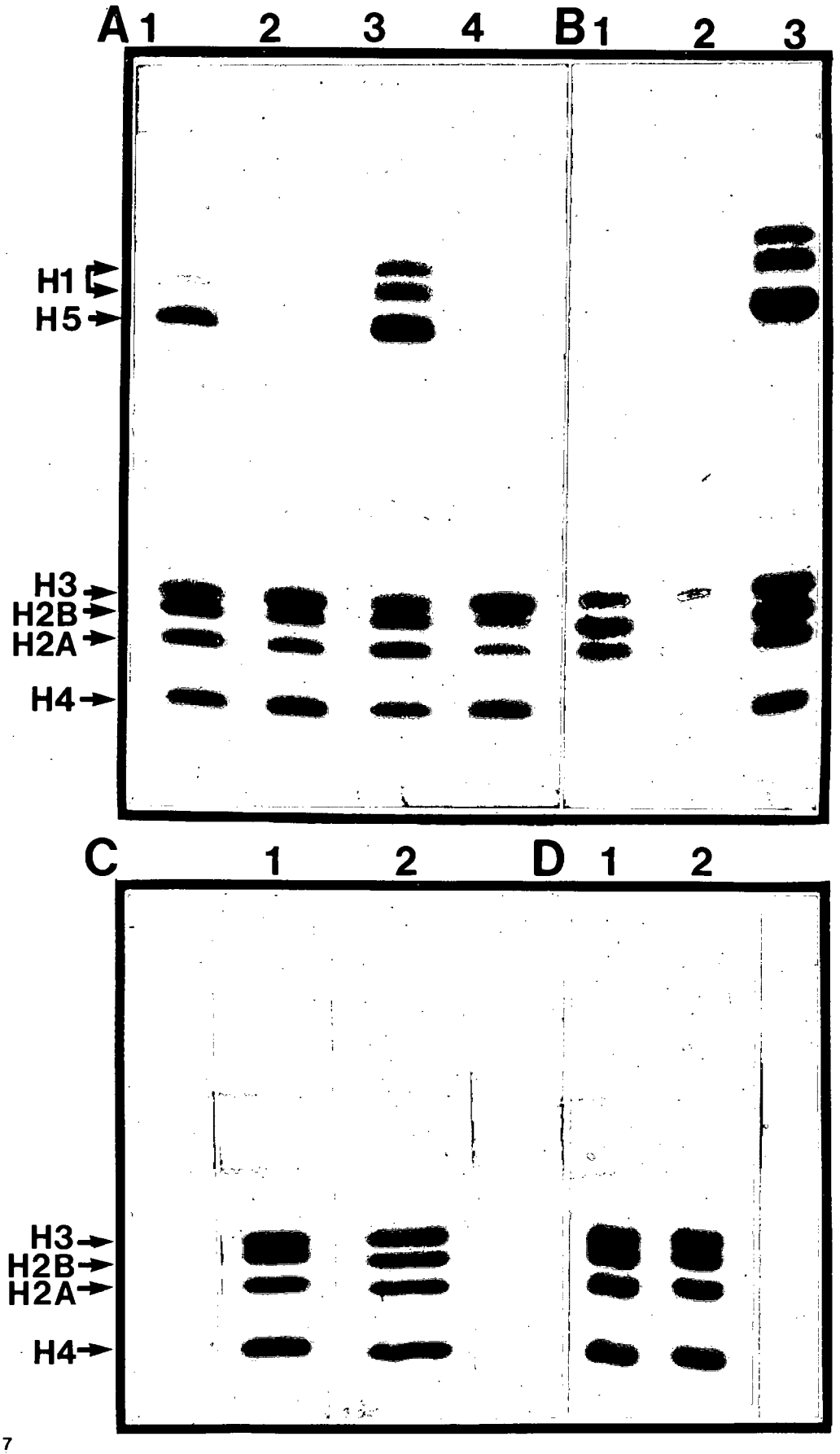


Fig. 7
SDS gel electrophoresis of histones.
Gel (A): Lane 1: total acid extracted histones. Lane 2: acid extracted core histones. Lane 3: total salt extracted histones. Lane 4: salt extracted core histones. **Gel (B):** Histones extracted by hydroxyapatite chromatography (Fig.). Lane 1: peak a. Lane 2: peak b. Lane 3: total salt extracted histones. **Gel (C):** Lane 1: Histone octamers purified by hydroxyapatite and exclusion chromatography. Lane 2: after storage for 4 years at -20 °C. **Gel (D):** Lane 1: histone octamers prior to assembly. Lane 2: histones extracted from assembled polycores purified by sucrose gradient centrifugation.

3.3 THE OPTIMISATION OF THE ASSEMBLY REACTION.

To optimise the assembly reaction, a number of parameters of the assembly reaction were investigated:

1) Time of preincubation of octamers and poly(glutamic acid)
2) The ionic strength at which the octamers are introduced to the system.
3) Temperature.
4) Nature and size of the polyanion.
5) Ratios of reactants

It has been suggested that the assembly reaction may take place in two distinct phases, first the interaction of the histones with the poly(L-glutamic acid), followed by the interaction of the histones with the DNA (Oohara et al. 1983).

The results in Fig. 8 show that there is no difference between the number of nucleosome cores assembled after preincubation of the histone octamers with poly(L-glutamic acid) for the times indicated:

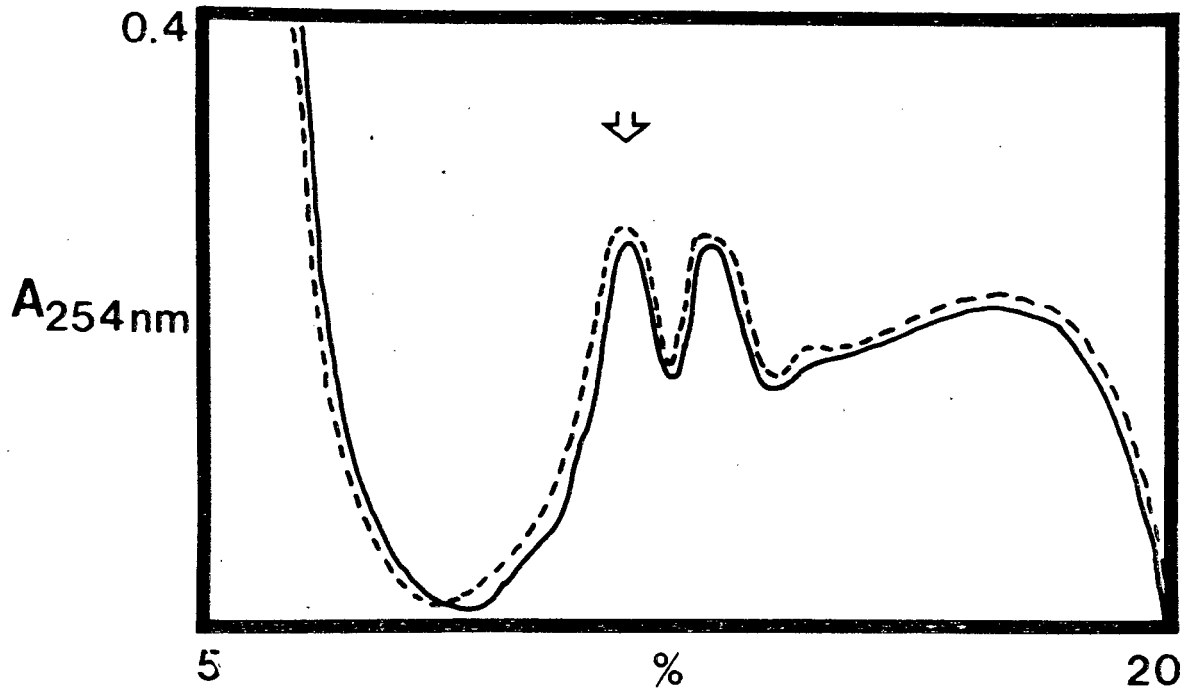


Fig. 8
Effect of pre-incubation on nucleosome core assembly.
Gradient: 5 - 20 % (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
The arrow indicates the nucleosome core position.
Assembly conditions:
DNA: Random
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Histone to DNA ratio (w/w): 1:1
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
Time of preincubation at 20°C:
——— 0 minutes
– – – – 30 minutes

Other authors reported that, upon mixing histone-poly(L-glutamic acid) initially insoluble complexes are formed (Cotten and Chalkley 1985) which only dissolve after several hours on shaking (Stein et al. 1979) or dialysis against decreasing ionic strength buffers (Oohara et al. 1983). I have never observed turbidity on mixing poly(glutamic acid) and histones provided undegraded histone octamers were used, and that poly(glutamic acid) and histones were mixed at approximately 2 M NaCl prior to dilution to low ionic

strength, before addition of the DNA. It is conceivable that the observation of other authors is due to partial denaturation of histones as a result of freezing the octamer preparation in buffers of high salt (Stein 1979), or low ionic strength (Oohara et al. 1983, Simpson and Stafford 1983 and Cotten and Chalkley 1985).

In order to prevent non-poly(glutamic acid) mediated assembly, (Stein 1979, Daban and Cantor 1982) the octamer solution was always first diluted in the presence of poly(glutamic acid) to low ionic strength before DNA was added to the system.

In gradient centrifugation experiments similar to that reported in Fig. 8 it was established that preincubation of the histones with poly(glutamic acid) at 1 M NaCl doubled the yield of assembly as compared to preincubation at 250 mM NaCl. Assembly takes place with nearly equal yields after incubation at +4°C and +37°C for 3 hours. Whereas the L-isomers of poly(glutamic acid) and poly(aspartic acid) with comparable molecular weight are approximately equally effective in promoting assembly, the D-isomer of poly(glutamic acid) appears to exibit a lower assembly promoting activity under identical conditions. Poly(glutamic acid) of a molecular weight range between 2 000-15 000 Daltons is approximately half as effective as the polymer in the range between 50 000-100 000 Daltons. Optimal assembly takes place at a histone:poly(glutamic acid) ratio of 1:1 (w/w) using the latter polymer size range. At a histone:DNA ratio of 0.5:1 (w/w) the yields of cores are higher than at a ratio of 2:1 (w/w) at which ratio presumably

predominantly tight dimers and polycores are formed (Fig. 9). These were also observed by (Stein et al. 1985).

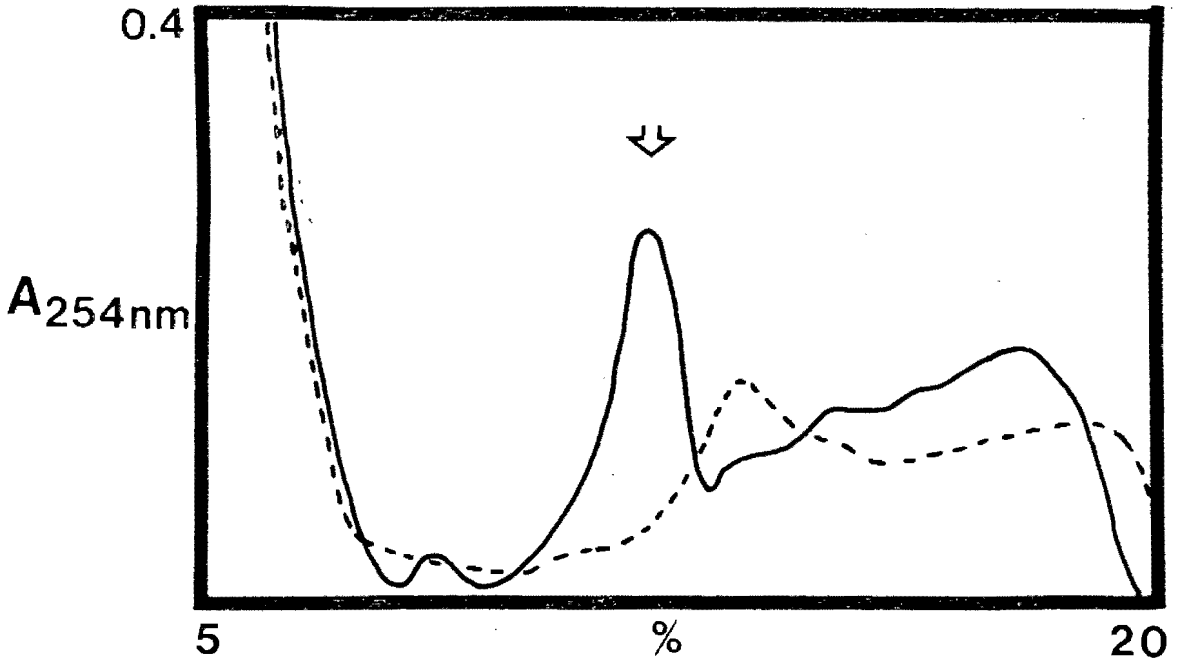


Fig.9
Effect of the histone to DNA ratio on nucleosome core assembly.
Gradient:5 - 20% (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
The arrow indicates the nucleosome core position.
Assembly conditions:
DNA: Random
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
Histone to DNA ratios used:
——— 0.5 : 1 (w/w)
– – – – 2.0 : 1 (w/w)

When core size DNA is used in the assembly reaction, at a histone to DNA ratio of 1:1.4 (w/w), the core peak contains 76% of all the recovered DNA (Fig. 10, Peak b). 11% of the DNA remained unbound (Fig. 10, Peak a) and 6% sedimented with an S value greater than nucleosome cores (Fig. 10, Peak c).

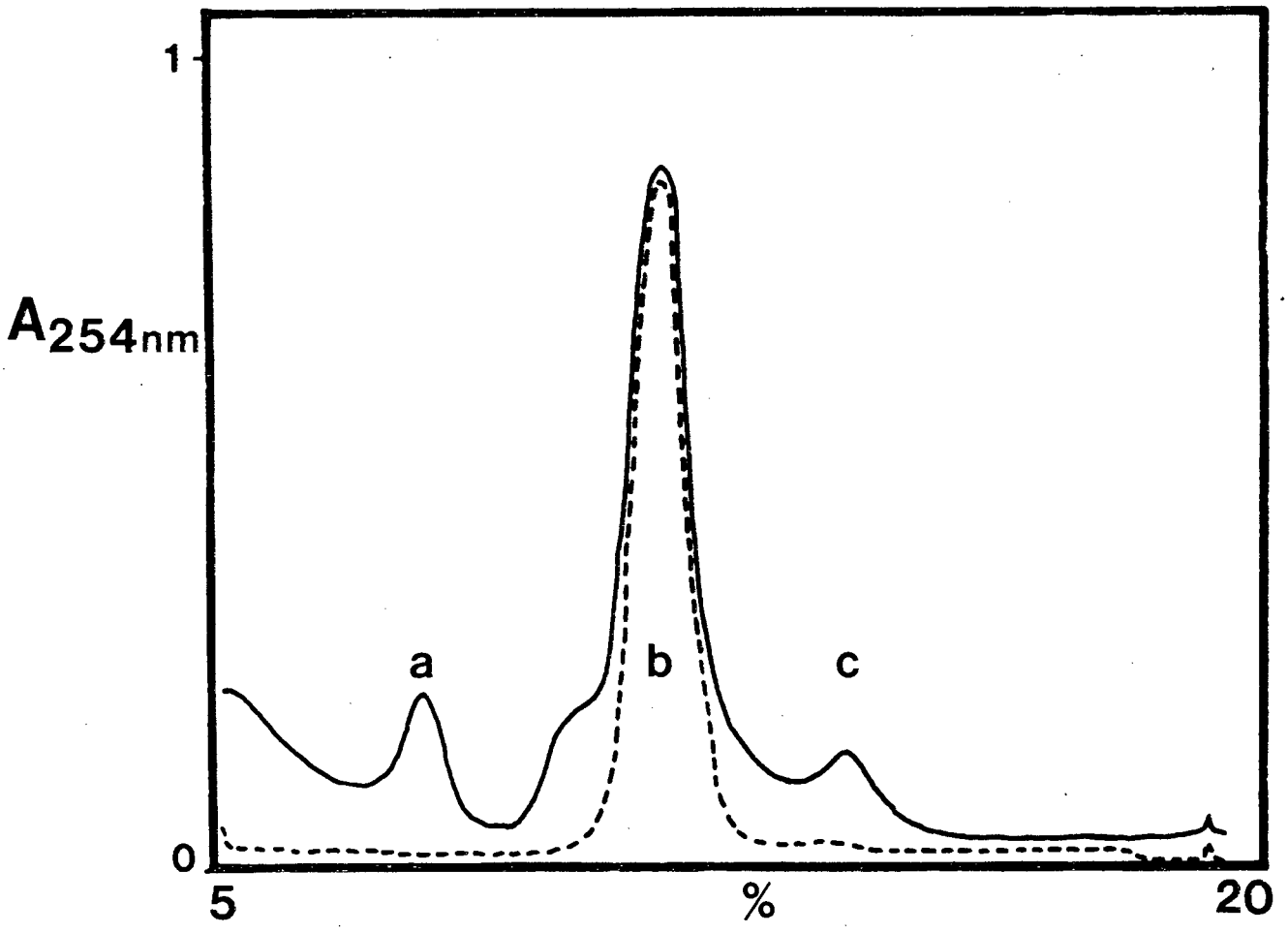


Fig.10
Cores assembled on 146 base pair DNA.
Gradient:5 - 20% (w/v) sucrose. Rotor: Beckman SW40Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
Assembly conditions:
DNA: 146 base pair.
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Histone to DNA ratio (w/w): 1.4:1
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
– – – – – Natural cores.
——— Reconstituted cores.
(a) Free DNA.
(b) Core peak.
(c) Large complex.

The optimisation of the assembly procedure has been achieved mainly by introducing the histones into the assembly reaction as purified octamers at high ionic strength and only then diluting the assembly reaction mixture in the presence of poly(glutamic acid) to low ionic strength prior to the addition

of DNA. This protocol allows the assembly of cores in high yield close to physiological ionic strength.

3.4 THE ROLE OF POLY(GLUTAMIC ACID):

Poly(glutamic acid) stabilizes the octamer in solution at low ionic strength (Stein et al. 1979 and Retief et al. 1984). In order to investigate whether a transient defined histone-poly(glutamic acid) complex forms in solution the assembly reaction was investigated using labelled poly(glutamic acid).

To this end the N-terminal amino group of the poly(glutamic acid) was labelled via acetylation. This label will not alter the overall charge characteristics of the molecule significantly.

Exclusion chromatography was undertaken of the assembly mixture containing [^{3}H]-labelled poly(glutamic acid) and [^{125}I]-labelled histone octamers, in the absence of DNA, on a column equilibrated and eluted with unlabelled poly(glutamic acid). Such an experiment will establish to which extent, if at all, the polyanion and the histone octamer interact to form a largely undissociated complex.

It is obvious from the experiment represented in Fig. 11 that the interaction between the polyanion and the histone octamer is transient. No stable complex is formed as each of the components of the mixture applied to the column elute with its characteristic elution volume, established previously in calibration runs.

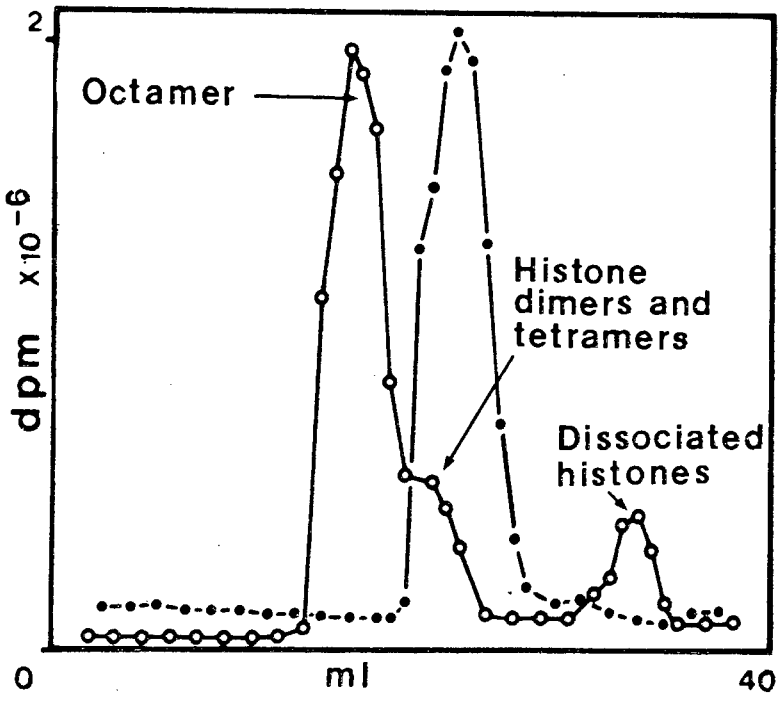


Fig.11
Exclusion chromatography of histone octamers stabilized by poly(glutamic acid).
Column: 1 cm diameter, 45 cm long.
Matrix: Ultra gel ACA 44.
Eluant: 0.2 M NaCl, 0.1 mM PMSF, 0.2 mM EDTA 0.1 mg/ml poly(glutamic acid) and 40 mM Tris/HCl pH 8.0.
Sample:
○—○—○—○ [^{125}I]-labelled histone octamers in the presence of unlabelled poly(glutamic acid).
●—●—●—● [^{3}H]-labelled poly(glutamic acid) incubated with unlabelled histones.

In the absence of poly(glutamic acid) the histone octamer dissociates into its component core histones.

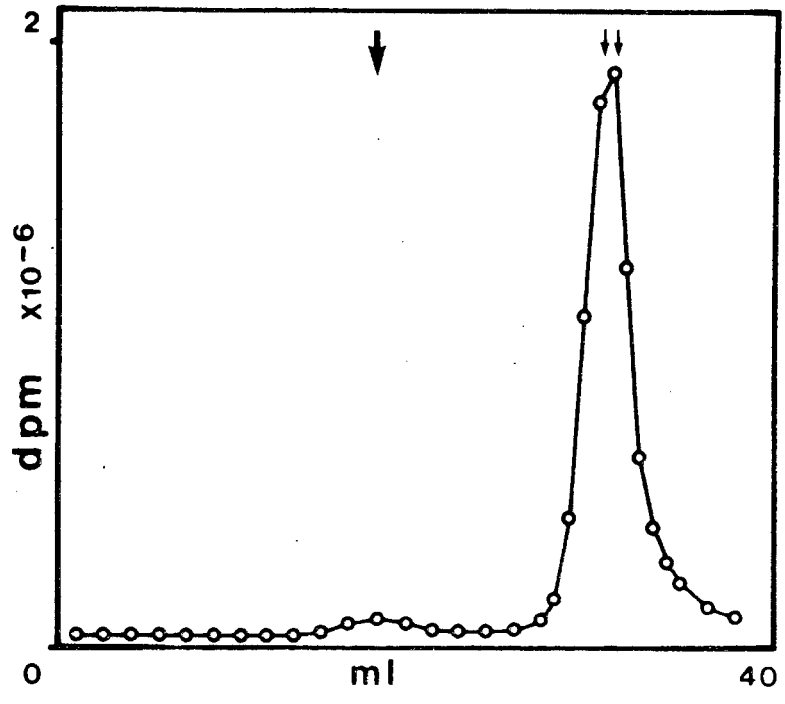


Fig. 12
Exclusion chromatography of histone octamers at low ionic strength.
Column: 1 cm diameter, 45 cm long.
Matrix: Ultra gel ACA 44.
Eluant: 0.2 M NaCl, 0.1 mM PMSF, 0.2 mM EDTA and 40 mM Tris/HCl pH 8.0.
Sample: [^{125}I]-labelled histone octamers.
→ Octamer
⇉ Dissociated histones

The absence of complex formation between octamers and the polyanion is also obvious from gradient centrifugation (Fig. 13). Interaction between DNA and poly(glutamic acid) as suggested by Yoshida (1983) is absent.

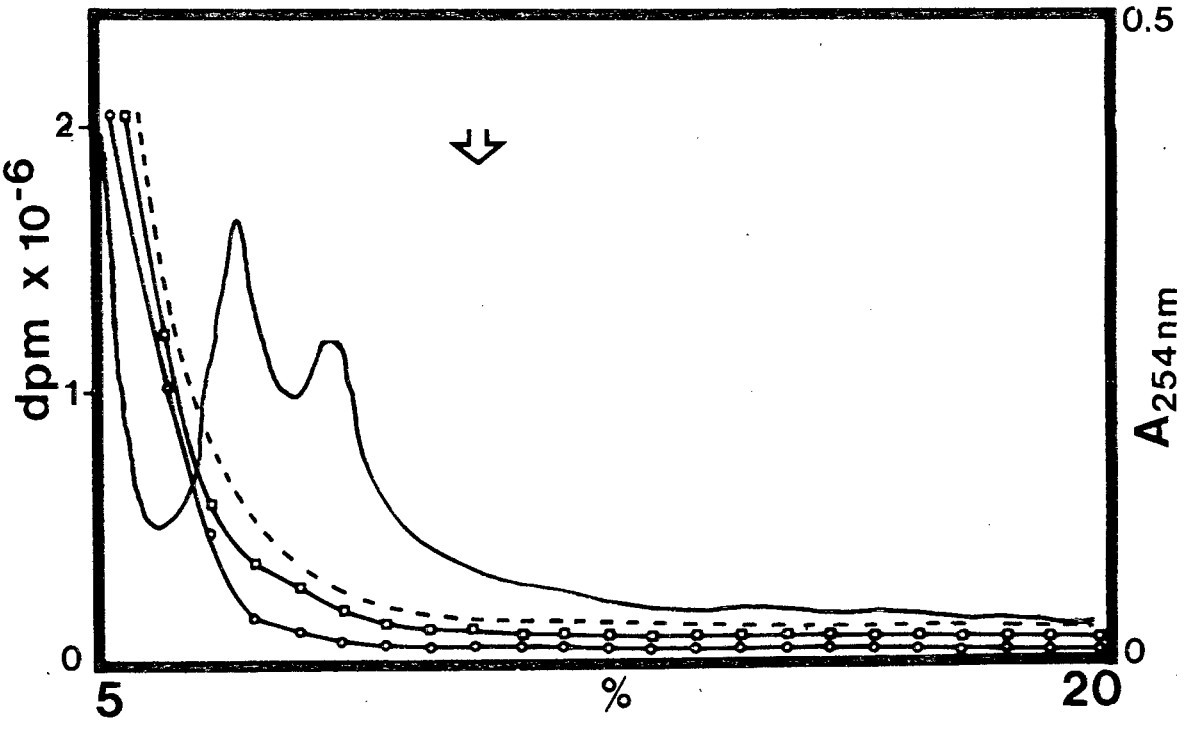


Fig. 13

Effect of histones and DNA on the sedimentation rate of poly(glutamic acid).

Gradient: 5 - 20 % (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
The arrow indicates the nucleosome core position.

Incubation conditions:

Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Poly(glutamic acid) to histone ratio (w/w): 2:1
Poly(glutamic acid) labelled by acetylation with [^{3}H]acetic anhydride.
Incubations: 3 hours at 37°C

o—o—o—o—o [^{3}H]poly(glutamic acid) only.
□—□—□—□—□ [^{3}H]poly(glutamic acid) incubated with histones.
- - - - - - - - [^{3}H]poly(glutamic acid) incubated with DNA.
——————— Absorbance at 254 nm of DNA incubated with [^{3}H]poly(glutamic acid).

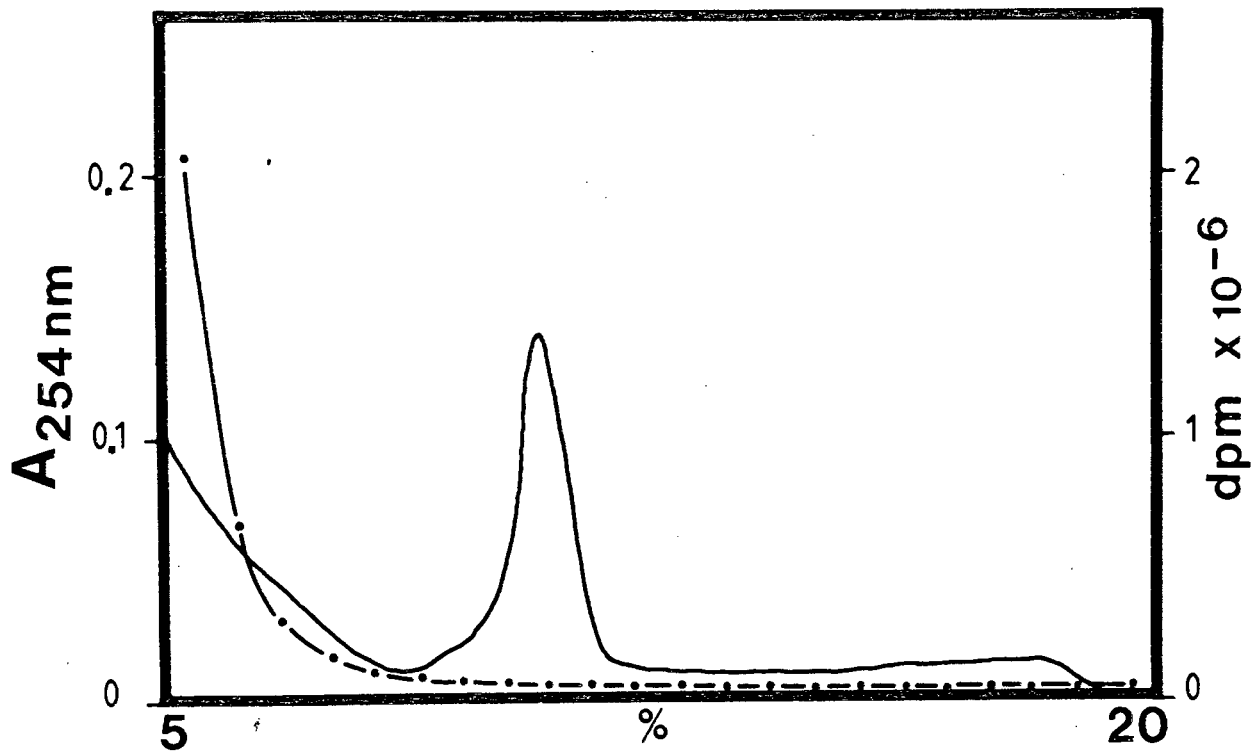


Fig.14

The effect of cores on the sedimentation rate of poly(glutamic acid).

Gradient:5 - 20 % (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
Incubation conditions:
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Poly(glutamic acid) to nucleosome core ratio (w/w): 2:1
Poly(glutamic acid) labelled by acetylation with ^{3}H-acetic anhydride.
Incubation: 3 hours at 37°C
— . — . — Poly(glutamic acid) incubated with nucleosome cores.
———— Absorbance at 254 nm.

As to be expected from the results this far, in the full assembly mixture the polyanion, the core and the polycore fraction sediment independantly.

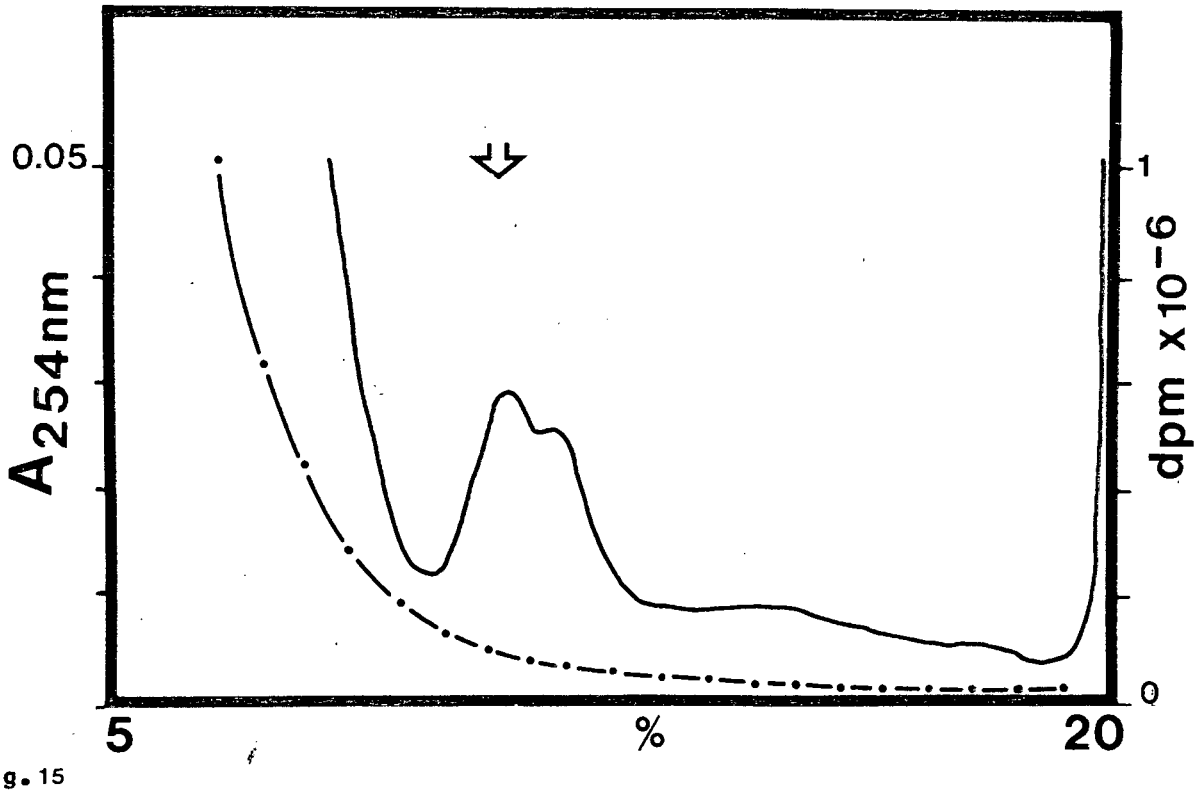


Fig.15

The fate of poly(glutamic acid) after assembly.

Gradient:5 - 20% (w/v) sucrose. Rotor: Beckman SW40Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
The arrow indicates the nucleosome core position.

Assembly conditions:

DNA: Long, redigested with Micrococcal nuclease after assembly.
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Histone to DNA ratio (w/w): 1:1
Poly(glutamic acid) to histone ratio (w/w): 2:1
Poly(glutamic acid) labelled by acetylation with [^{3}H]acetic anhydride.
Assembly: 3 hours at 37°C
—·—·— Poly(glutamic acid)
——— Absorbance at 254 nm

This is also obvious from the experiment in Fig. 14 in which previously assembled cores were mixed with poly(glutamic acid) and then subjected to gradient centrifugation.

The electrophoretic analysis of the assembled polycores (Fig. 16) reveal essentially the same information as the sucrose gradient centrifugation experiment.

The protection of DNA against micrococcal nuclease is the result of the interaction of the octamers with DNA to form mono-, di- and oligomers of core particles. The poly(glutamic acid) itself has no DNA protection properties. It is also evident from the gel electrophoresis that the core formation is crucially dependent on the presence of poly(glutamic acid) in the assembly cocktail.

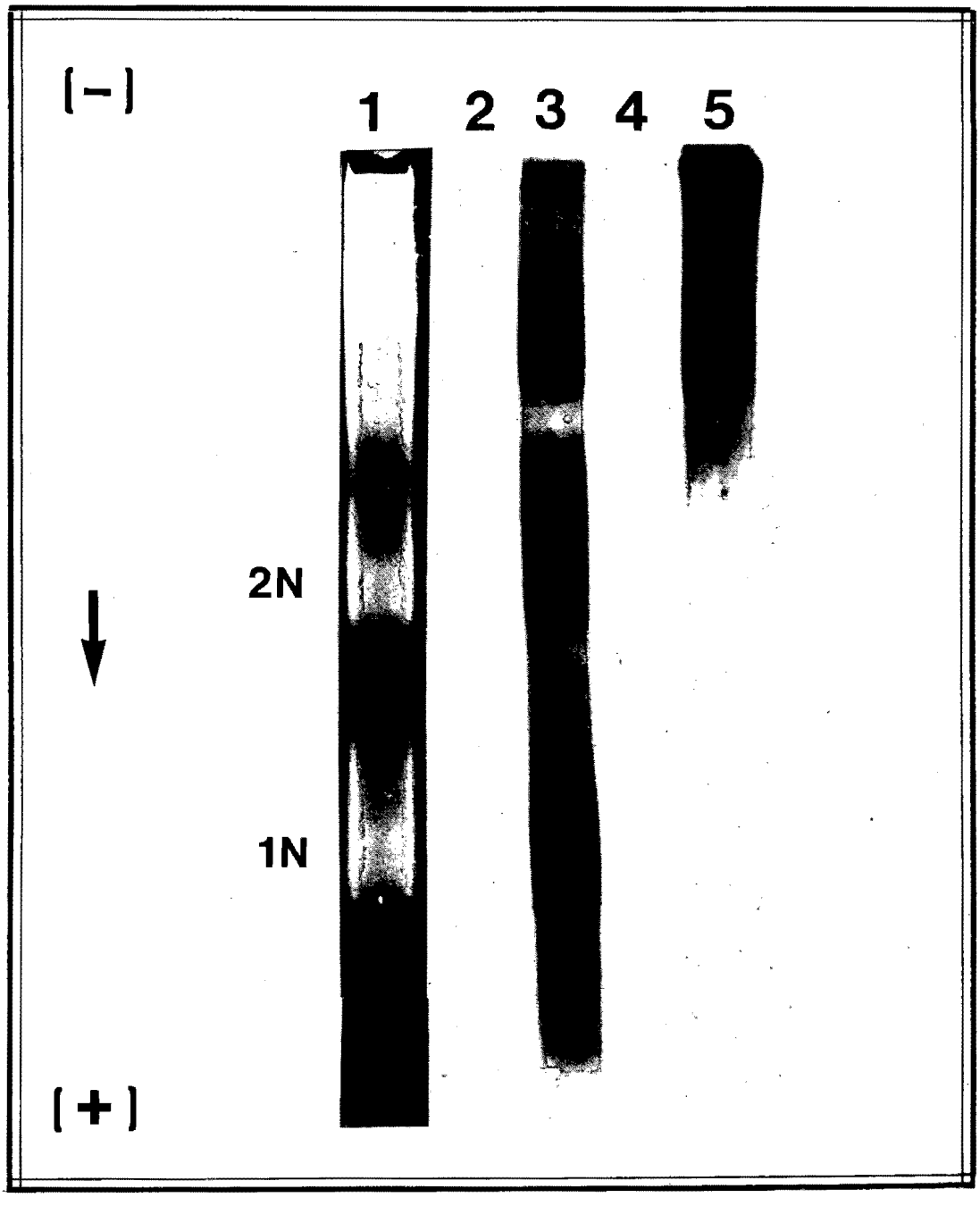


Fig. 16
Low ionic strength electrophoresis of histone-DNA complexes.
Lane 1: chicken erythrocyte chromatin partially digested with Micrococcal nuclease, stained with ethidium bromide. 1N = natural cores and monosomes. 2N = dimers. Lanes 2-5: autoradiography of nick-labelled random DNA. Lane 2: DNA. Lane 3: DNA assembled with a histone to DNA ratio of 0.9:1 (w/w) in the presence of poly(glutamic acid). Lane 4: DNA incubated with poly(glutamic acid) in the absence of histones. Lane 5: DNA incubated with histones in the absence of poly(glutamic acid).

3.5 KINETICS OF ASSEMBLY

In order to get an approximation of the kinetics of octamer assembly on linear random DNA, aliquots were withdrawn from the assembly mixture, digested quickly to core size particles and the yields of particles for each time interval determined by gradient centrifugation.

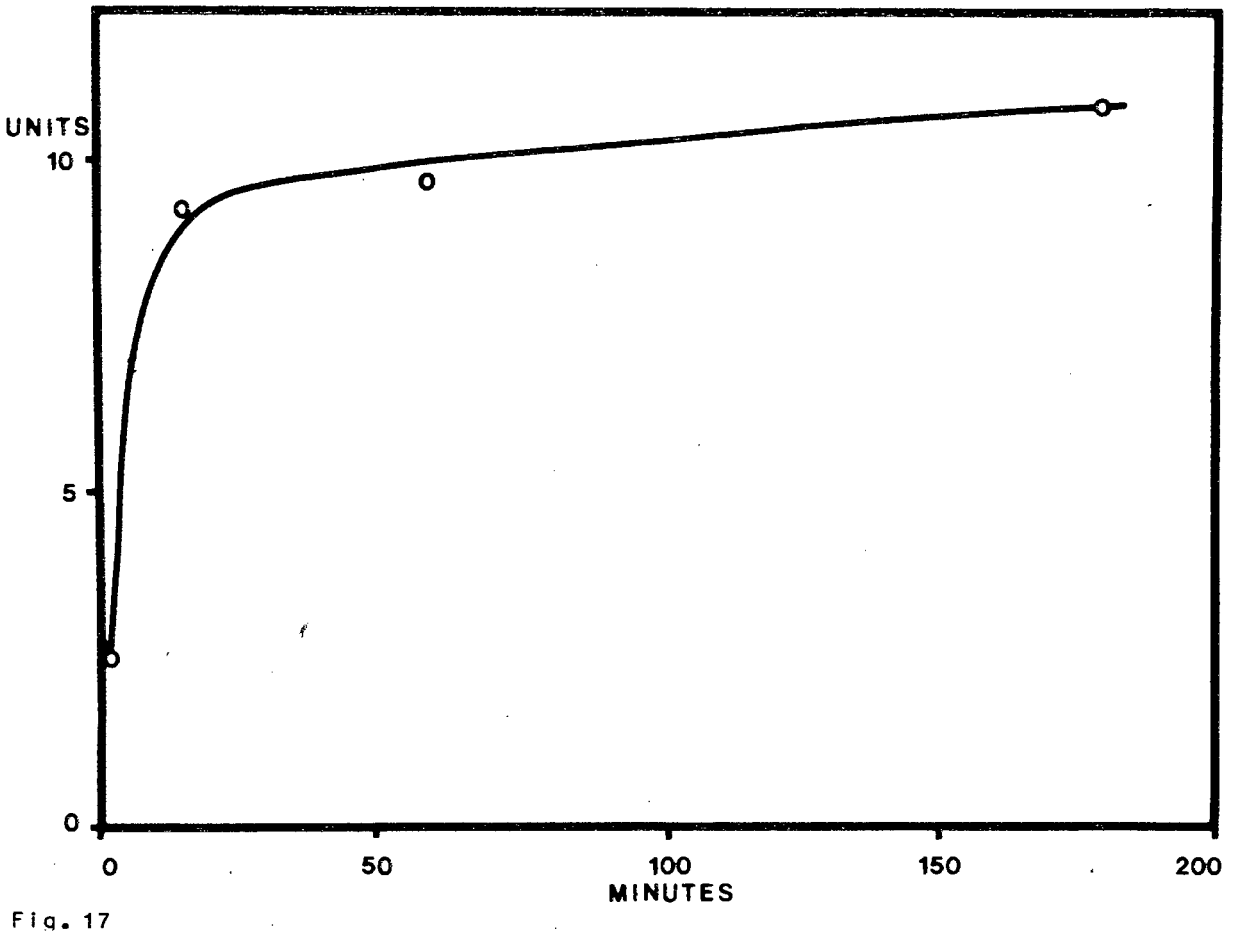


Fig. 17
Kinetics of the assembly reaction, determined by rapid Micrococcal nuclease digestion and quantitation of the cores produced on sucrose gradients.

Initially the reaction proceeds rapidly, 84% of the cores produced are formed within the first 15 minutes, the yield then increases to 88% after 60 minutes. These results are similar to those reported by authors using other assays (Stein et al. 1979 and Earnshaw et al. 1980). The poly(glutamic acid) assisted assembly is thus considerably faster than assembly by dialysis

which yields 80% after 24 hours (Tatchell and Van Holde 1977), direct mixing with 55% in 16 hours (Stein et al. 1979) and the salt jump method with 80% in 24 hours (Stein 1979).

4. THE NATURE OF THE CORE AND ITS MULTIMERS ON RANDOM DNA

This section deals with the establishment of the credentials of the core particle assembled "in vitro" on random DNA.

4.1 SUCROSE GRADIENT CENTRIFUGATION:

Centrifugation reveals that the major products of the assembly on long DNA are rapidly sedimenting large complexes (Fig. 18). After micrococcal nuclease digestion a range of fractions appear, corresponding to cores, tight dimers and tight trimers respectively (Fig 18) (See section 4.2). These results are not influenced by the method of octamer preparation employed.

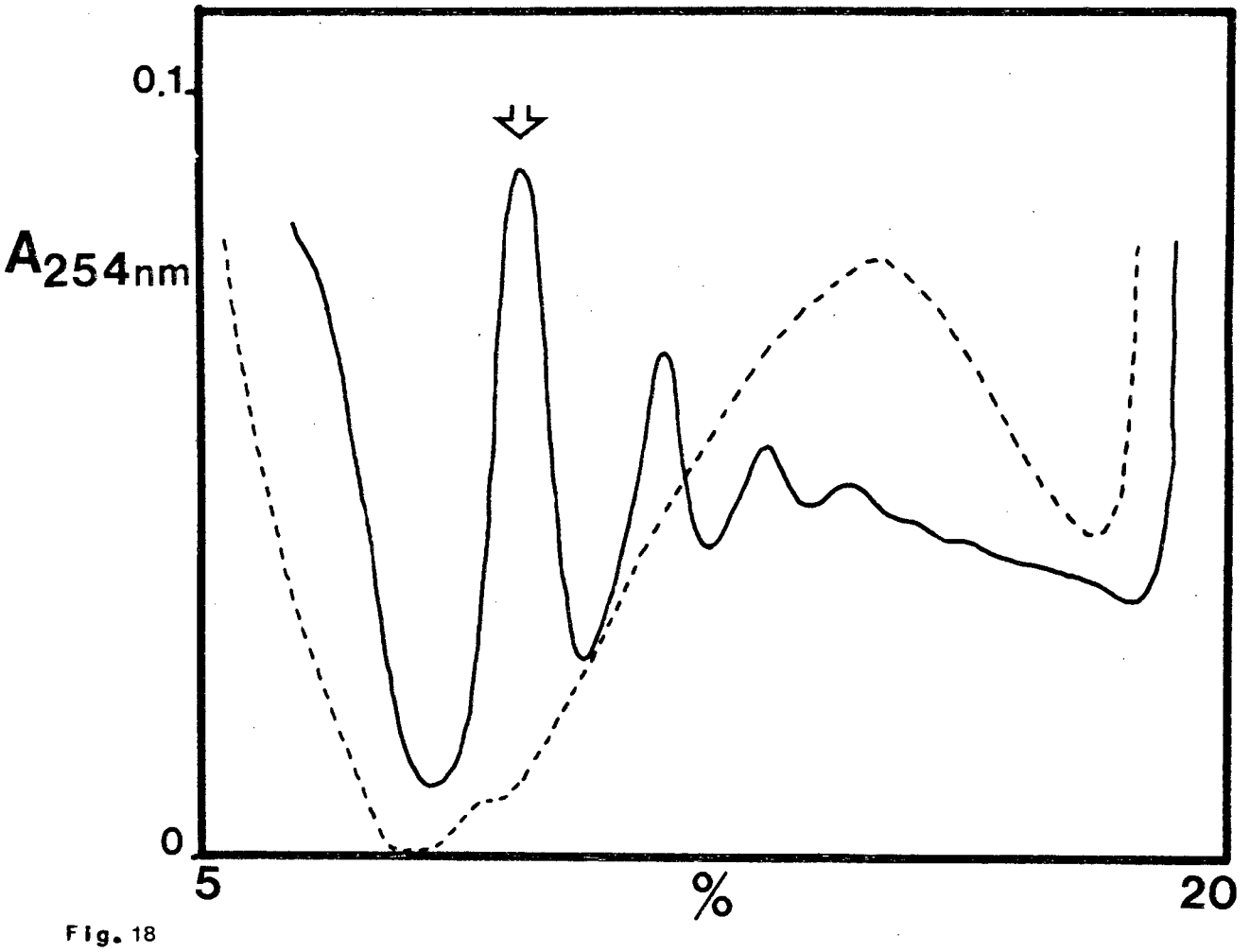


Fig. 18
The effect of Micrococcal nuclease digestion on assembly products.

Gradient: 5 - 20 % (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
The arrow indicates the nucleosome core position.

Assembly conditions:

DNA: Long
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Histone to DNA ratio (w/w): 1:1
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
— — — — Undigested polycores
———— Polycores after digestion

The sedimentation velocity of the assembled and digested core particles is identical to similarly treated natural cores. Of a total of 121 gradients run in the various experiments, the average elution volume on gradient fractionation was 6.3 ml with a standard deviation of 0.1 ml. The standard deviation of the difference between the elution volume of native and

assembled cores was 0.03 ml (native cores were run in each centrifugation as a standard to monitor the sucrose gradient).

4.2 MICROCOCCAL NUCLEASE DIGESTION:

The natural nucleosome core is associated with 145 base pairs of DNA which is protected against micrococcal nuclease digestion. The peaks produced on sucrose gradient analysis, after micrococcal nuclease digestion of assembled cores and multimers, were collected and the DNA analyzed by denaturing DNA gel electrophoresis. The size of the DNA associated with the core was found to be 145 ±4 base pairs. In the large DNA-histone complex assembled, the cores are situated close to each other as tight dimers and trimers are formed without unprotected linker DNA, as is obvious from the repeat length.

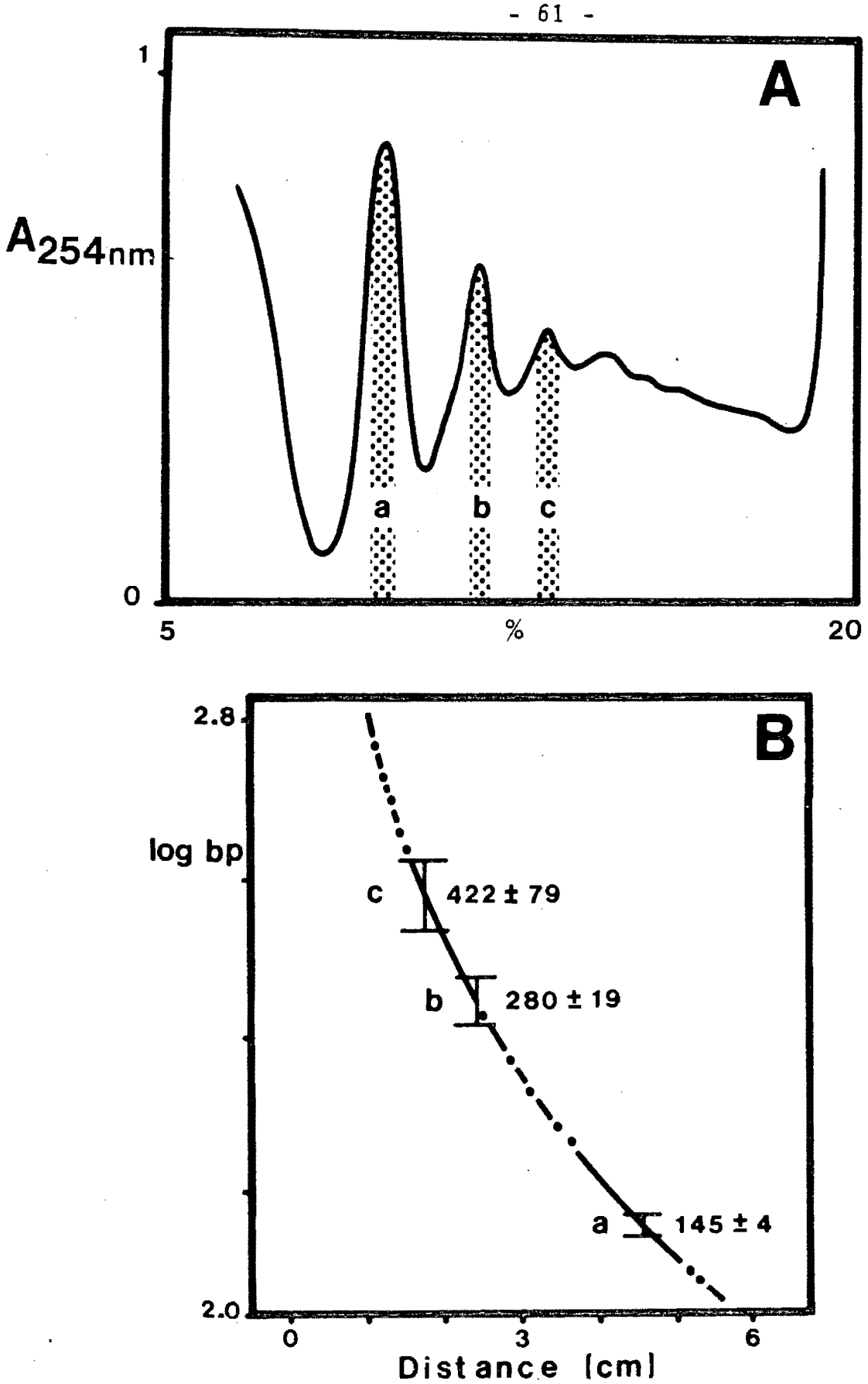


Fig 19
(A) 5-20% sucrose gradient ultracentrifugation of nucleosome cores after assembly on long DNA and digestion with Micrococcal nuclease. The peaks labelled a, b, and c were collected and the DNA extracted.
(B) The DNA from peaks a, b, and c was analysed by denaturing DNA gel electrophoresis and the gel calibrated with a Hae III digest of pBR322.

On SDS gel electrophoresis assembled nucleosome cores contained all four of the core histones in approximately stoichiometric ratios. (Fig. 7 D, Lane 2.) This indicates that the bulk of the assembled nucleosome cores must contain a full complement of core histones.

4.3 DNASE I DIGESTION:

The DNA on the surface of natural cores is protected from the action of DNase I in a very characteristic fashion (Lutter 1978). The different susceptibilities at the 10 base pair spaced cutting sites is conceivably the result of local conformational changes of the DNA caused by the shape of the underlying protein. A DNase I digestion pattern of assembled cores should reveal subtle differences between natural and assembled cores. To this end natural nucleosome cores, cores assembled on 145 bp DNA and free DNA from one isolate were compared (Fig. 20).

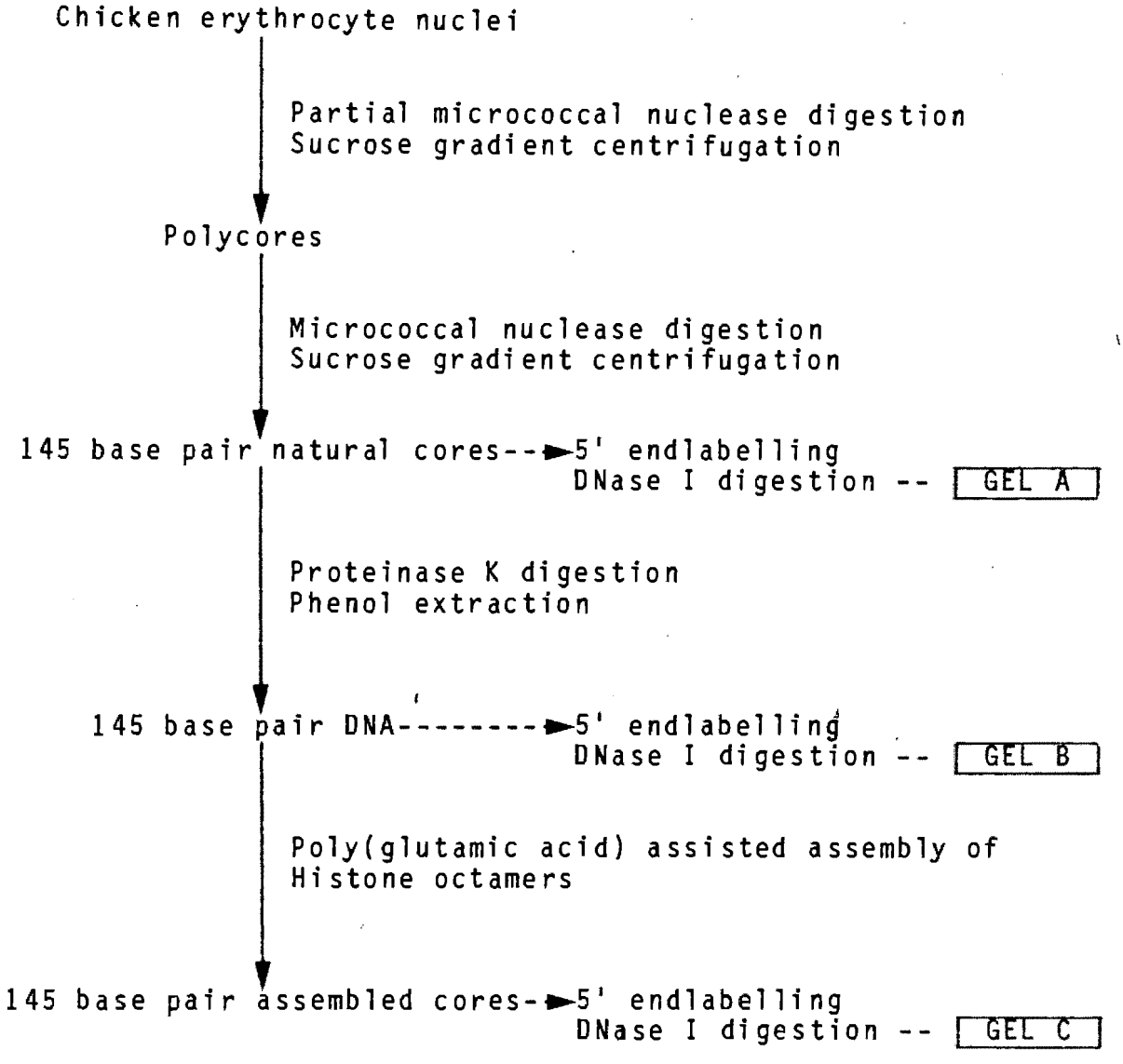


Fig. 20
Experimental protocol for the production of cores, 145 base pair DNA and assembled cores.

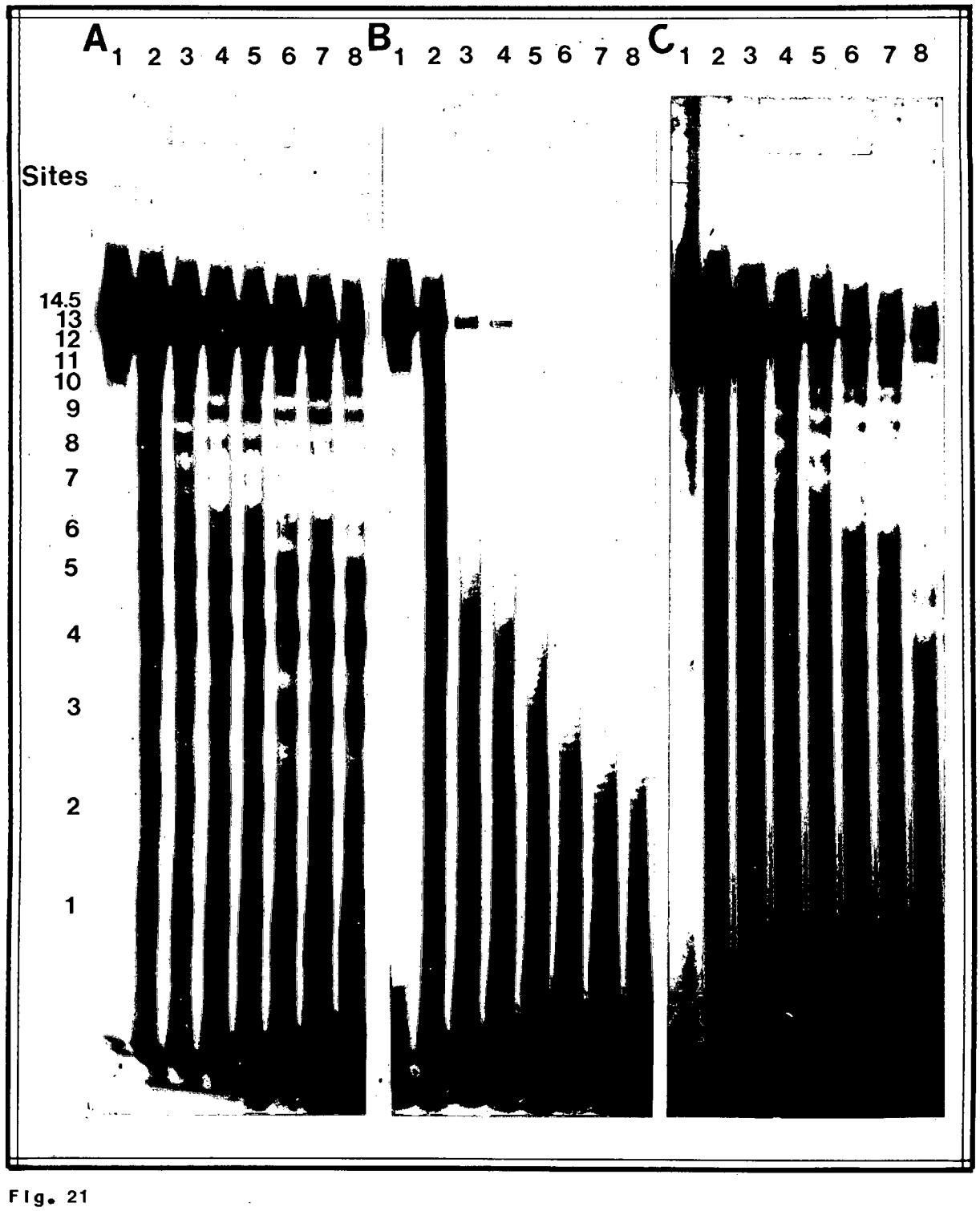


Fig. 21
DNase I digestion of 5' end-labelled cores.
Gel (A): natural nucleosome cores. Gel (B): 145 base pair DNA. Gel (C): Nucleosome cores assembled on 145 base pair DNA. Gels (A, B and C), Lanes 1-8: DNase I digestion for 0, 10, 20, 30, 40, 60, 100, 180 seconds.

The three digestion patterns produced from natural cores, free DNA and associated cores reveal for the assembled cores the typical 10 base pair protection ladder, indistinguishable from that produced from natural cores. This ladder in absent is a digest of free DNA.

Cores were also assembled on long DNA with two different octamer preparations and trimmed to 145 base pairs to achieve better resolution of the DNase I cutting sites. In this type of experiment precise trimming of the DNA, associated with the core particle, with micrococcal nuclease is necessary prior to end-labelling and DNase I digestion (Figs. 22 and 23). Again the 10 bp ladders of natural and assembled cores are indistinguishable.

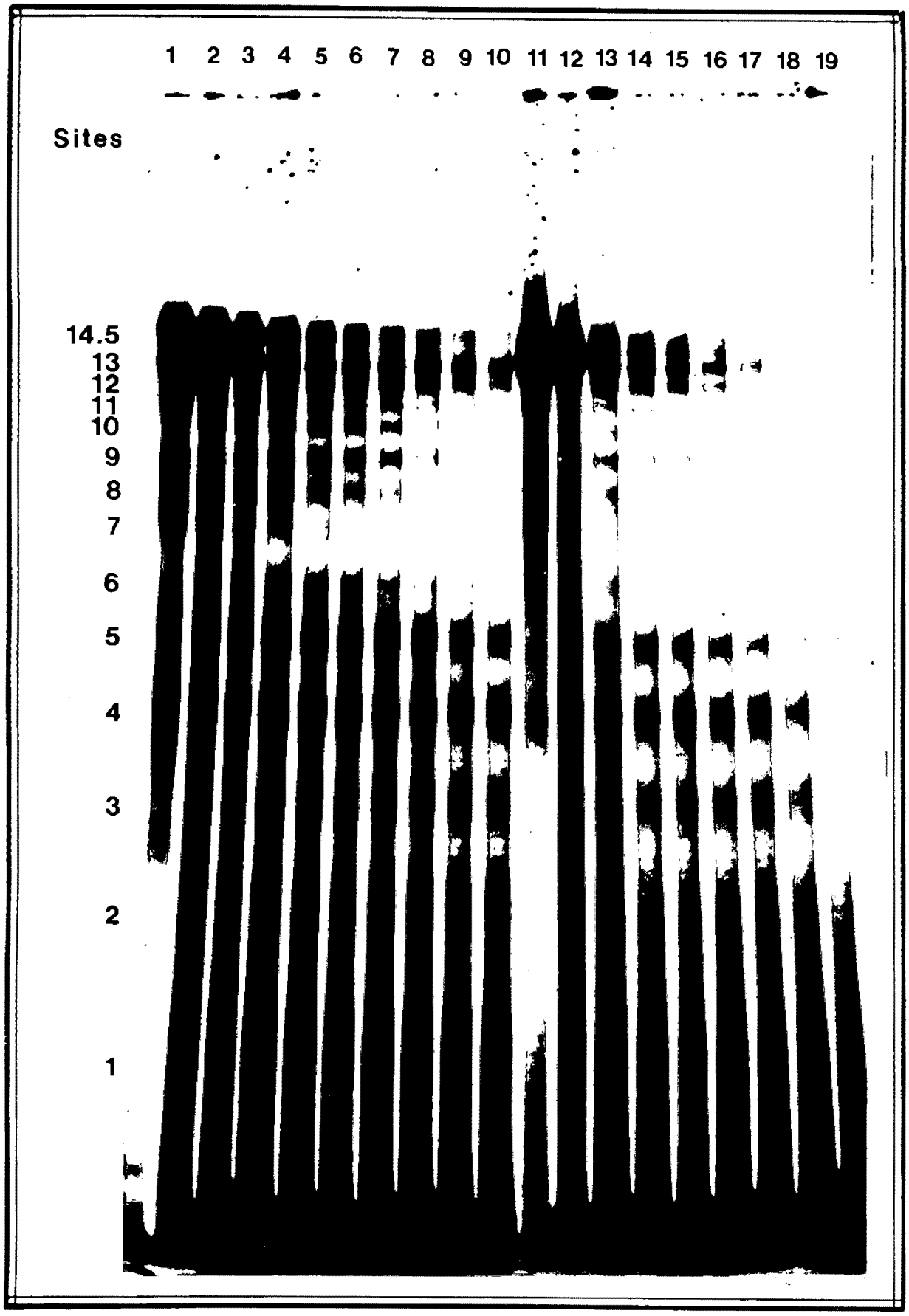


Fig. 22
DNase I digest of [^{32}P] 5' end-labelled natural and assembled cores. Lanes 1-10: end-labelled natural cores digested for 0, 7, 20, 33, 45, 57, 68, 82, 93 and 105 seconds. Lanes 11-19: cores assembled on long DNA with octamers extracted according to Ruiz-Carillo and Jorcano (1979). The cores were trimmed with Micrococcal nuclease and the resultant cores end-labelled and digested with DNase I for 0, 19, 33, 48, 60, 72, 84, 97 and 110 seconds.

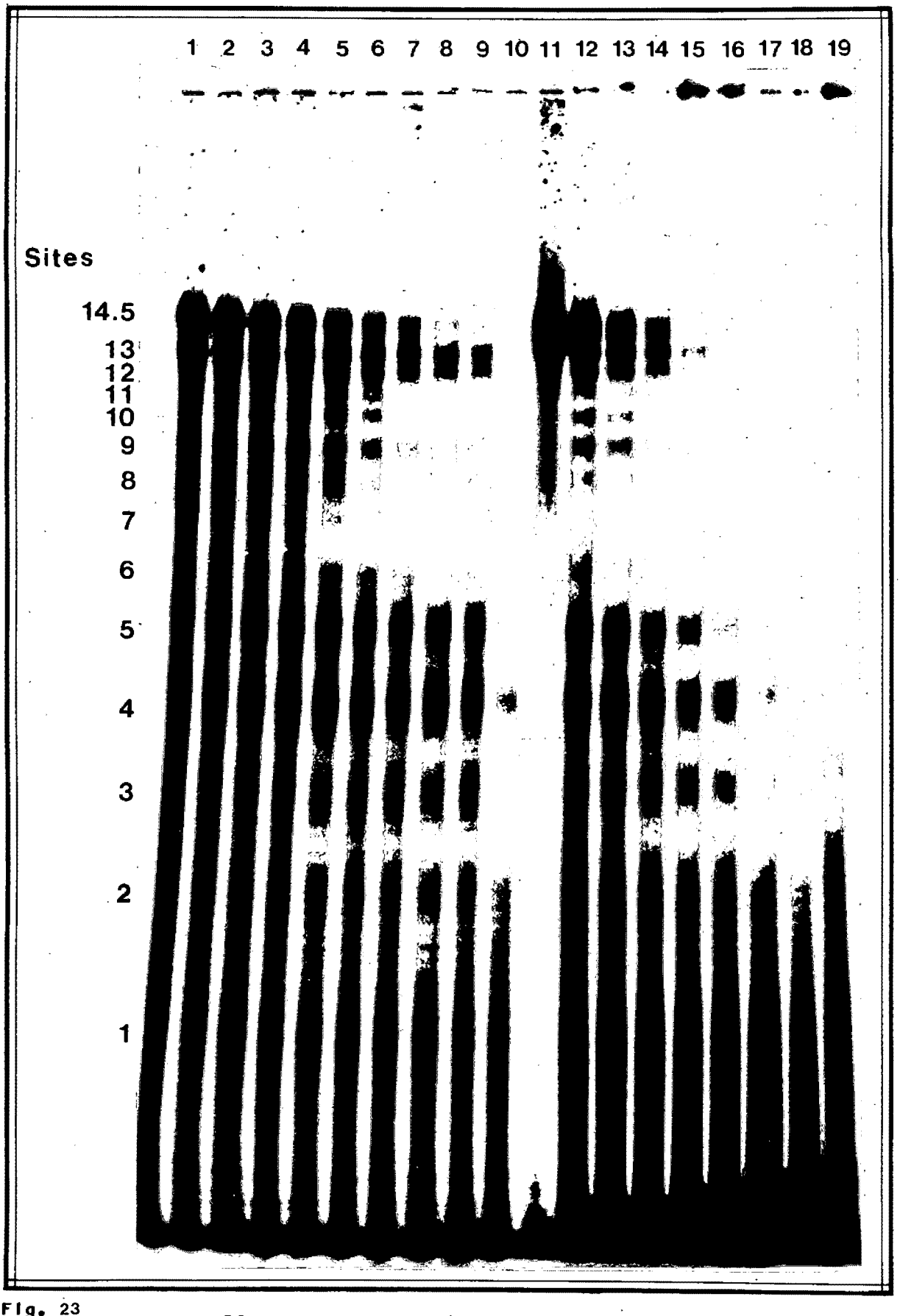


Fig. 23
DNase I digest of I ^{32}P] 5' end-labelled natural and assembled cores. Lanes 1-10: end-labelled natural cores digested for 0, 8, 21, 33, 46, 58, 71, 83, 94 and 105 seconds. Lanes 11-19: cores assembled on long DNA with octamers purified by hydroxyapatite chromatography. The cores were trimmed with Micrococcal nuclease and the resultant cores end-labelled and digested with DNase I for 0, 13, 24, 36, 49, 62, 76, 89 and 100 seconds.

The impression gained from the inspection of the autoradiograph of the gels, namely that the ladders produced by DNase I digestion of natural and assembled cores are virtually identical is confirmed by the more detailed analysis of the cutting rate constants. The small differences between the rate constants for the assembled and native cores are possibly due to differences in the degree of trimming by the micrococcal nuclease apparent in Figs. 22 and 23. The results of both native and assembled cores agree well with those for natural cores obtained by Baer and Rhodes (1983).

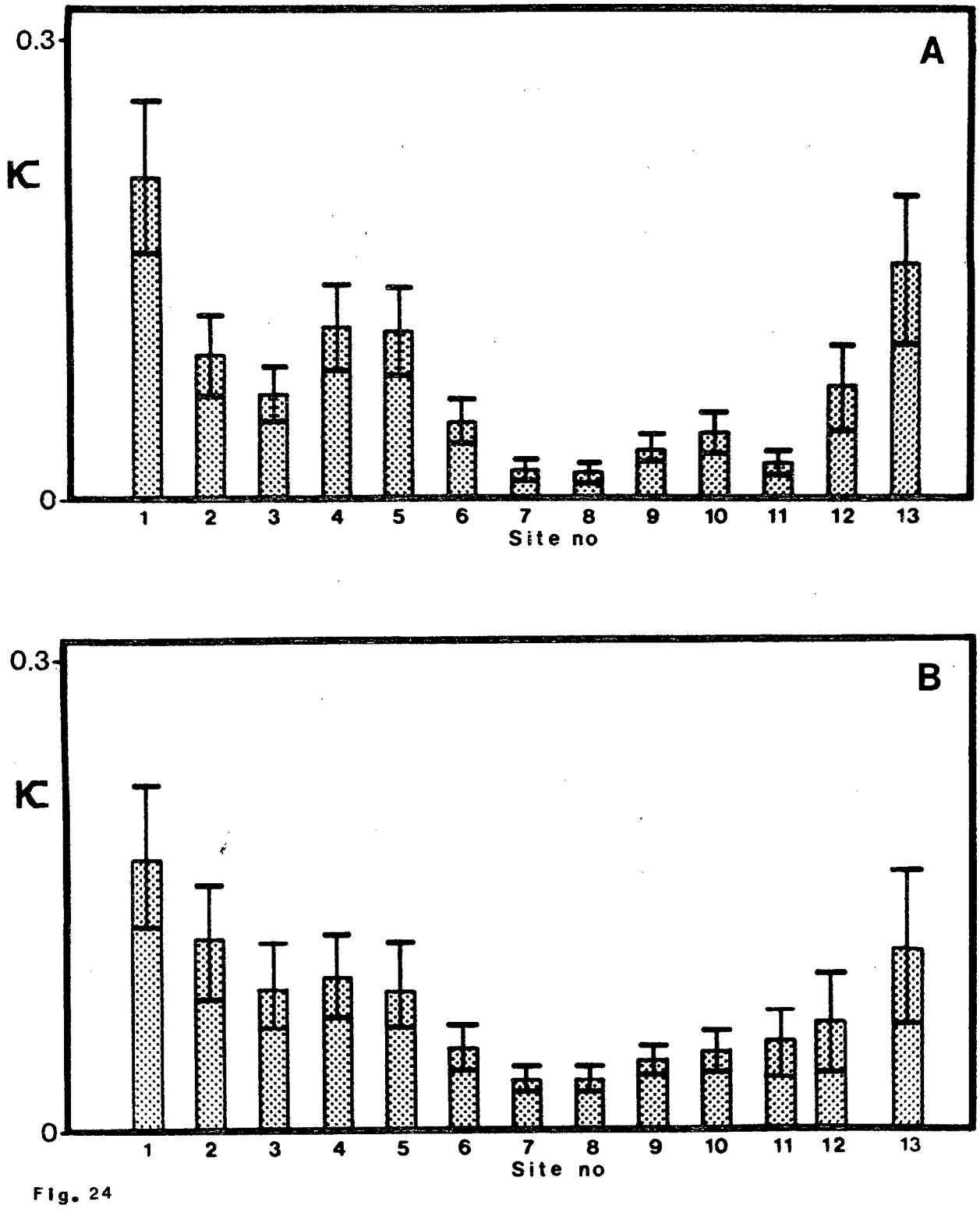


Fig. 24

DNase I cutting rate constants of nucleosome cores:

(A) Natural cores. The cutting rate constants, the mean and standard deviations for each site was determined for each site in 6 different natural core preparations.

(B) Assembled cores. The rate constants were determined for 6 core assembly preparations in which different core preparations were used; two preparations of sodium chloride extracted histones, two histone octamer preparations eluted from hydroxyapatite, one from renatured acid-extracted histones and one from salt extracted histones stored for 2 months in 1M NaCl, 50% w/v glycerol at -20 °C. The cutting rates at the respective susceptible sites in any of the artificially assembled cores did not differ significantly from preparation to preparation.

The relative rate constants were determined as described previously (Lutter 1978, Retief et al. 1984).

Bars: standard deviation of 34%

4.4 ELECTRON MICROSCOPY:

The electrom microscopic appearance of these polycore types, assembled at different histone:DNA ratios, is compared in Fig. 25. The density of cores assembled on the DNA corresponds well to histone:DNA ratios employed in the assembly reactions, and to the sedimentation characteristics of the polycore population (Fig 26). The polycores have the same general size and appearance as that of H1 depleted native chromatin (Oudet et al. 1975). However the cores are not evenly spaced.

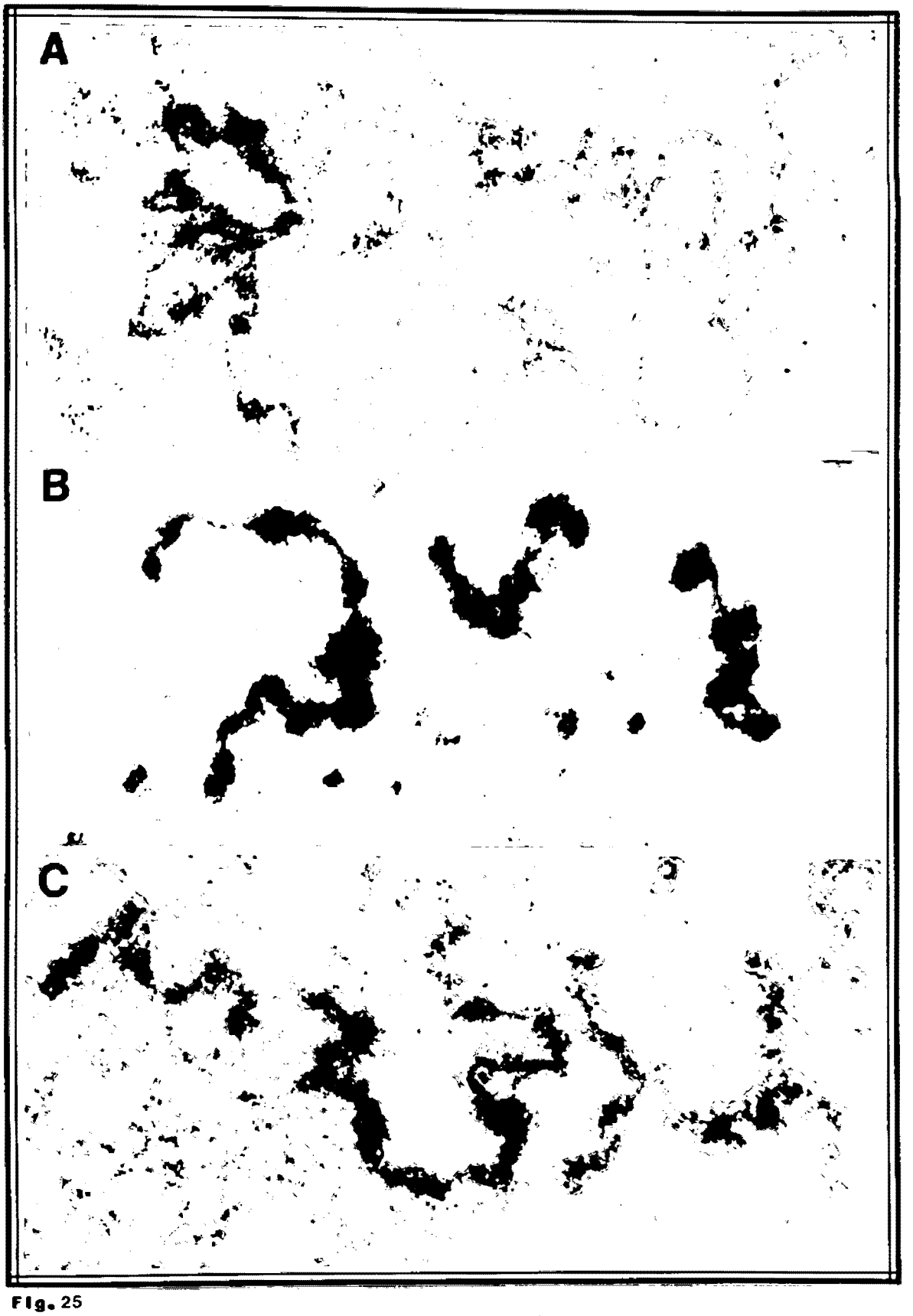


Fig. 25
Dark field electron microscopy of assembled polycores.
Polycores assembled on long DNA, purified by sucrose gradient centrifugation (Fig.26). Histone to DNA ratios (w/w): (A) 0.5:1 (B) 1:1 (C) 2:1

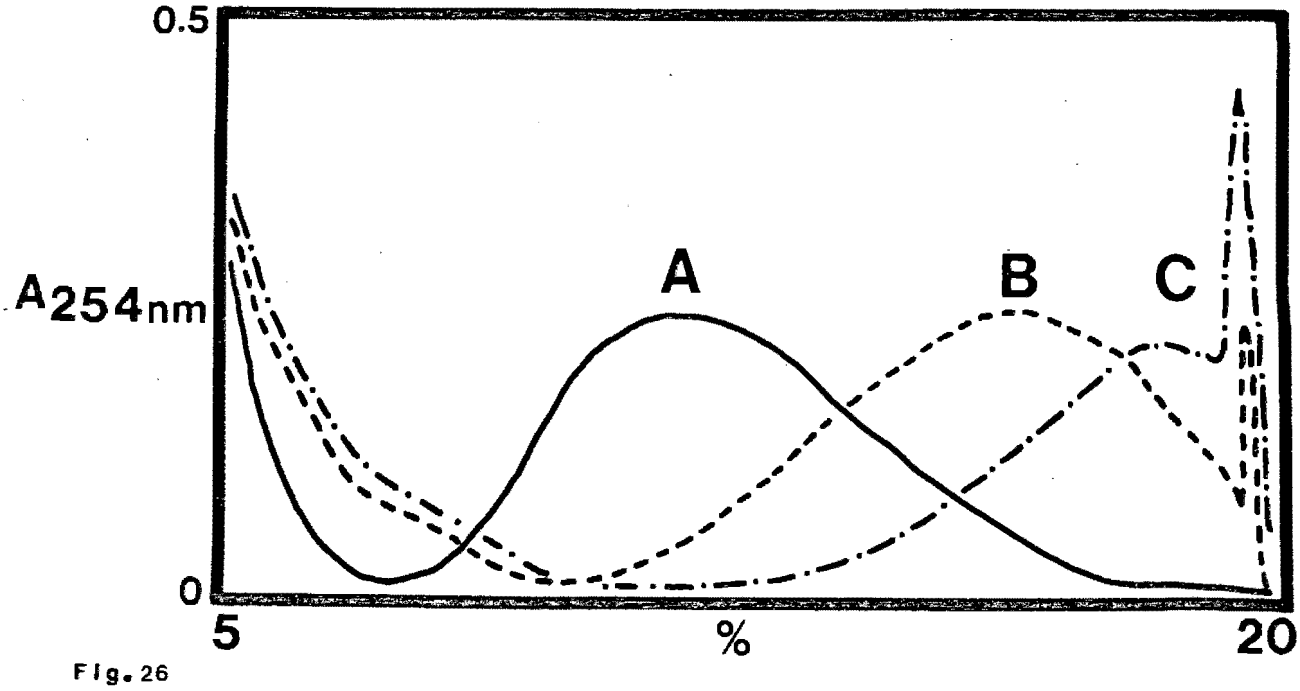


Fig.26

Preparation of polycores for electron microscopy.

Gradient:5 - 20 % (w/v) sucrose. Rotor: Beckman SW40Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and 10 mM TEA/HCl pH 7.6.

Assembly conditions:

DNA: Long
Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
Histone to DNA ratio:

(A) 0.5 : 1 (w/w)
(B) 1 : 1 (w/w)
(C) 2 : 1 (w/w)

5. MODIFICATION OF THE ASSEMBLY PROTOCOL FOR THE ASSEMBLY WITH NANOGRAM AMOUNTS:

The assembly of nucleosome cores on unique DNA has to be undertaken at very low DNA concentrations for a number of reasons, dictated by the methodologies employed in the production of unique DNA and the subsequent high and low resolution mapping. Due to the low concentrations of histones and DNA in these experiments they have to be radioactively labelled to monitor the success of the assembly reaction.

[^{125}I] was chosen as a tracer to label tyrosine residues of the histones. The tyrosines in the histone octamer are buried (Michalski-Scrive et al. 1982) and presumably not involved in the histone-DNA interactions, but play a role in the histone-histone interaction of the hydrophobic areas in the octamer. To avoid interferance with such interactions during iodination, the histone octamer was labelled at 2 M NaCl with either lactoperoxidase, immobilised on Sephadex, or Iodobeads (N-Chloro-benzenesulfonamide [sodium salt], immobilised on non-porous polystyrene beads, Pierce). Immobilized lactoperoxidase proved to be inefficient at 2 M NaCl. Reducing the ionic strength to 1 M NaCl yielded a specific activity of only 0.0039 mCi/mg. The immobilised enzyme also showed only limited stability under storage conditions. On the other hand, iodination of histone octamers at 2 M NaCl with Iodobeads resulted in a quantitative incorporation of the radioactive iodine, yielding octamers with a specific activity of 0.236 mCi/mg. (Fig. 27), sufficient for the required measurements.

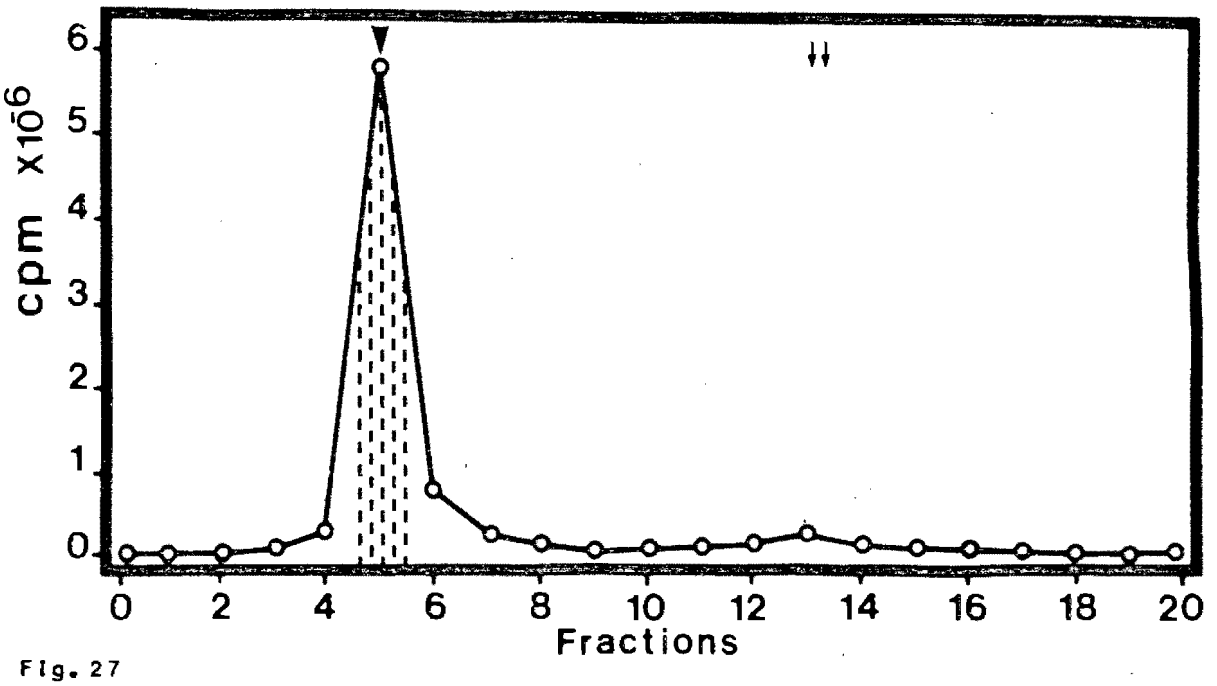


Fig. 27
Exclusion chromatography of labelled histones.
Column: 0.5 cm diameter, 10 cm long.
Matrix: Sephadex G50 medium.
Eluant: 2 M NaCl, 20 mM Tris/HCl pH 8.0.
Sample: Histone octamers labelled with Iodobeads.
→ Octamer position
⇉ Position of dissociated core histones
The shaded area indicates the fraction pooled for subsequent experiments.

The polycore assembly using [125-I] labelled octamer and the h22 clone of the sea urchin histone gene quintet can be monitored either via exclusion chromatography (Fig. 28) or elution from hydroxyapatite (Fig. 29). In the latter protocol free DNA, histones and assembled polycores elute at characteristic NaCl concentrations.

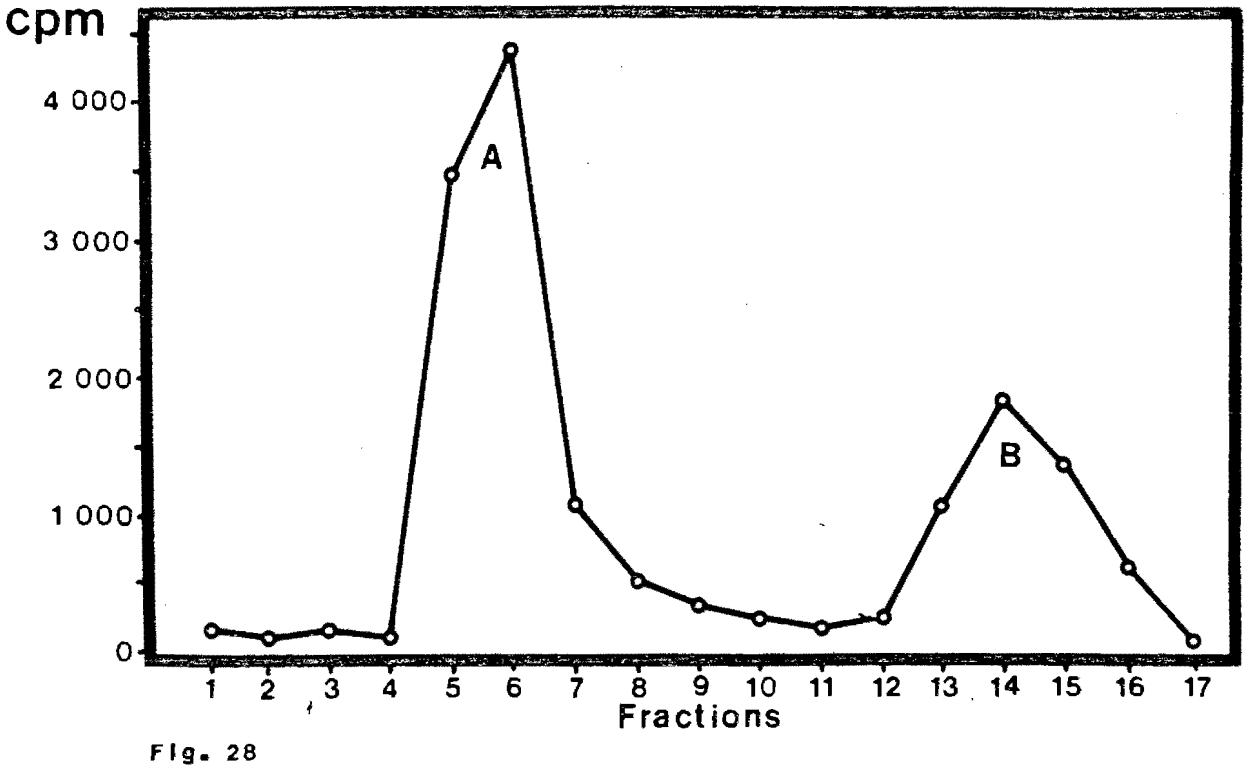


Fig. 28
Exclusion chromatography of assembled polycores.
Column: 0.5 cm diameter, 10 cm long.
Matrix: Sepharose CL 4B.
Eluant: 20 mM NaCl, 1 mM EDTA and 10 mM Tris/HCl pH 7.6.
Sample: ph22 assembled with [^{125}I]-labelled histones.
(A): assembled polycores
(B): free histones

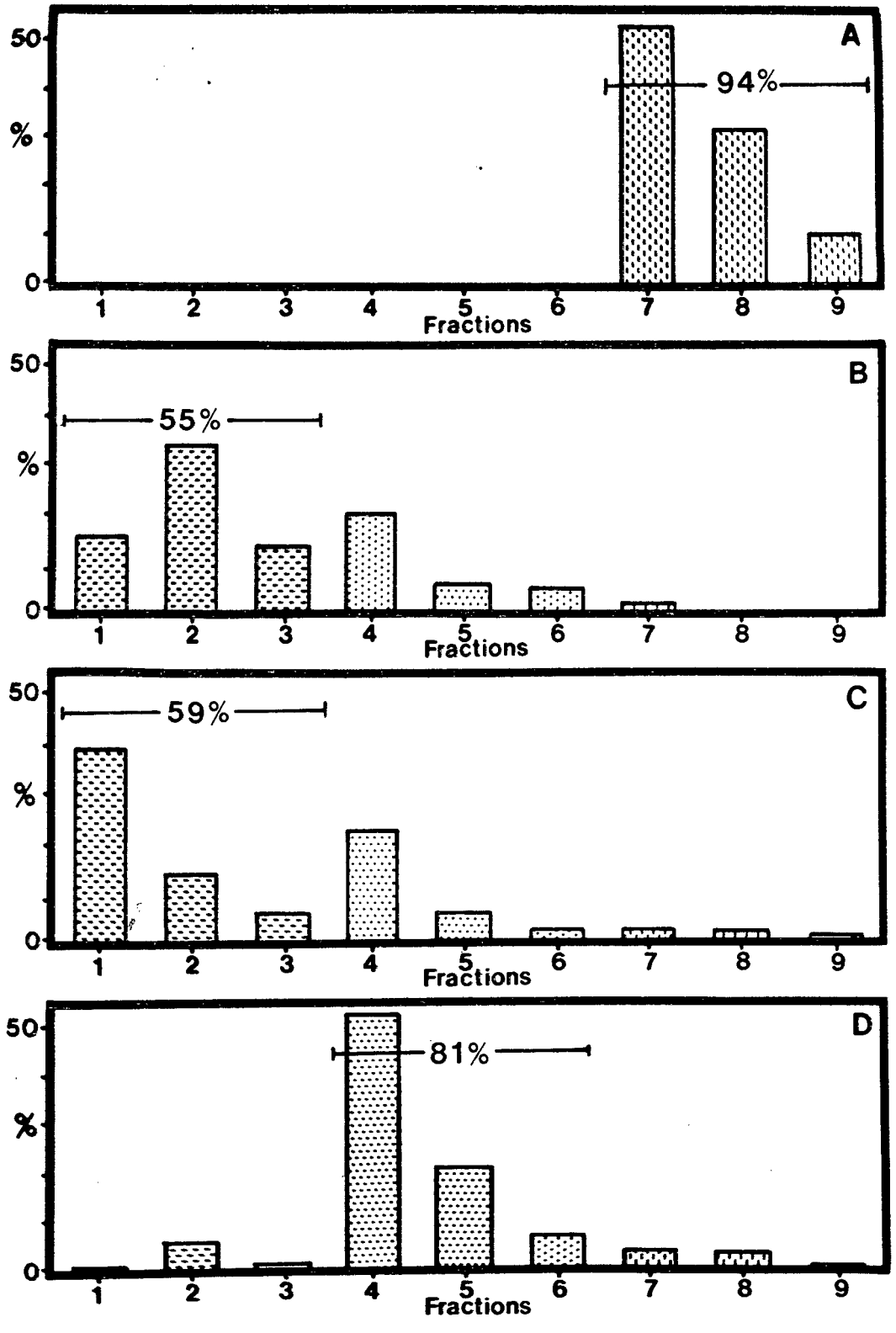


Fig. 29

Hydroxyapatite chromatography of assembled polycores.

Column: Miniature column with a volume of 0.2 ml

Matrix: Hydroxyapatite

Eluant: A step gradient with 0.2 ml buffer/fraction.

Fractions 1-3 0.5 M NaCl, 10 mM phosphate buffer pH 6.9.

Fractions 4-6 2 M NaCl, 10 mM phosphate buffer pH 6.9.

Fractions 7-9 0.5 M phosphate buffer pH 6.9.

Sample:

(A) [^{32}P] nick-labelled ph22 DNA.

(B) [^{125}I] labelled histone octamers.

(C) [^{125}I] labelled histone octamers incubated with poly(glutamic acid)

(D) ph22 assembled with [^{125}I] labelled histone octamers.

6. THE ASSEMBLY OF CORES ON UNIQUE DNA

6.1 SELECTION OF A SUITABLE STRETCH OF DNA

To investigate the structural rules governing the assembly of nucleosome cores, a stretch of DNA complying with a number of requirements should be selected. It is important that the complete sequence of the fragment be known. This facilitates the selection of restriction sites, functional areas, such as coding and spacer areas, and allows prediction of putative DNA structures. Such a stretch of DNA should comprise structural genes and linker areas. This should make it possible to investigate whether conformational characteristics of these two functionally different DNA areas play an important role in the putative sequence selectivity of the core assembly (Mengeritsky and Trifonov 1983, 1984). Furthermore, it is desirable to investigate "in vitro" assembly on genes which are also expressed under defined conditions "in vivo". The interpretation of the results may be facilitated if the organisation of the genes are simple, namely transcribed from the same strand without any introns. As a technical consideration, the size of the genomic DNA should be short enough to be propagated in common plasmids such as pBR322, and contain suitable restriction sites to allow the construction of probes and to allow the excision of small fragments for high resolution mapping.

I have selected the h22 histone gene quintet from the sea urchin Psammechinus miliaris. Many reports on the structure, function and sequence of histone gene repeats have

been published by Birnstiel and his co-workers (Birnstiel et al. 1974, 1977, Portmann et al. 1976, Schaffner et al. 1978, Busslinger et al. 1979, 1980, Hentschel et al. 1980, Aulehla-Scholtz and Jacob 1980, Hentschel 1982). For reviews see Kedes (1979), Hentschel and Birnstiel (1981). The pBRh22/4 clone, (Fig. 30) and its complete sequence, had been kindly provided by Professor M.L. Birnstiel, University of Zürich.

The histone genes are reiterated several hundred fold in the genome of the sea urchin (Kedes and Birnstiel 1971). These genes constitute some 0.5-0.8% of the total genomic DNA of the sea urchin. The structural areas of the five histone genes are adjacent to each other and connected via a spacer area. The Psammechinus quintet has been characterised in detail (Schaffner et al. 1978). For a review see Kedes (1979). In all the highly reiterated early histone gene families of Psammechinus miliaris, Strongylocentrotus purpuratus, Psammechinus lividus, Echinus esculentus and Lytechinus pictus that have been studied, there are striking organisational similarities (Holt and Childs 1984). All five of the histone genes are present in the same order and the genes are transcribed from the same strand with the same transcriptional polarity. Within that general framework there exists some structural variety between these gene batteries. For review see Hentschel and Birnstiel (1981).

The histone genes are present in the genome as extended reiterated units, each containing one gene coding for each of the various histones. The high degree of reiteration has been interpreted as a structural prerequisite to provide the large number of histone mRNA molecules necessary during early embryogenesis. However, a considerable number of histone variants are expressed during the life cycle of the sea urchin. These variants are expressed in a programmed fashion (Cohen et al. 1975, Brandt et al. 1979, Childs et al. 1979, Arceci and Gross 1980 and Von Holt et al. 1984). On the evidence available at present it appears that the gene repeat comprises the early histones. On the other hand, the many closely related variants of the early embryo up to gastrula stage have not been unequivocally correlated with specific genes. In addition to the overall program, the turnover of the early as well as the late histones H2A and H2B in the nucleosomes is almost twice that of H3 and H4 (Schwager et al. 1985). These findings point to the possibility that histone quintets may contain, in addition to the structural genes and the consensus sequences for initiation and termination (Schaffner et al. 1978), information controlling the regulation (Busslinger et al. 1979, 1980) and timing of expression. The presence of such control sequences and the possibility of analysis of the gene products "in vivo" and "in vitro" make the investigation of the h22 histone gene repeat particularly interesting as a substrate for the study of DNA-chromosomal protein interactions.

The chromatin structure of the h22 histone gene repeat at various developmental stages has been studied by indirect end-labelling (Bryan et al. 1983). The h22 histone gene repeat is transiently expressed at the 5 hr (128 cell) blastula stage. During this stage the promoter areas of all five of the histone genes are sensitive to nuclease digestion. The inverted DNA repeats necessary for processing the RNA molecules remain resistant to micrococcal nuclease cutting. The sensitivity of the promoter regions disappears abruptly at the hatching blastula stage. These changes in nuclease susceptibility have however not been ascribed by the authors to changes in the nucleosome positioning.

In common with most histone genes the h22 histone gene repeat has GC-rich coding areas and AT-rich spacer areas (Schaffner et al. 1978). A striking feature of the sequence in the non-coding area, is the occurrence of a monotonous sequence $(5'G\text{-}A3')_{16}$ in the H1-H4 spacer area and its complementary sequence $(5'C\text{-}T3')_{23}$ in the H2A-H1 spacer (Hentschel 1982) Similar sequences are also found in the H2A-H1 spacer area in S. pupuratus and a similar but shorter one in the H3-H4 spacer area of Drosophila (Tautz and Renz 1984). These sequences are involved in a unique slippage mechanism demonstrated by Hentschel (1982). The possible functions of these structures have aroused considerable interest. These sites may be preferred nucleation sites for recombination events (Kedes 1979), or a transposon type of rearrangement of the H1 gene may occur through such monotonous sequences (Portmann and Busslinger 1979).

6.2 LITERATURE REVIEW OF MAPPING TECHNIQUES FOR NUCLEOSOMES:

Mapping protocols can be broadly divided into two categories, namely low resolution (±10 base pairs) or high resolution (±1 base pair). For review see Igo-Kemenes et al. (1982).

Wasylyk et al. (1979) have attempted to determine the positions of "in vitro" assembled nucleosome cores on SV40 DNA using electron microscopy. In principle, electron microscopy should allow objective determination of the nucleosome core spacing. A difficulty arises when it comes to the unequivocal determination of the reference point from which to start the measurements. Electron microscopic investigation of the SV40 minichromosome (Saragosti et al. 1980), and "in vitro" reconstituted minichromosomes (Hiwasa et al. 1981) pointed to an area of free DNA around the origen of replication. Rearrangement of the nucleosomes during the preparation of the samples for lectron microscopy can occur using certain fixation protocols. Glutaraldehyde fixation alone is unsuitable for this purpose (Sewell et al. 1984).

A frequently used method to determine "in vivo" positioning involves the isolation of nucleosome cores, followed by digestion of the DNA with an appropriate restriction enzyme and probing for periodicity using a suitable probe (See section 2.1.2). If cores are randomly distributed these gels reveal a smear of DNA. Specifically positioned cores give a range of discrete DNA fragments. Since the mapping of the fragments is undertaken on DNA fragments of nucleosome or polysome size,

determined by the natural "in vivo" distribution of cores, adequate control experiments with naked DNA are not possible. In addition, this type of mapping is also subject to a subtle artifact, in that a population of cores trimmed with micrococcal nuclease will always be enriched in GC sequences because of the sequence preference of the enzyme, which leads to the distruction of cores in AT-rich regions, thus yielding a biased population of cores (McGhee and Felsenfeld 1983).

The mapping of nucleosome positions over longer stretches of chromatin has been undertaken using restriction enzymes (Varshavsky et al. 1979, Pfeiffer and Zachau 1980). Similar to micrococcal nuclease the former will differentiate between protected and unprotected sites, such as the area around the origin of replication in SV40 minichromosomes (Varshafsky et al. 1979). Mapping the positions of nucleosome cores with restriction enzymes has, however, serious limitations because of the uneven distribution of suitable restriction sites.

The indirect end-labelling method introduced by Wu (1980) has become the most successful and widely applied low resolution method for the mapping of nucleosome positions. Briefly, from a partial DNase I digest of chromatin, the DNA is isolated, restricted, analysed by agarose gel electrophoresis and probed with a small DNA fragment that will hybridise to one end of the restricted DNA. This allows the measurement of the DNase I cutting sites relative to that end of the DNA. Control experiments to determine the background produced by DNase I on free DNA can easily be carried out (Samal and Worcel 1981,

Bryan et al. 1983, Worcel et al. 1983).

A number of protocols for the high resolution mapping of nucleosome cores have been developed:

To unequivocally determine the size of the DNA fragment it must be labelled at one end only, as in the chemical DNA sequencing protocols. This is achieved by labelling at the 5' or the 3' end and digestion at an asymmetrically positioned restriction site to produce one large and one small fragment, which can be separated by gel electrophoresis. Each of these gel fragments is uniquely labelled at one end and can be used as a substrate for assembly. This method has been used by Simpson and Stafford (1983) to establish the position of a nucleosome core on a 260 base pair DNA fragment. Only small quantities of labelled DNA can be prepared by agarose gel electrophoresis. In addition DNA produced in this way is often contaminated by impurities from the agarose (Smith 1980). Such contamination makes additional purification steps necessary as it may effect the histone-DNA interactions.

The combination of 5' end-labelling of nucleosome cores, digestion of the 3' ends with an exonuclease, followed by electrophoretic separation of the strands, also allows the establishment of the positions of nucleosome cores (Chao et al. 1979). However, since the position of the nucleosome core is mapped simultaneously from both ends of the DNA fragment, this method can yield ambiguous results when cores are positioned in multiple frames.

The positions of nucleosome cores can be determined by identifying the sequence of the core associated DNA. This is achieved by cloning the associated DNA, after trimming with Exo III and S1 nucleases, into M13 phages and subjecting the resultant clones to Sanger sequencing protocols (Sanger and Caulson 1975). This method has been applied to satellite DNA chromatin (Böck et al. 1984). Fifty clones had to be sequenced to accurately position two cores (Böck et al. 1984). Since the cores initially produced by micrococcal nuclease digestion may represent a biased population (McGhee and Felsenfeld 1983) the interpretation of results may require caution.

Ideally, positioning of nucleosome cores on a fragment of DNA should initially be investigated at low resolution. Once the approximate positions of the nucleosome cores have been determined, high resolution techniques can be used to investigate specific nucleosome positions in detail.

A number of considerations for both types of mapping are important. The enzymes used to map core positions should not be applied under conditions of limit digestion (McGhee and Felsenfeld 1983). Also, a large number of cuts are undesirable as these will generate small fragments of DNA with the possibility of octamer migration.

6.3 LOW RESOLUTION MAPPING

The pBRh22/4 plasmid contains two complete identical histone gene quintets of the sea urchin Psammechinus miliaris, as an inverted tandem repeat inserted into the Hind III site of pBR322 (Sutcliff 1978).

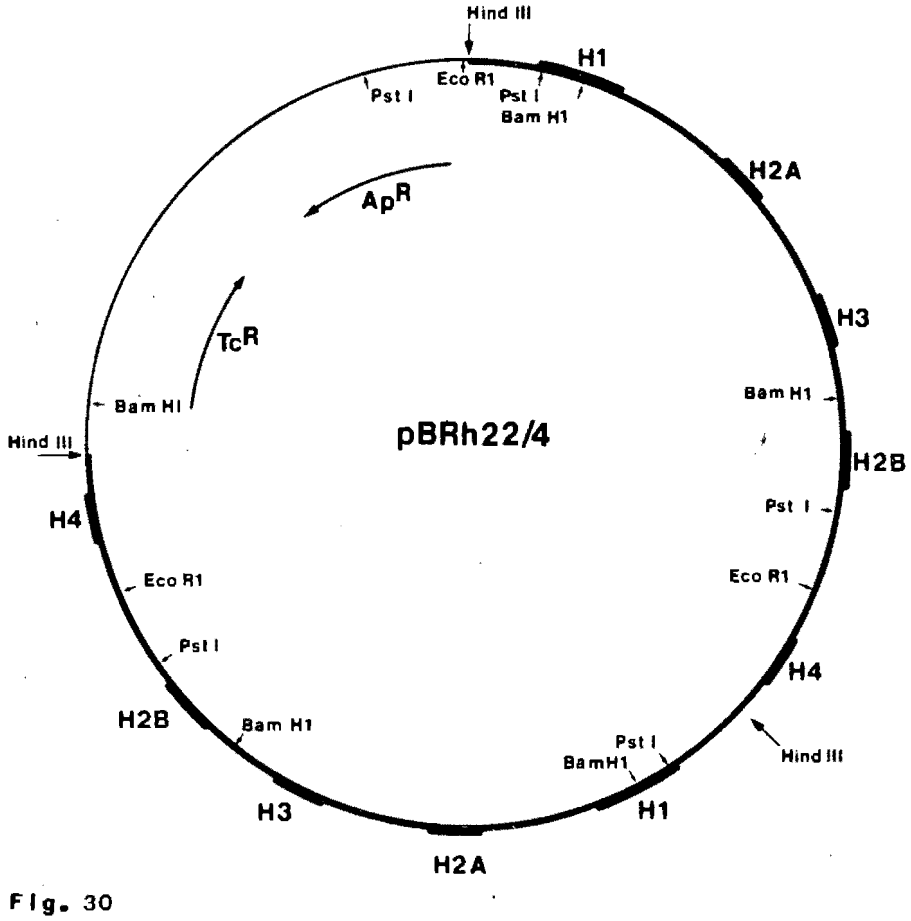


Fig. 30
ph22 plasmid.
Thick lines: Protein coding areas of the histone genes.
Thin line: pBR322 vector.
Tc_R: Tetracycline resistant gene.
Ap_R: Ampicillin resistant gene.

The pBRh22/4 plasmid was transported from Zürich as purified DNA and propagated in transformed HB101 cells. The integrity of the histone gene repeat isolated, and the orientation of the insert, was confirmed by restriction enzyme digestion.

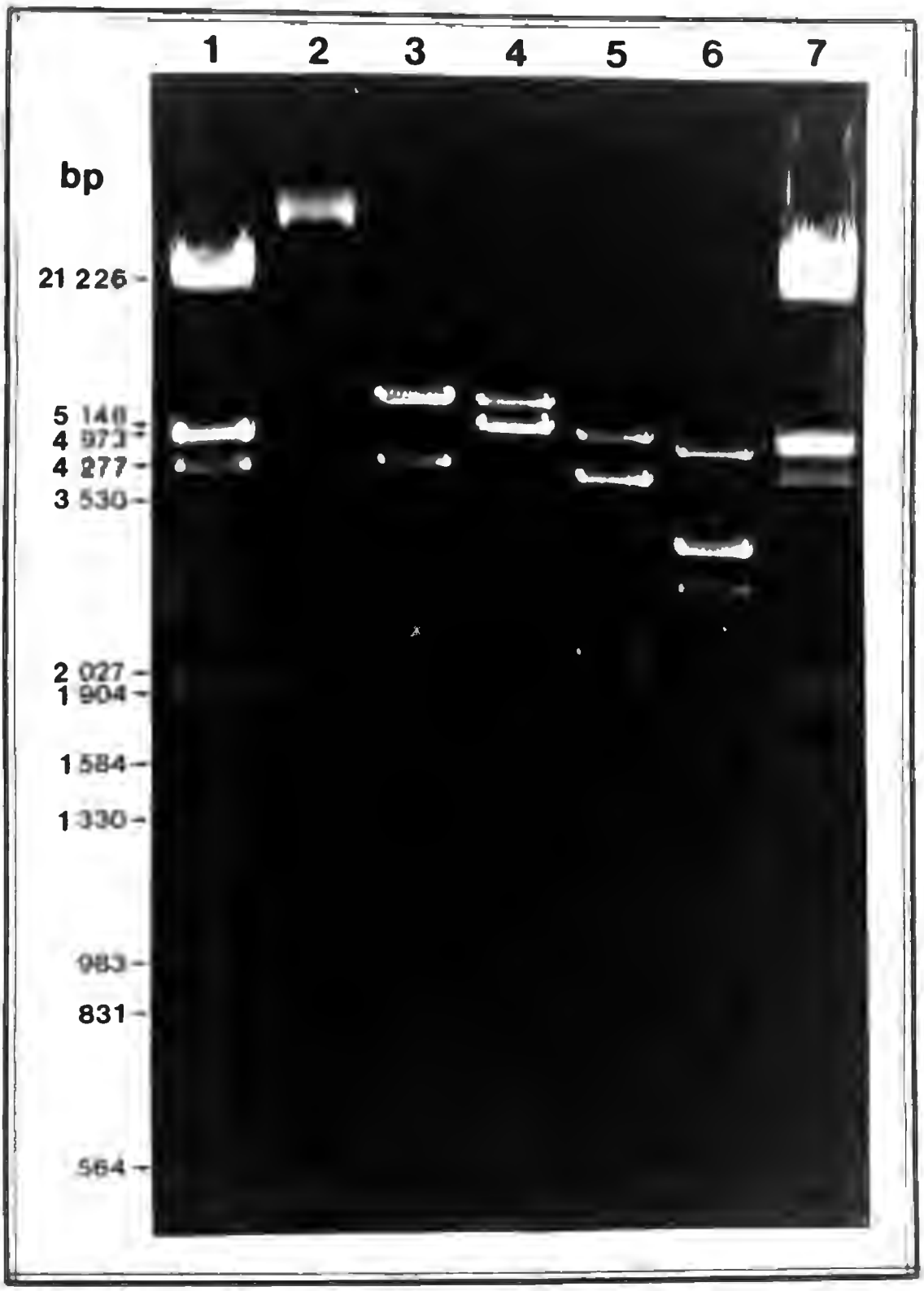
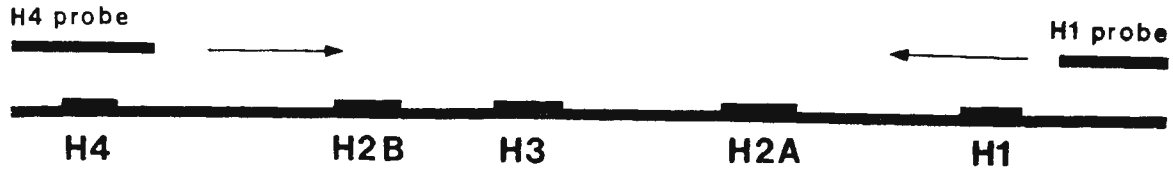


Fig. 31
Restriction enzyme digest of pBRh22/4.
0.8% agarose gel electrophoresis. Lane 2: uncut. Lane 3: Hind III (6356, 4363) Lane 4: Eco RI (5380, 6356, 5339). Lane 5: Pst I (1354, 4164, 2192, 4164, 5201). Lane 6: Bam HI (4875, 3135, 3221, 3135, 2709). The numbers in brackets indicate the expected sizes of the fragments in base pairs. Lanes 1 and 7: Eco RI.Hind III digest of Lambda DNA as size markers.



H22

Fig. 32
Probing strategy used for indirect end-labelling.
Probes adjacent to the ends of the h22 histone gene quintet uniquely label that end of the DNA. This allows the position of the cutting sites to be determined.
Thick lines: protein coding areas

The H4 probes were constructed from the fragment of the h22 between the Pst I and Hind III sites, comprising the coding region of the H4 gene and some of the spacer area between H4 and H2B. This fragment has been inserted between the Pst I and Hind III sites in pBR322. The insert can then be selected for by screening for tetracycline sensitivity. The complete insert is too large to use conveniently for indirect end-labelling. During the insertion into pBR322, the Cla I site in pBR322 is removed. Therefore, the internal Cla I site in the insert offers itself for further restriction by Hind III and Cla I. In a similar way a slightly larger fragment can be generated by restricting with Hind III and Eco R1.

The authenticity of the probes was verified by restriction enzyme mapping with the following enzymes: Hind III and Cla I, Pst I and Eco RI. The sizes of all these fragments are as predicted.

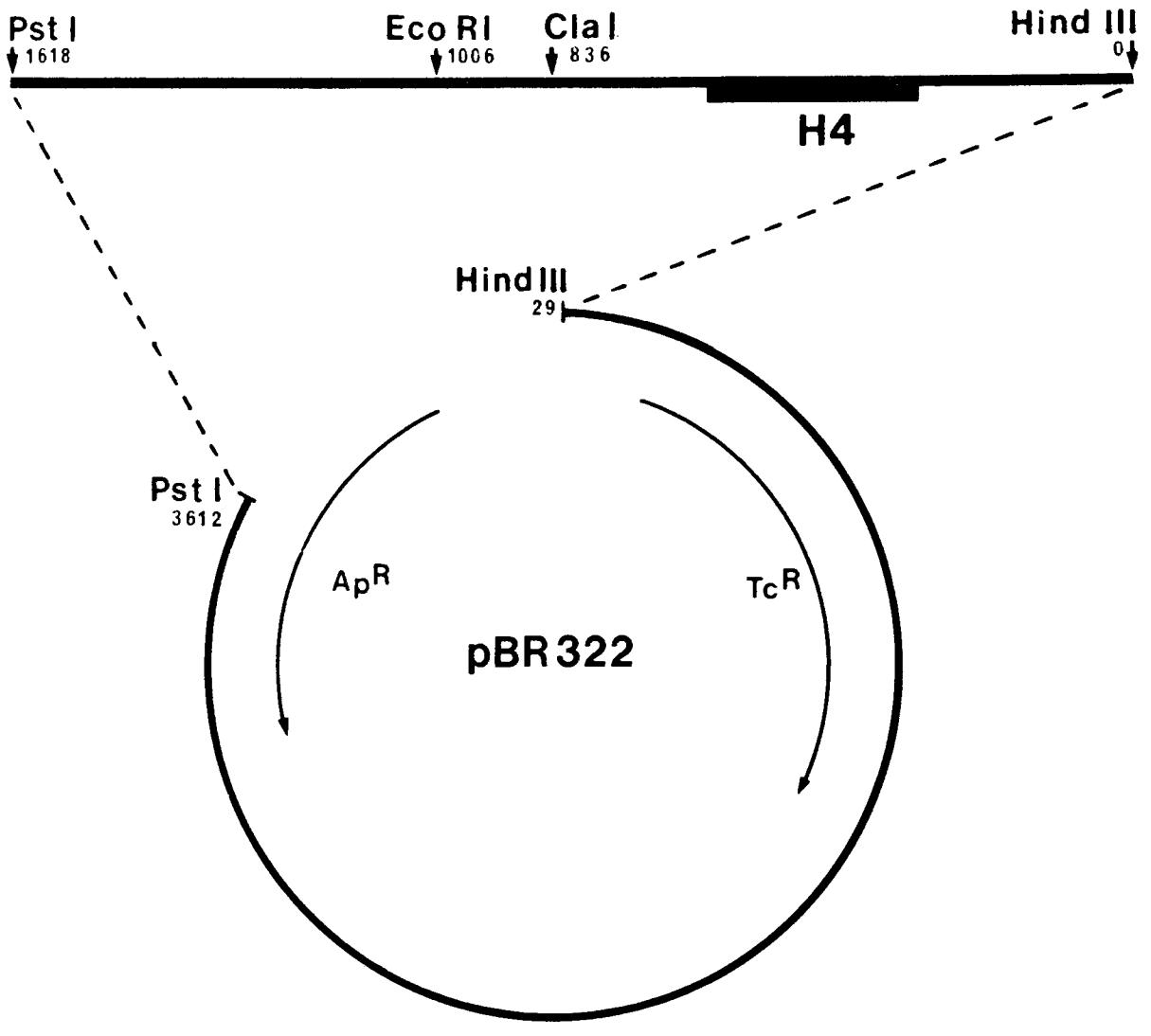


Fig 33
Construction of the H4 probe.
The plasmid ph22 was digested with Pst I and Hind III. The DNA fragments were separated by agarose gel electrophoresis and the relevant band, 1618 base pairs long, was extracted and purified. The vector pBR322 was prepared in a similar way by digestion with Pst I and Hind III. The fragments were electrophoresed and the vector extracted. The DNA concentrations of the insert and the vector were measured and a ligation mixture was prepared using a 100 fold excess of vector. The mixture was ligated, transformed and screened for tetracycline sensitivity. One of the colonies exibiting tetracycline sensitivity was grown up to litre culture and screened for the presence of the insert.

To map the gene from the 3' end, there is a convenient Pst I site that, on restriction, would provide a suitable probe to map the gene from this end. However, this fragment defeated all attempts to insert it between the Pst I and Hind III sites in pBR322. Therefore a larger piece of DNA flanked by the restriction sites Hind III and Bam H1 was eventually inserted in the corresponding sites in pBR322.

The H1 probe, consisting mostly of the H1 downstream spacer, was constructed in a similar way to the H4 probe but using the restriction enzyme sites Hind III and Bam H1. This fragment was then inserted into the corresponding Hind III and Bam H1 sites in pBR322. The clone can be screened by its sensitivity to ampicillin. The insert is relatively large but a smaller probe can be excised by digestion with Pst I and Hind III.

The authenticity of the resultant plasmids was confirmed by restriction enzyme digestion.

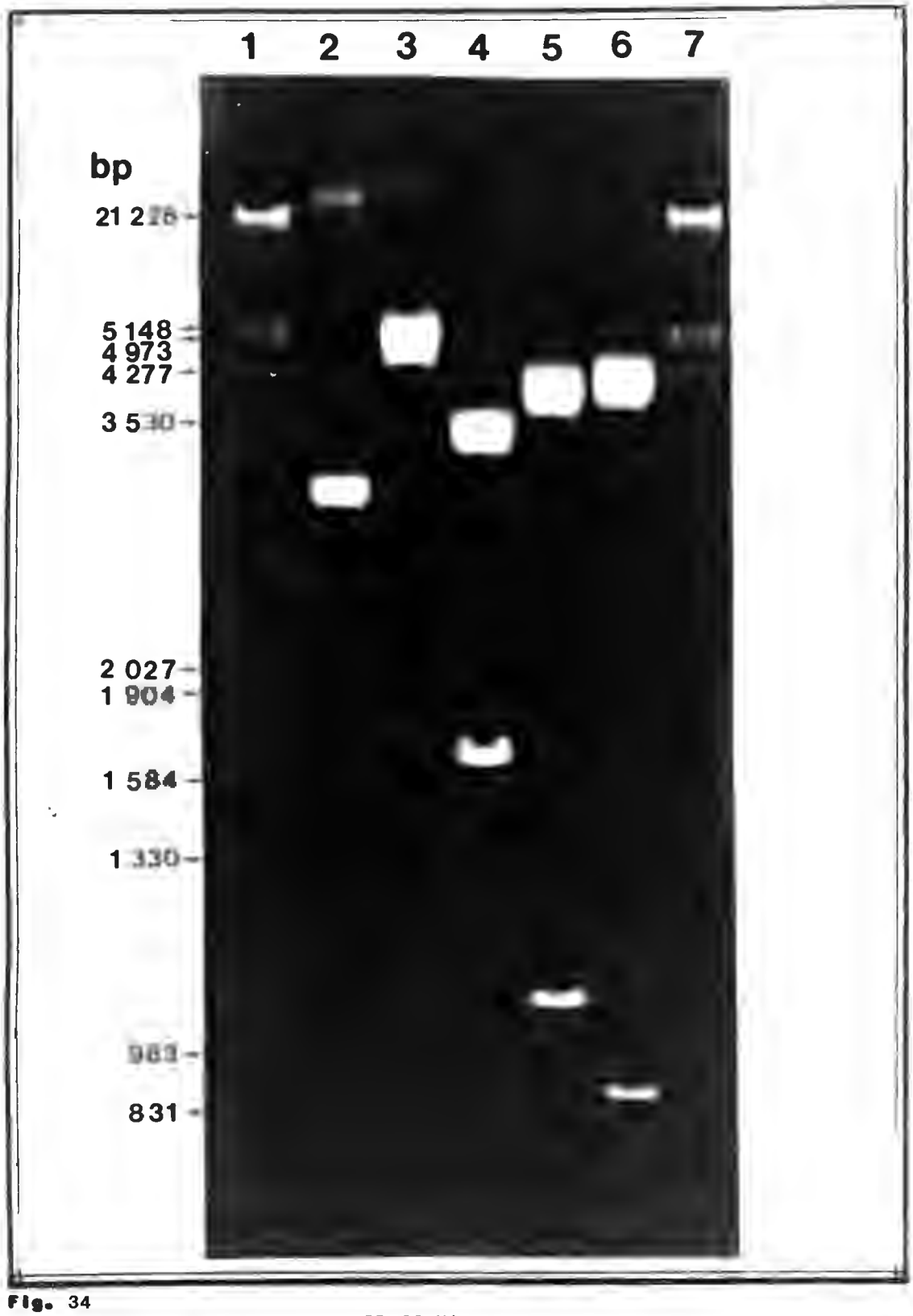


Fig. 34
Restriction enzyme digest of pBR322 H4.
1.5% agarose gel electrophoresis. Lane 2: uncut. Lane 3: Hind III. Lane 4: Hind III and Pst I. Lane 5: Hind III and Eco RI Lane 6: Hind III and Cla I. Lanes 1 and 7: Hind III.Eco RI digest of Lambda DNA as size markers.

To map the gene from the 3' end, there is a convenient Pst I site that, on restriction, would provide a suitable probe to map the gene from this end. However, this fragment defeated all attempts to insert it between the Pst I and Hind III sites in pBR322. Therefore a larger piece of DNA flanked by the restriction sites Hind III and Bam H1 was eventually inserted in the corresponding sites in pBR322.

The H1 probe, consisting mostly of the H1 downstream spacer, was constructed in a similar way to the H4 probe but using the restriction enzyme sites Hind III and Bam H1. This fragment was then inserted into the corresponding Hind III and Bam H1 sites in pBR322. The clone can be screened by its sensitivity to ampicillin. The insert is relatively large but a smaller probe can be excised by digestion with Pst I and Hind III.

The authenticity of the resultant plasmids was confirmed by restriction enzyme digestion.

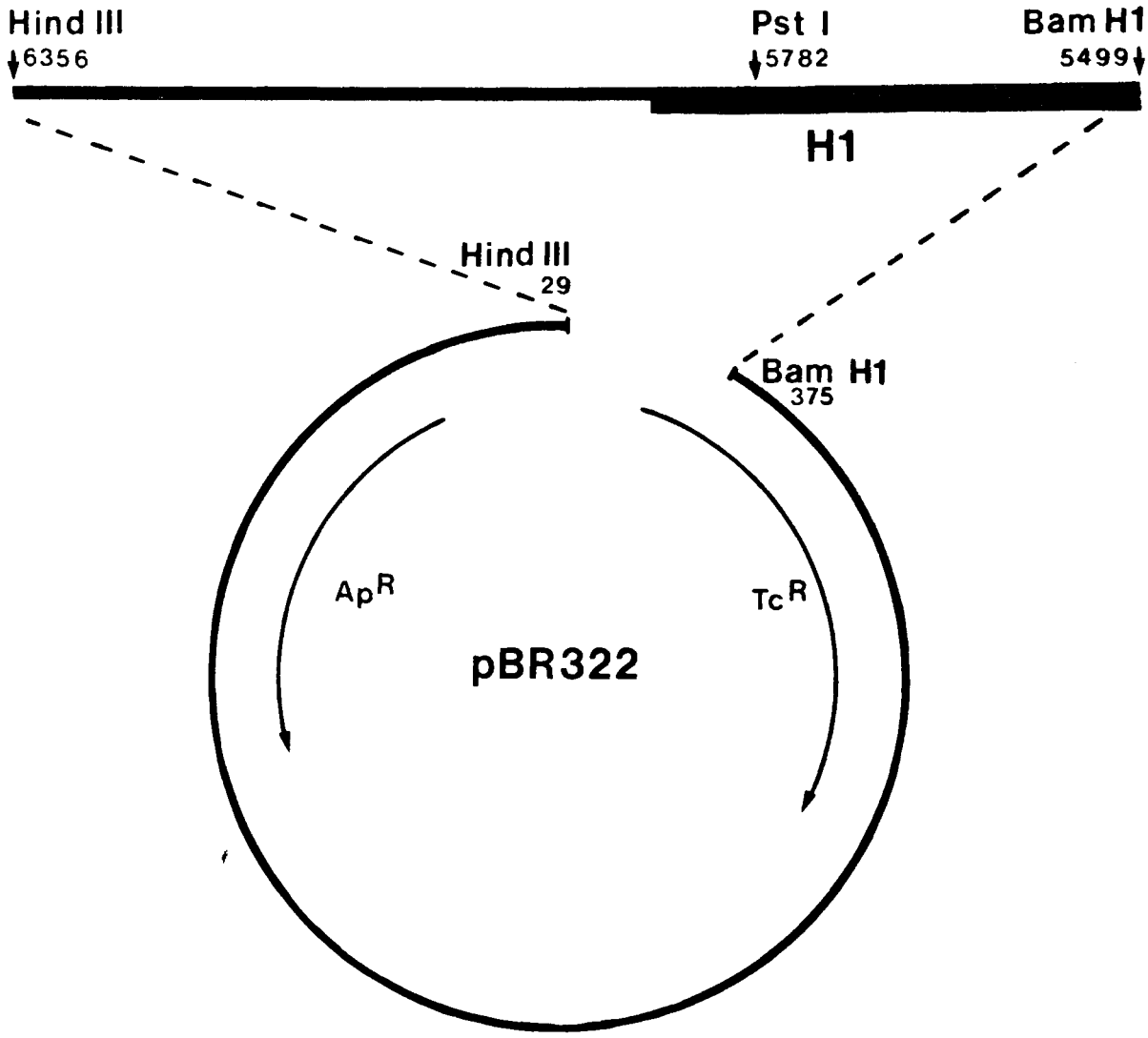


Fig. 35
Construction of the H1 probe:
The the ph22 plasmid was digested with Hind III and Bam H1. The DNA fragments were purified by agarose gel electrophoresis and the relevant band, 857 base pairs long, purified. The vector was prepared by digestion with Bam H1 and Hind III and purified by agarose gel electrophoresis. The vector and insert were mixed at a 100 to 1 molar excess of vector. After ligation, transformation and screening for ampicillin resistance a colony was picked and the plasmid prepared in milligram quantities.

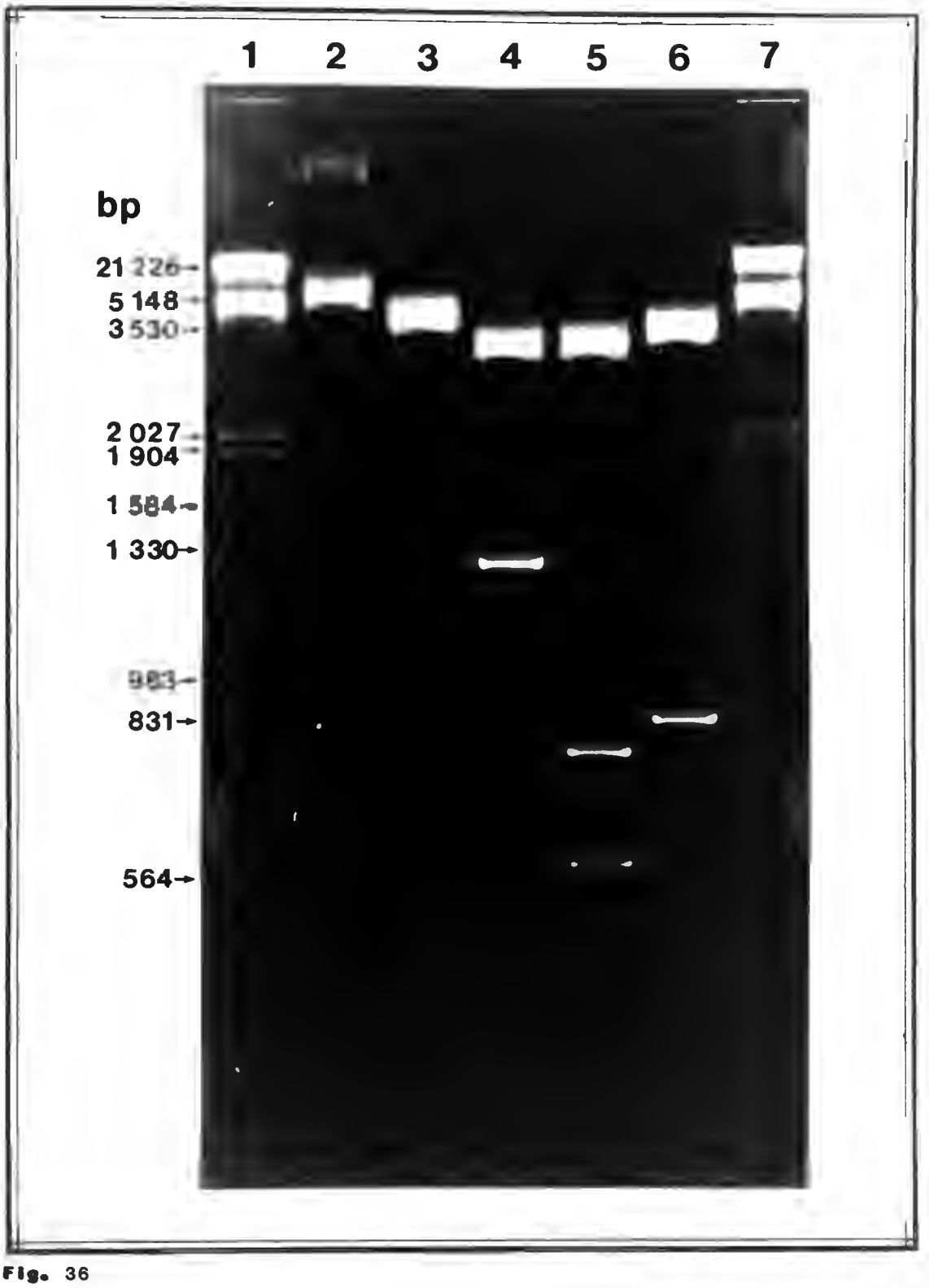


Fig. 36
Restriction enzyme digest of pBR322 HI.
1.5% agarose gel electrophoresis. Lane 2: uncut. Lane 3: Hind III. Lane 4: Pst I. Lane 5: Hind III and Pst I. Lane 6: Hind III and Bam HI. Lanes 1 and 7: Hind III.Eco RI digest of Lambda DNA as size markers.

The small size of the probes limits the amount of radioactivity that can be hybridised to the h22 sequence. In order to have available probes of higher activity, single stranded M13 phages have been constructed (Hu and Messing 1982). These phages have been labelled using a probe primer (Amersham) and the Klenow fragment of DNA polymerase. The large size of the synthesized strand allows the incorporation of more radioactive nucleotides and since the synthesized strand is doublestranded non specific hybridisation is reduced. Denaturation of the probes, with the possibility of renaturation, is also not required and the purification of probes by gel electrophoresis is avoided.

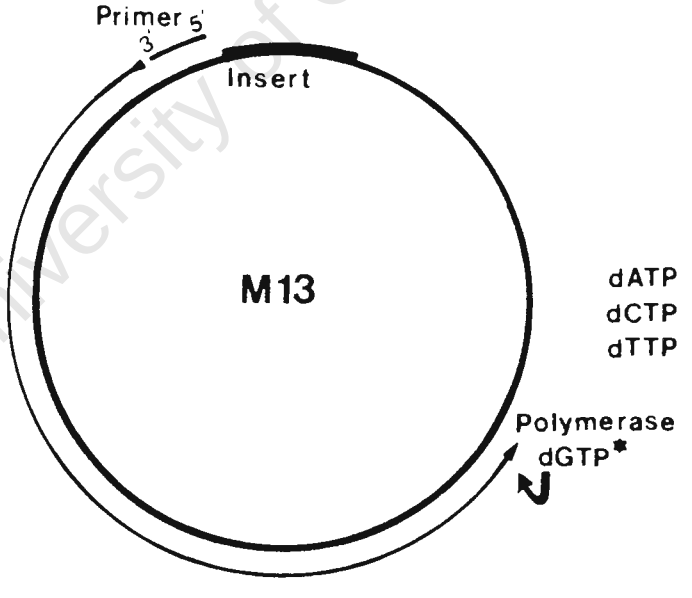


Fig. 37
Labelling of M13 probes:
A primer is hybridised to the (+) strand of the M13 phage and the Klenow fragment of DNA polymerase is used to synthesize the (-) strand. The insert remains single stranded and can be used as a probe, without denaturation, provided the synthesis is not carried to completion.

To construct a single stranded phage probe, the Hind III-Cla I fragment containing the H4 gene was inserted between the Hind III and Acc I sites of the M13mp9 replicative form.

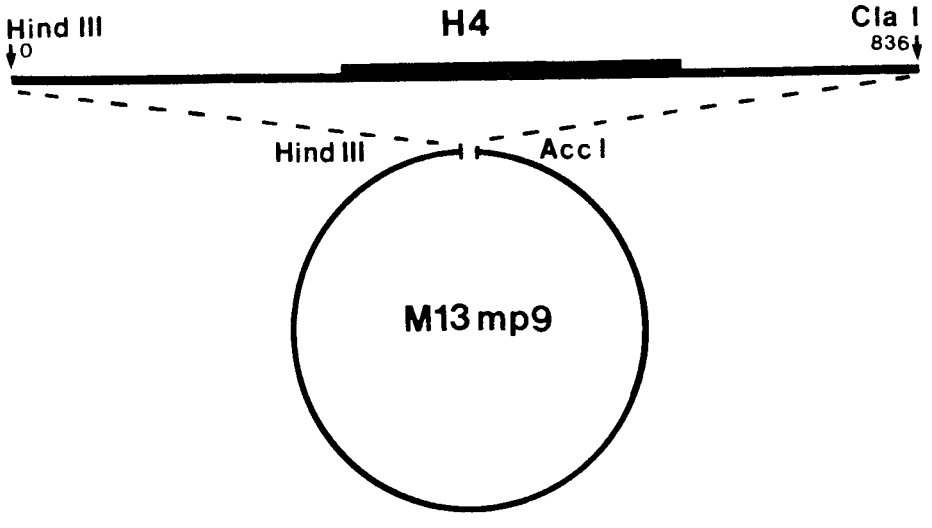


Fig. 38
Construction of M13/H4 probes.
The Hind III-Cla I fragment of ph22 (836 base pairs), was inserted into the Hind III-Acc I sites of the replicative form of M13 mp9.
Thick line: coding areas of histone H4.
Numbers: restriction sites in the h22 histone gene quintet.

Two sizes of DNA fragments from the H1 spacer area were inserted into M13 probes. The Pst I-Hind III fragment and the larger Bam H1-Hind III fragments were inserted into the corresponding restriction sites in the M13mp9 replicative form (Fig. 39) and the presence of the correct insert verified by probing restricted pBRh22. (Figs. 40 and 41)

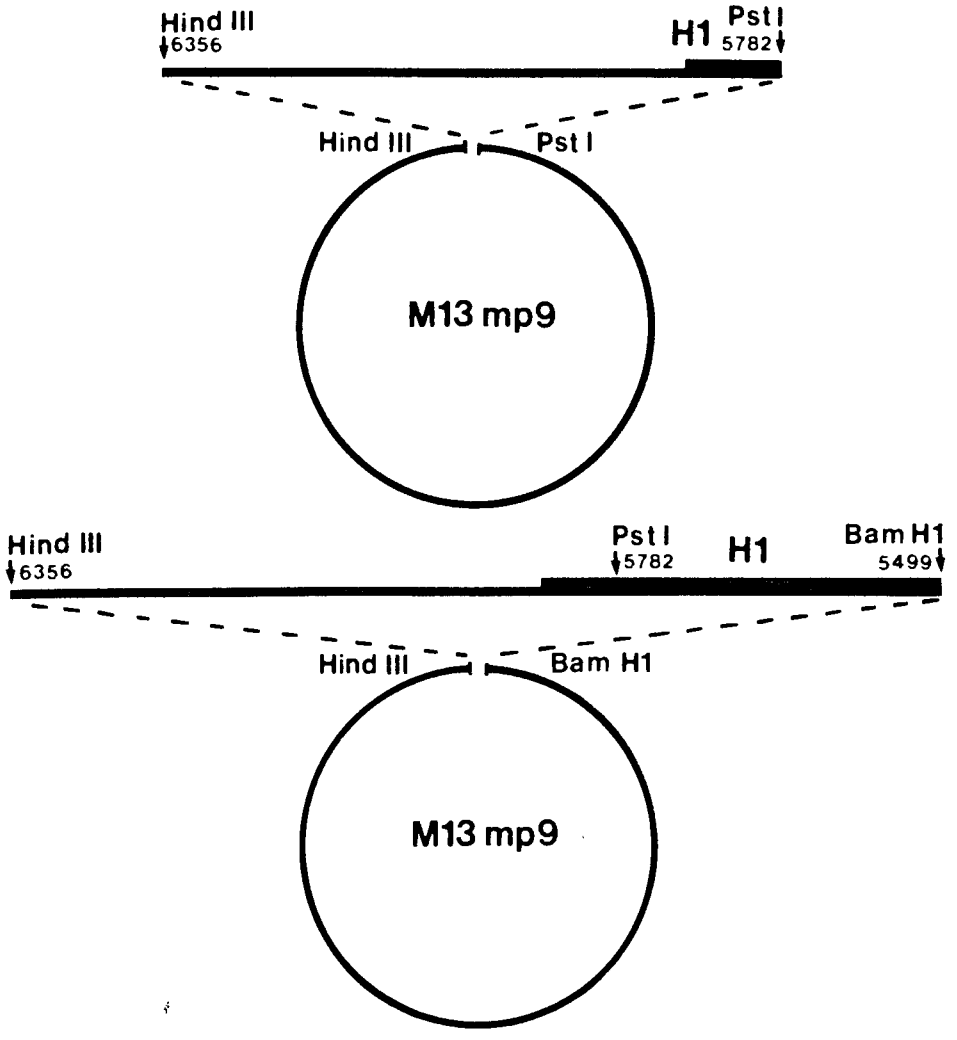


Fig. 39
Construction of M13/H1 probes.
The Hind III-Pst I and Hind III-Bam H1 fragments of ph22 were inserted into the corresponding sites of the replicative form of M13 mp9.
Thick line: coding areas of histone H1.
Numbers: cutting sites in the h22 histone gene quintet.

After transfection and isolation of the single stranded form the probe was verified by probing restricted fragments of pBRh22.

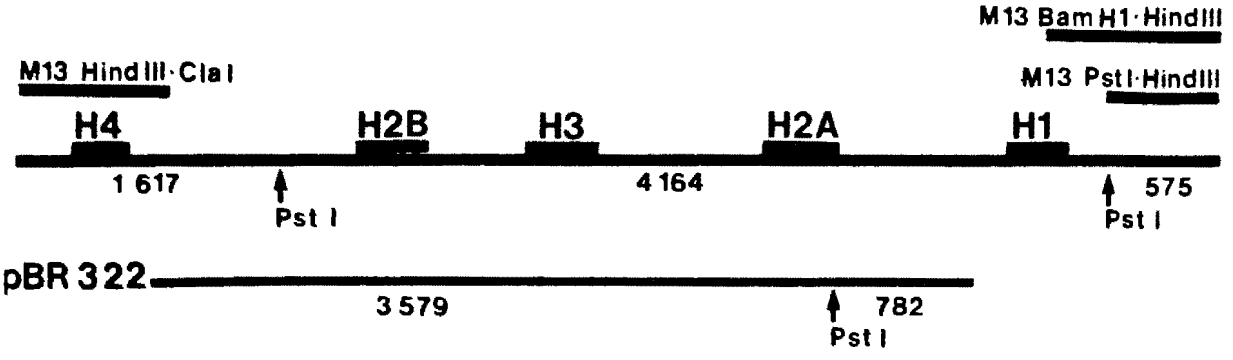


Fig. 40

The probing strategy used to verify the probes inserted into M13.

The h22 and the pBR322 represent a ph22 plasmid after digestion with Hind III. Thick lines: protein coding areas of h22. The numbers indicate the sizes of the fragments produced by Pst I and Hind III digestion. After electrophoresis these fragments can be probed to verify the M13 probes. Note: The M13 probes indicated represent only the complementary sequences inserted into the M13 phage.

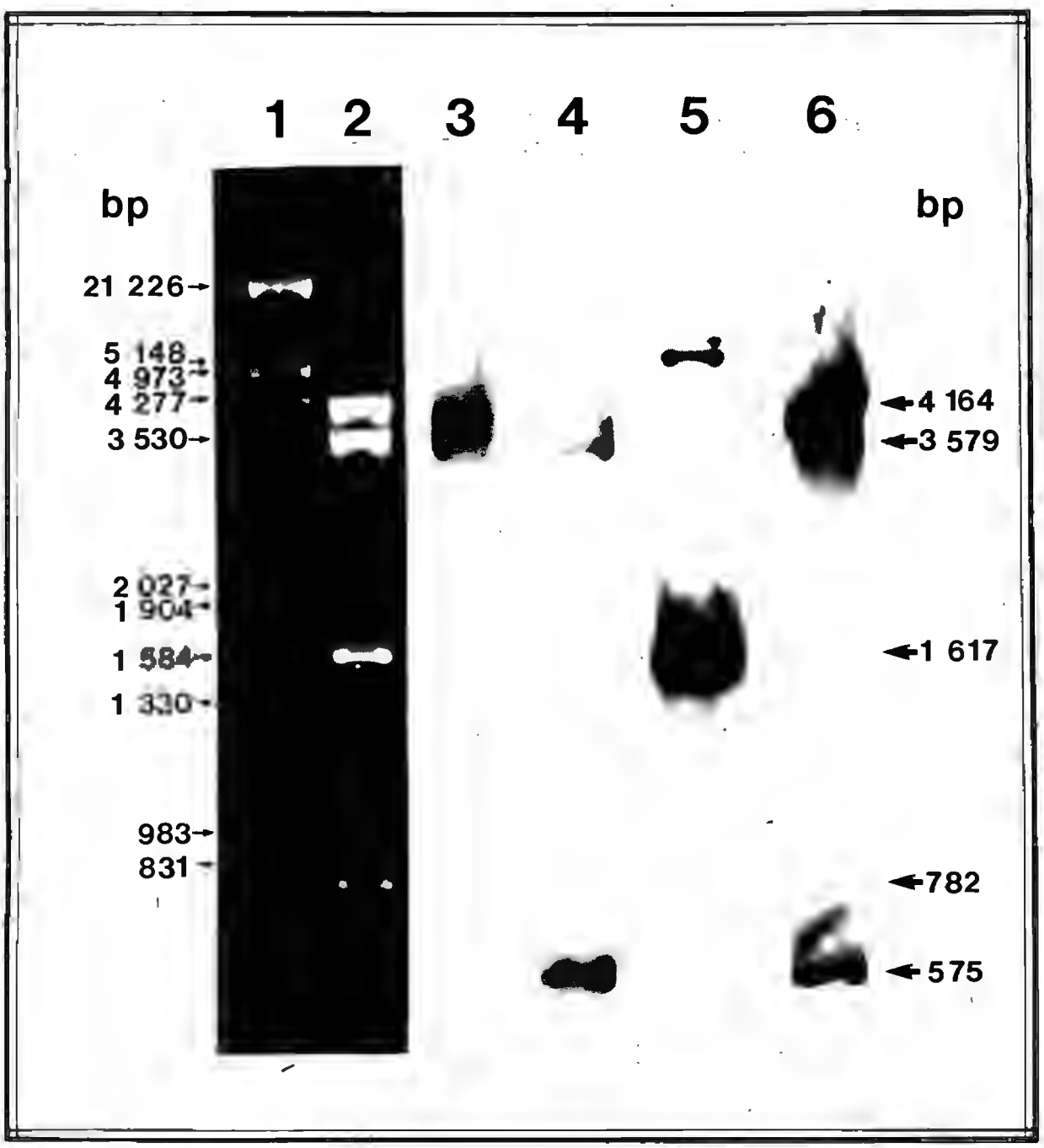


Fig. 41

Hybridisation with probes inserted into M13.

Lane 1: Eco RI.Hind III digest of lambda DNA. The fragment sizes are indicated on the left. Lanes 2 to 6: Pst I.Hind III digest of ph22. The numbers on the right (large arrows) are the expected sizes of the DNA fragments. Lane 2: ethidium bromide stained. Lanes 3 to 6 were excised, Southern blotted and hybridised with the following probes: Lane 3: nick-labelled pBRh22. Lane 4: M13(Hind III-Pst I)H1 probe. Lane 5: M13(Hind III-Cla I)H4 probe. Lane 6: M13(Hind III-Bam H1)H1 probe. The faint, approximately 1100 bp, bands in lanes 4 and 6 may be due to the hybridisation of two 575 bp fragments.

6.3.2 Assembly on the h22 histone gene repeat:

Cores were assembled on Hind III restricted, electrophoretically purified, h22 quintets. To test for the presence of nucleosome cores the assembled gene repeat was digested with micrococcal nuclease under limit digest conditions, and the DNA probed with the complete h22 gene repeat. A range of fragments with predominantly 145, 290 and 435 base pair sizes was produced. These DNA fragment sizes are characteristic of the presence of nucleosome cores, closely spaced dimers and trimers respectively. The 145 base pair fragment confirms the presence of nucleosome cores on the DNA while closely spaced dimers and trimers indicate a population of cores positioned adjacent to each other (Steinmetz et al. 1978), called tight dimers and trimers. Since tight dimers and trimers have not yet been demonstrated to be present in natural chromatin, at least some of the cores cannot be positioned comparable to the natural state. Free h22 DNA digested as a control did not yield any discrete DNA fragments.

Fig.42
Micrococcal nuclease digest of cores assembled on the h22 histone gene repeat. The pBRh22 plasmid was Hind III digested, assembled with nucleosome cores and digested with 2 units Micrococcal nuclease/µg for 0, 15, 30, 60, 120, 300 and 600 seconds. The fragments were electrophoresed on a 2% agarose gel, Southern blotted and probed with nick-labelled purified h22. Lanes 1-7: h22 DNA. Lanes 9-15:assembled h22. Lane 8: 5' end-labelled Eco RI.Hind III digest of Lambda DNA as size standards.

In order to probe for any sequence specific protection sites due to the presence of cores, the polycores assembled on the h22 quintet were partially digested with DNase I and probed with one of the H4 probes. The band pattern produced on free and assembled DNA appears to be identical. No change in the pattern that can be ascribed to the presence of nucleosome cores could be seen.

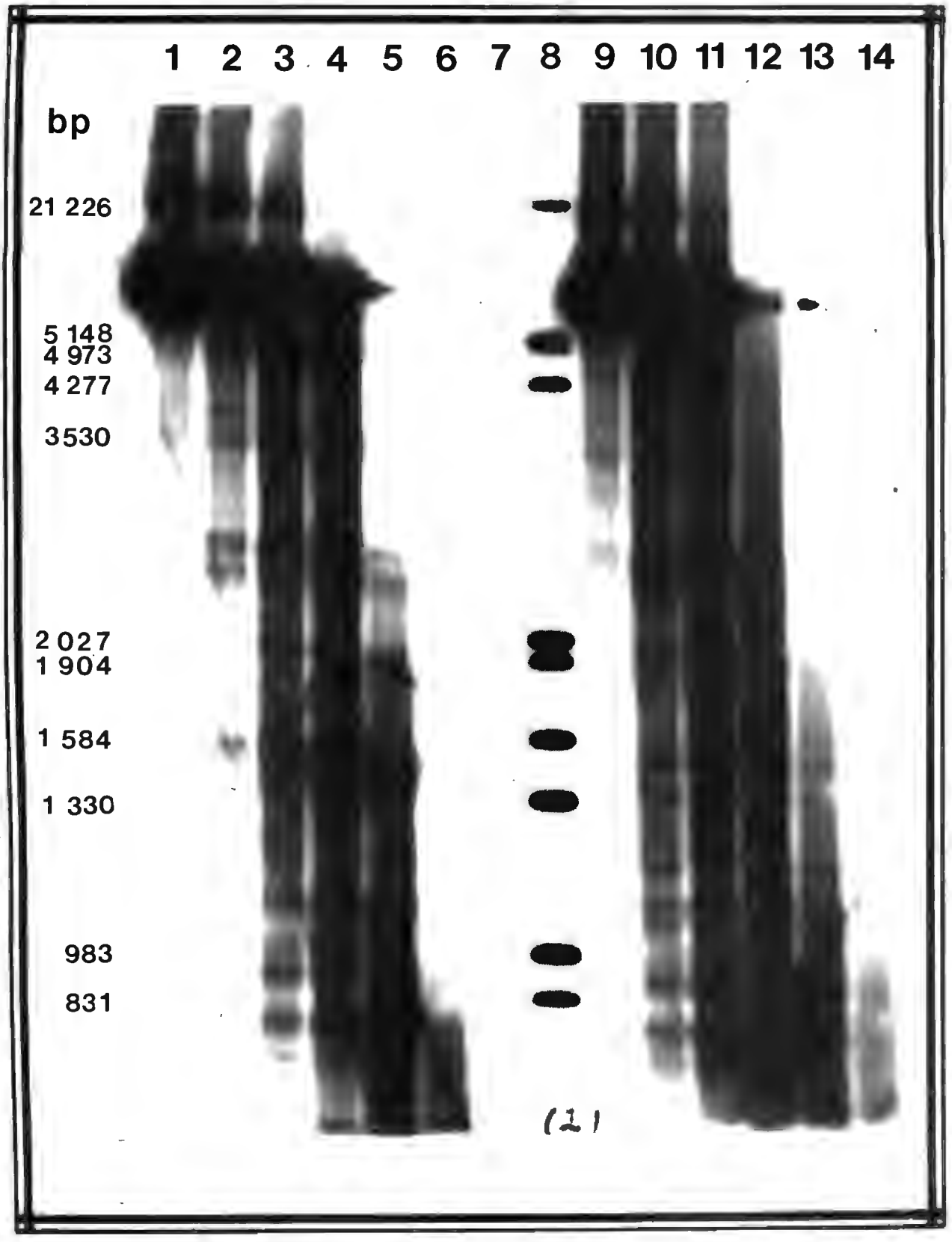


Fig. 43
DNase I digestion and indirect end-labelling of the h22 histone gene repeat. Nucleosome cores were assembled on Hind III digested h22 histone gene battery at a histone to DNA ratio of 0.64:1 (w/w). The polycores were digested with DNase I for 0, 15, 30, 60, 120, 300 and 600 seconds, followed by 0.8% agarose gel electrophoresis, Southern blotting and hybridisation with the purified H4(Cla I-Hind III) fragment. Lanes 1-8: free DNA. Lanes 9-16: assembled ph22. Lane 17: 5' end-labelled, Hind III.Eco R1 digest of Lambda DNA.

Assembled and free h22 DNA, digested with micrococcal nuclease and probed with the H4 probe, produced similar patterns. Compared to the free DNA (Fig. 44; lane 7), it appears that the sequences 500 - 1 000 base pairs and 1 500 - 2 000 base pairs of the assembled quintet (Fig. 44; lane 17) are more protected than the remainder. The overall rate of digestion of the assembled DNA however, was significantly lower than that of the free DNA.

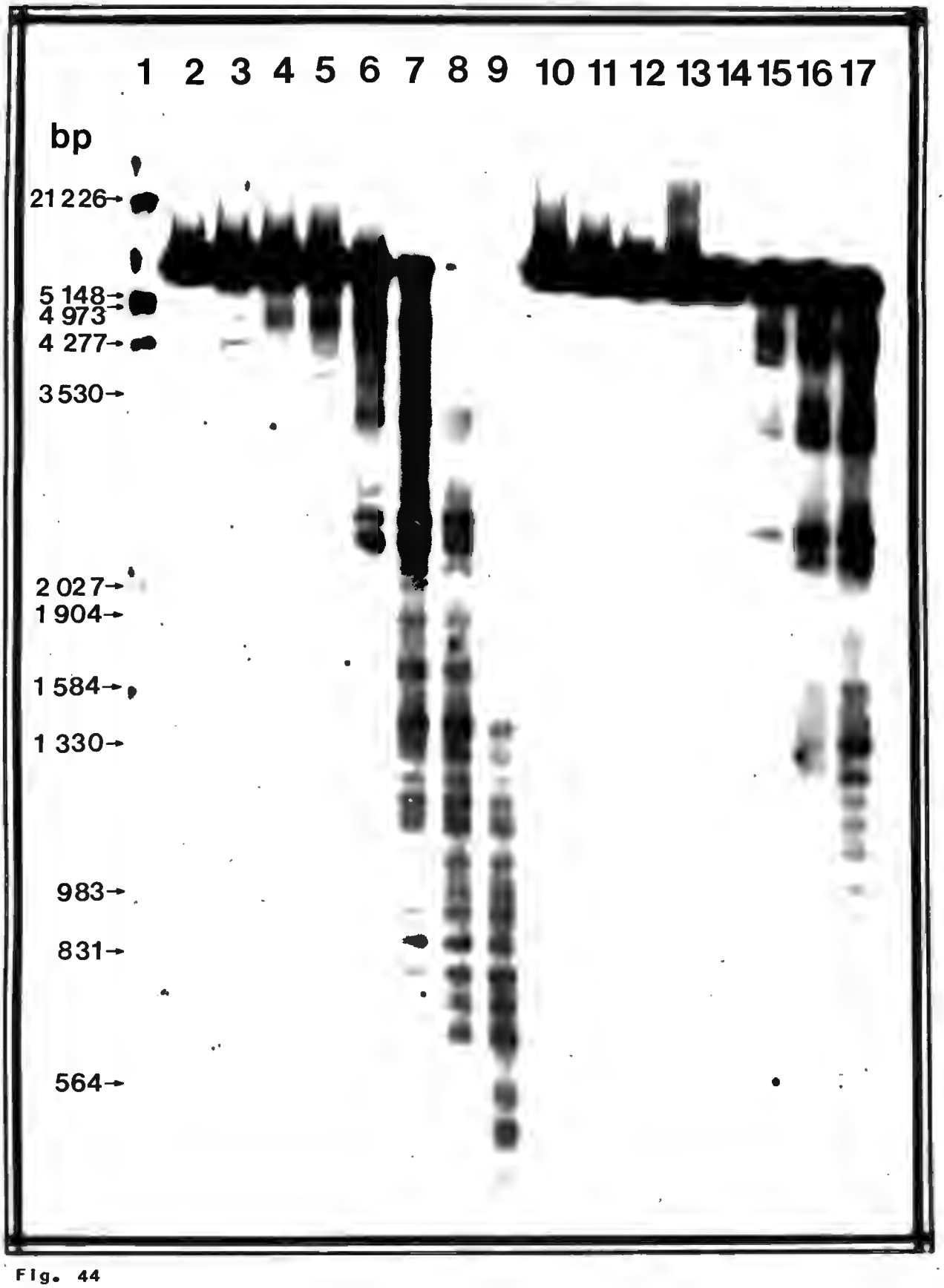


Fig. 44

Micrococcal nuclease digestion and indirect end-labelling of the h22 histone gene repeat.

Nucleosome cores were assembled on Hind III digested h22 histone gene battery at a histone to DNA ratio of 0.64:1 (w/w). The polycores were digested with 0.03 units Micrococcal nuclease/µg DNA for 0, 15, 30, 60, 120, 300, 600 and 1200 seconds, followed by 0.8% agarose gel electrophoresis, Southern blotting and hybridisation with the purified H4(Cla I-Hind III) fragment. Lanes 2-9: free DNA. Lanes 10-17: assembled ph22. Lane 1: 5' end-labelled, Hind III.Eco R1 digest of Lambda DNA.

It is concievable that the similarity in the pattern of naked and assembled DNA is the result of extended tracts of tightly packed cores which could form at a high histone to DNA ratio. To test this polycores assembled at half the previous histone to DNA ratio were investigated. The pattern produced is identical to those produced at higher histone to DNA ratios. The only difference is that the rate of digestion is more rapid than those produced at the higher histone to DNA ratio.

In view of the previous results with the pBR322H4 probe the other H4 and H1 probes have not been used.

The low resolution mapping of polycores assembled "in vitro" does not reveal preferred positioning of cores.

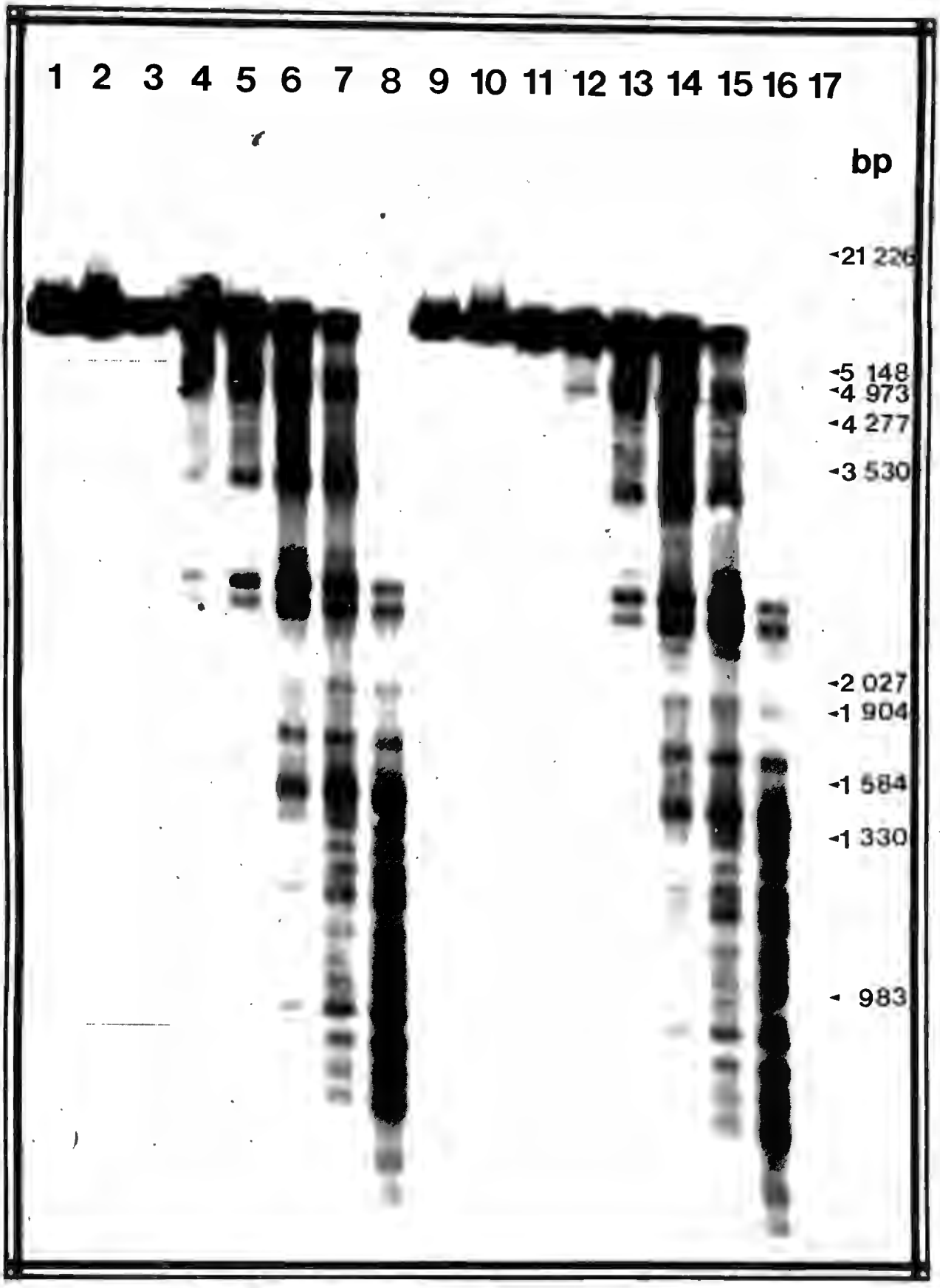


Fig. 45

Micrococcal nucleae digestion and indirect end-labelling of the h22 histone gene repeat assembled at a low histone to DNA ratio.

Nucleosome cores were assembled on Hind III digested h22 histone gene battery at a histone to DNA ratio of 0.32:1 (w/w). The polycores were digested with 0.03 units Micrococcal nuclease/µg DNA for 0, 15, 30, 60, 120, 300, 600 and 1200 seconds, followed by 0.8% agarose gel electrophoresis, Southern blotting and hybridisation with the purified H4(Cla I-Hind III) fragment. Lanes 1-8: free DNA. Lanes 9-16: assembled ph22. Lane 17: 5' end-labelled, Hind III.Eco R1 digest of Lambda DNA. The accurate positions were determined on an overexposed autoradiogram.

6.4 HIGH RESOLUTION MAPPING

Although there is no evidence for nucleosome core postioning on the h22 histone gene quintet, I proceeded with high resolution mapping of some selected DNA fragments because the h22 histone gene quintet contains prominent stuctural features, that may not necessarily cause unique positioning of the nucleosome cores, but may have other local influences on the distribution of the assembled nucleosome cores, namely provide frames for preferential assembly (Linxweiler and Horz 1985). The low resolution of the indirect end-labelling method did not allow such detailed analysis of the assembled cores.

In view of the limitations inherent in the high resolution mapping methods hitherto used (See literature review), I have used a novel approach, making use of pGV403 plasmids. These plasmids had originally been developed to simplify direct end-labelling of DNA fragments for Maxam and Gilbert chemical sequencing reactions (Volkaert et al. 1984).

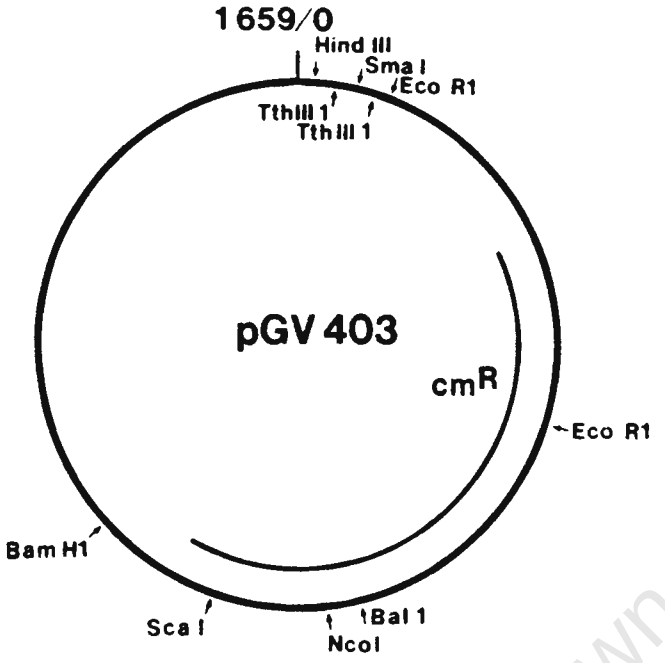


Fig. 46
pGV403 plasmid.
The plasmid size is 1659 base pairs. The main cutting sites are indicated. The plasmid was obtained from Amersham, Sma I digested and alcaline phosphatase treated.
cm^R : chloramphenicol resistant gene.

These plasmids possess a Sma I site for blunt ended insertion. This site is flanked by two different Tth 111 I sites.

Tth 111 I site, general sequence:	G-A-C-N|N-N-G-T-C C-T-G-N-N|N-C-A-G
Tth 111 I site upstream of Sma I:	G-A-C-T|A-A-G-T-C C-T-G-A-T|T-C-A-G
Tth 111 I site downstream of Sma I:	G-A-C-T|C-A-G-T-C C-T-G-A-G|T-C-A-G

Restricting these Tth 111 I sites yield a one base 5' overhang at the four ends of the two fragments produced. Each 5' overhang contains one unique base, A,T,C and G respectively. When the corresponding recessed ends are filled in with a single radioactively labelled nucleotide, only one of the four generated ends is labelled. The fragment containing the insert

is flanked by G and A 5' overhangs. This allows the inserted fragment to be labelled uniquely at either end by using [α-^{32}P]dCTP or [α-^{32}P]dTTP for the fill-in reaction. Since the one base 5' overhangs produced by the vector will not be labelled, the vector DNA does not have to be removed and can act as carrier DNA in the system.

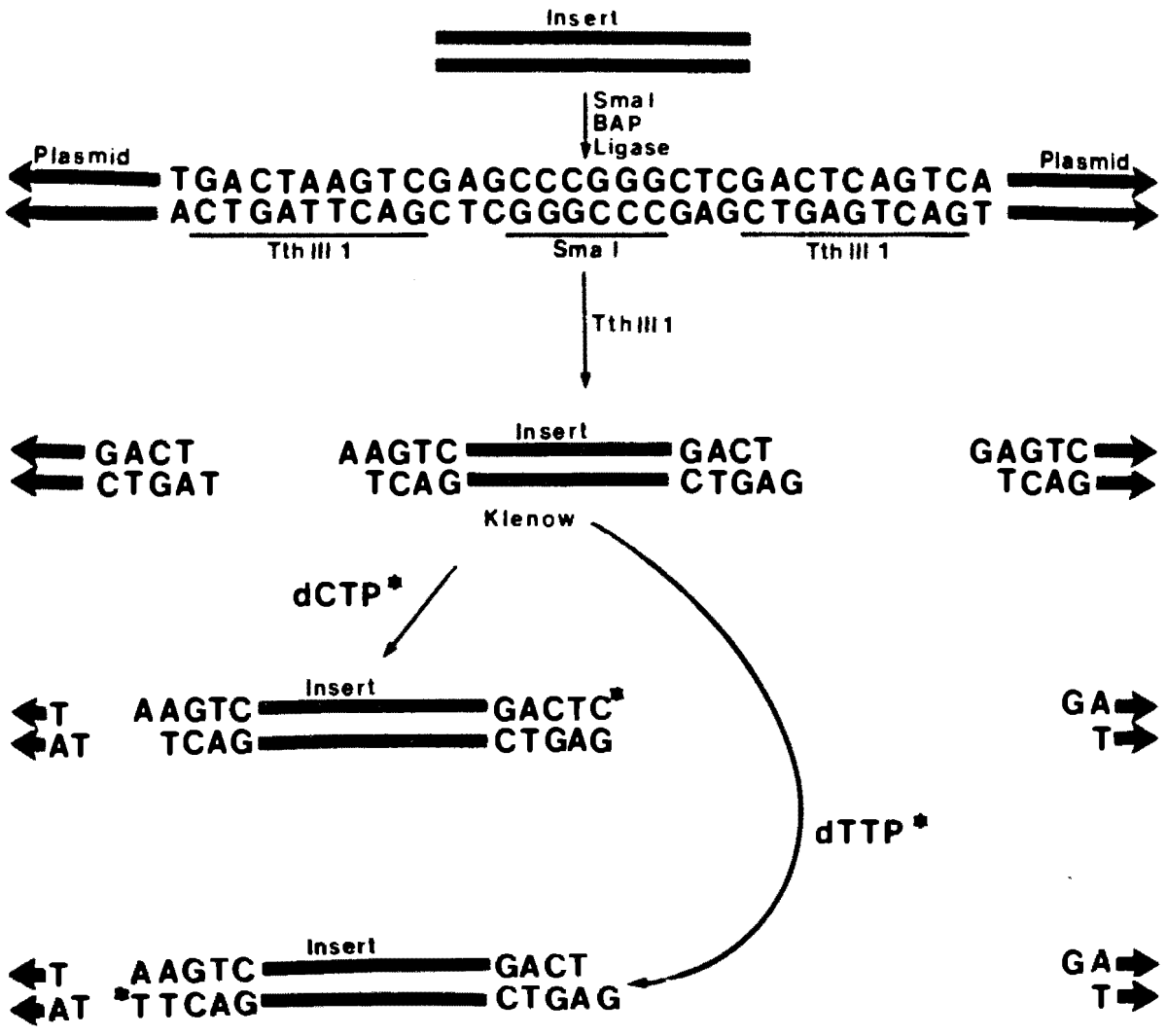


Fig. 47
Direct end-labelling using pGV plasmids:
A bluntended fragment is ligated into the bacterial alcaline phosphatase (BAP) treated Sma I site. The transformed plasmid is propagated in HB101. The purified plasmid is then digested with Tth 111I at 65°C. This is followed by a Klenow repair of the generated sticky ends in the presence of either $[^{32}P]$dCTP or $[^{32}P]$dTTP. Since only one of the four possible sticky ends can be labelled the insert is labelled uniquely at one end.

The use of the pGV403 plasmids removes the methodological difficulties imposed by other methods of preparing end-labelled DNA for purposes of core assembly. The plasmids can be produced and purified in milligram quantities on caesium chloride gradients. The labelling protocols have been tuned to rapidly produce sufficient amounts of uniquely end-labelled DNA. Since only a single restriction enzyme digest at 65°C, and a fill-in

reaction using the Klenow fragment are needed, DNA degradation and exposure to radioactivity is reduced to a minimum.

The high resolution mapping of the DNA can be carried out optimally only over short stretches, approximately 300 base pairs. The previous low resolution mapping had not revealed preferred assembly sites for cores in the h22 polycore that could be of interest. I have therefore selected sequences from the h22 quintet which, in the light of our present knowledge of octamer-DNA interactions, are of special interest.

It has been shown that certain DNA sequences can influence the assembly of cores, for example, cores can be assembled on poly[d(A-T)].poly[d(T-A)] and poly[d(G-C)].poly[d(C-G)] but will not form on poly(dA).poly(dT) and poly(dG).poly(dC) fragments (Simpson and Kunzler 1979). This has been related to the bending stiffness of the polymers (Hogan et al. 1983). A poly(dA).poly(dT) fragment, 97 bases long, inserted into a plasmid inhibits assembly while the same polymer, only 20 bases long, does not significantly influence the assembly (Prunell 1982). In these investigations assembly was achieved by dialysis from high to low ionic strength. Similar results were reported on the assembling of extensive tracts of poly(dA).poly(dT) inserted into a recombinant plasmid, using poly(glutamic acid) or RNA as assembly factors (Kunkel and Martinson 1981). It appears therefore, that the presence of poly[d(purine)].poly[d(pyrimidine)] sequences in the DNA may inhibit core formation (Haeutle and Martinson 1983). Such structural features in natural DNA are therefore of specific

interest.

Two sequences containing poly[d(purine)].poly[d(pyrimidine)] tracts are striking characteristics of the h22 gene quintet. The first is a poly[d(G-A)].poly[d(C-T)]$_{16}$ stretch in the H1-H4 spacer and the second a poly[d(C-T)].poly[d(G-A)]$_{23}$ stretch in the H2A-H1 spacer (See section 6.1). The two strands of the homocopolymers can, depending on the ionic stregnth, slip over each other resulting in the formation of single stranded loops abutting the polymers. These single stranded loops result in S1 nuclease sensitive sites in the histone gene battery (Hentschel 1982).

The inclusion of such repeat sequences into the DNA fragments, to be analysed by high resolution mapping, offer a special methodological advantage. The digestion of such a monotonous stretch of DNA sequence produces a characteristic motif in the nuclease digestion pattern marking the orientation of the DNA insert. Furthermore, the dinucleotide repeat is expected to result in an unmodulated pattern of nuclease cutting because the conformation of the DNA is expected to be monotonous throughout this stretch. A change in the digestion pattern after assembly, due to selective protection, should be clearly visible over the background of digestion produced by these free DNA sequences.

In addition to the features considered above, the H2A-H1 stretch also contains the post H1 mRNA 3' termination sequence (Hentschel et al. 1980).

Plasmid construction:

The selected fragments were inserted into the Sma I site of the pGV plasmids by blunt ended ligation.

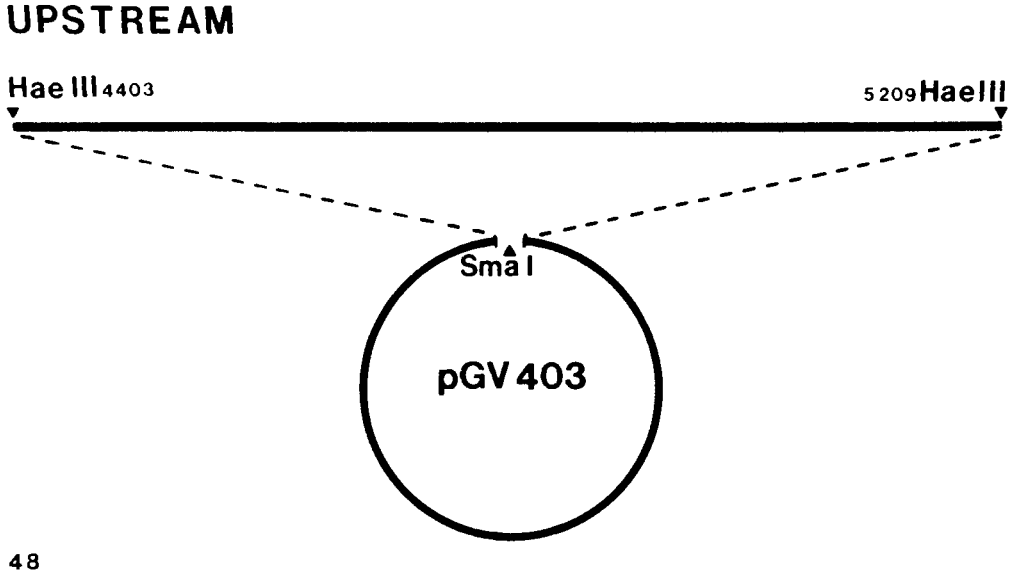


Fig. 48
Construction of pGV403 Upstream plasmid:
The fragment situated between the Hae III (4402) and the Hae III (5209) sites upstream of the H1 coding area was inserted into the Sma I site of pGV403. The numbers in brackets indicate the restriction sites in h22.

STOP

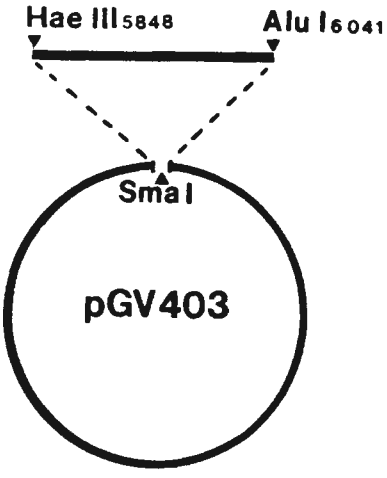


Fig. 49
Construction of pGV403 Stop plasmid:
The fragment coding for the stop signal of the H1 coding area situated between Hae III (5848) and Alu I (6041) sites was inserted into the Sma I site of pGV403. The numbers in brackets indicate the restriction sites in h22. The orientation of this fragment was not established.

END

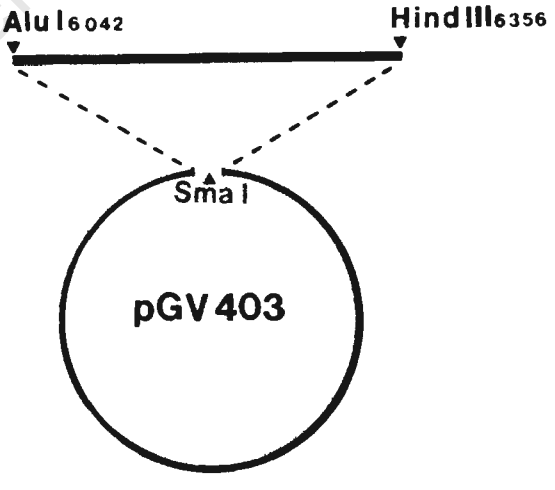


Fig. 50
Construction of pGV403 END plasmid:
The fragment situated downstream from the h22 H1 histone gene between sites Hind III (6356) and Alu I (6042) was inserted into the pGV403 plasmid after the recessed 3' end was filled in the Klenow fragment of DNA polymerase. The numbers in brackets indicate the restriction sites in h22.

HB101 host cells were transformed with the ligated plasmids and screened for chloramphenicol resistance. The presence of inserted histone gene sequences was confirmed by colony hybridisations using nick-labelled h22 as a probe. The positive colonies were picked into liquid media and the plasmids purified by cell lysis and caesium chloride centrifugation.

The purified plasmids were digested with Tth 111 I and labelled by a fill-in reaction using the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP. The labelled fragments were analysed by denaturing DNA electrophoresis. The sizes of the inserts determined in this way are 22 bases larger than the original inserts due to the inclusion of the fragments between the Sma I site and the Tth 111 I sites of the pGV plasmids. At the 5' end of the fragment 10 bases of the pGV plasmid are included plus the 1 base 5' overhang. At the 3' end the pGV plasmid contributes 10 bases. During the fill-in labelling reaction an additional base, the radioactive nucleotide is added.

```
        10         20         30         40         50         60
AGTCGAGCCC CCACCAAATA TTCAAGAAAG ATAAAAGTCT CTGTAATGAA ATCAAGCATC

        70         80         90        100        110        120
AACAACAAAA TAAAATAATA AATAAATAAA TATATAAAAC AAAAATGAAC CAAAGTAATA

       130        140        150        160        170        180
ATAATAGTAA AGAAAATAGA CCACCCACAC ACACACACAC ACACACTCTC TCTCTCTCTC

       190        200        210        220        230        240
TCTCTCTCTC TCTCTCTCTC TCTCTCTCTC TGTGCCCTTC TCTCAATCTC ATTTTTAATT

       250        260        270        280        290        300
AGTCATTCAA TGAAGCAAAT ATAGTAAGTG CCTAGTAAGA TATCTCTCAT CTCCTTTAAA

       310        320        330        340        350        360
CTATATGAAC TATGCTGATA TTCAACGAAT ATAACACGAG CCTATACAGA AACCGCGAGA

       370        380        390        400        410        420
AGAAACAATT TGCTTATATT ATTCAAATGC TATTATGTTC TGGATGTTGC ATATTAATAA

       430        440        450        460        470        480
CTCTCAGGTA AAACGATAAC ACGTTGTCAA ACTTGAAGCC TGCAACGAGG GTATTAAGTG

       490        500        510        520        530        540
TTTCAAAGAA ACATGGATCT ATAACAAATA TTCAATTCAA TCGCTTTTGG GAAGGGGGTG

       550        560        570        580        590        600
ACGAACATTT AACTTTGCAC ACATTTACGT TTCGGTTTTT TTTCCTCTCA TCCCAAGAAG

       610        620        630        640        650        660
ACGGTTTTAA GACTGGTATA TGGTTATCAT AATCATTGCA ATGATTCTTG GCGAGGGCAA

       670        680        690        700        710        720
AGTTTTCTGT TATAATCATC TGCTTGTCAG CAATTTCAAC AAAGTTTCGA TCTGCAAACA

       730        740        750        760        770        780
CACGCTGATC GGCAGTGAGG CAAAACAGAC AAATCGGACA AATTGTTTCC CACCACGTAC

       790        800        810        820
GCAACCGCGC GGGATATAGG TGAGGTTGCC GTGAGGGGGG CTCGACT
```

Fig. 51

Sequence of the Upstream fragment:

The sequence was taken from the complete h22 sequence provided by M. Birnstiel.

Underlined: Sections of the fragment belonging to the pGV403 plasmid.

```
        10          20          30          40          50          60
AGTCGAGCCC CCAAGAAGGT TGCAAAATCG AAATGATGTT GCACGTCCTA CTCGTGTCAC

        70          80          90         100         110         120
CACAACACAA CGGCTCTTTT CAGAGCCACC ACATTTCCAC GAAAGACCAA TGCCTTATTT

       130         140         150         160         170         180
TGATACGTAA TGATTAATGT AGAGAAACAG CAAAATATTC ATATTACTGC TGCTAATTAA

       190         200         210
AGAGAGACAA AACAAAACAA GAAGGGGCTC GACT
```

Fig. 52
Sequence of the Stop fragment:
The sequence was taken from the complete h22 sequence provided M. Birnstiel.
Underlined: Sections of the fragment belonging to the pGV403 plasmid.


```
        10          20          30          40          50          60
AGTCGAGCCC CTAACACACG CTTCAAACTA CTAATAAAAC CAACGCATGA AATTCATTGT

        70          80          90         100         110         120
TTATCTATTA ATGTGCCAAC TCTGCAAAGG TGGCACCCAG GAAAGGAGAT TTACAGAAAT

       130         140         150         160         170         180
AAGCAACACG TAGAGGAAAA GAGAGTTATA CCACTCCTGA CATGAAACAC ACTCAATTCA

       190         200         210         220         230         240
ACATATTTAG AGGAAGGGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG GGGGGGGGGG

       250         260         270         280         290         300
AGGGAGAATT GCCCAAAACA CTGTAAATGT AGCGTTAATG AACTTTTCAT CTCATCGACT

       310         320         330
GCGCGTGTAT AAGGATGATT ATAAGCTGGG CTCGACT
```

Fig. 53
Sequence of the End fragment:
The sequence was taken from the complete h22 sequence provided M. Birnstiel.
Underlined: Sections of the fragment belonging to the pGV403 plasmid.
Enclosed: Bases contributed by the fill-in reaction of the Hind III site.

Upon labelling and analysis by denaturing DNA gel electrophoresis the sizes of these labelled fragments are as predicted (Fig. 54 and 55). Digestion of the labelled fragments with DNase I, followed by denaturing DNA gel electrophoresis, produces a complex band pattern. The simple sequence motifs are striking features of these patterns and make the identification and establishment of fragment orientation easy. The change in the positions of these motifs on the DNA gels when [α-^{32}P]dCTP and [α-^{32}P]dTTP are used to label the fragments confirms the rationale of the labelling protocol.

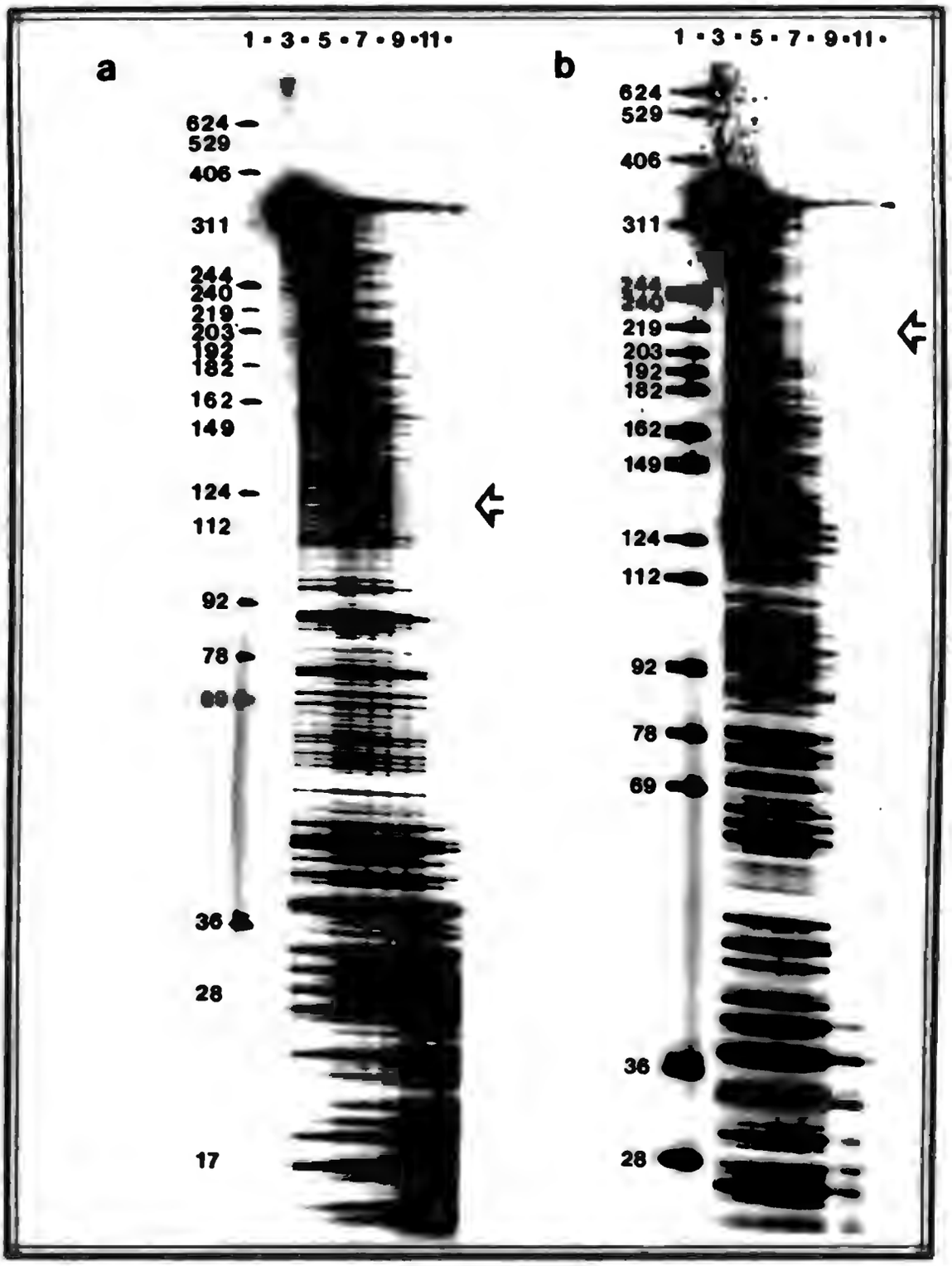


Fig.54
End fragment, DNase I digestion of free DNA.
Denaturing DNA gel electrophoresis of free DNA labelled and digested as follows: Gel a: α^{32}PdCTP. Lane 1: size markers. Lane 2 sample buffer. Lanes 3-12: 1.25 units DNase I/ug DNA for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds. Gel b: α^{32}PdTTP. Lanes 1 and 2: size markers. Lanes 3-12: 1.25 units DNase I/ug DNA for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds.
Arrows: approximate positions of simple sequence motif.

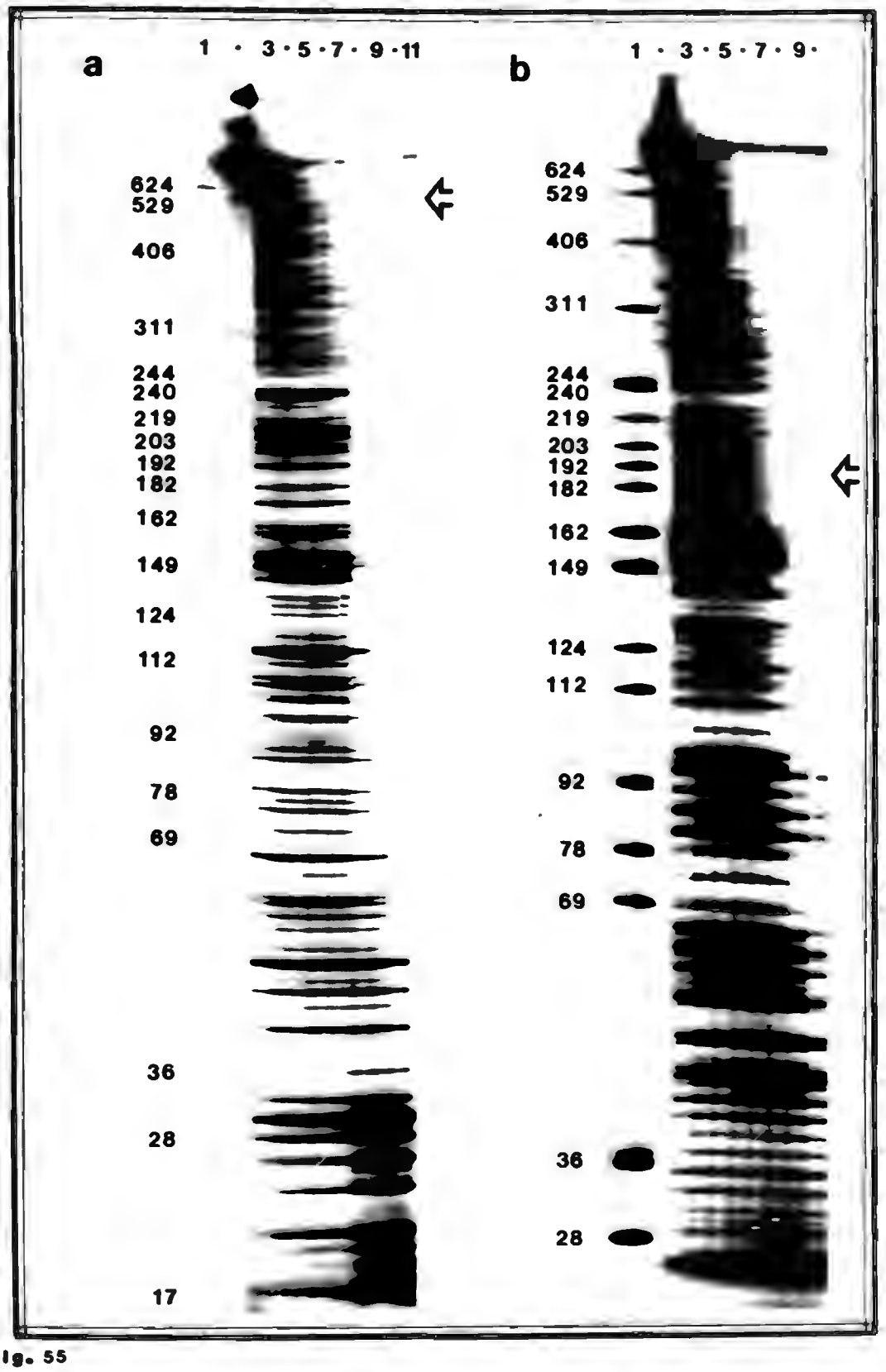


Fig. 55

Upstream fragment, DNase I digestion of free DNA.

Denaturing DNA gel electrophoresis of free DNA labelled and digested as follows: Gel a: [^{32}P]dCTP. Lane 1: size markers. Lanes 2-11: 1.25 units DNase I/ug DNA for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds. Gel b: [^{32}P]dTTP. Lane 1: size markers. Lanes 2-10: 1.25 units DNase I/ug DNA for 0, 15, 30, 60, 90, 120, 240, 480 and 720 seconds. Arrows: approximate positions of simple sequence motif.

The autoradiographs contain a considerable amount of information impossible to assess by visual inspection only. In order to extract this information a scanning densitometer, capable of scanning large autoradiograms at high resolution and the necessary computer programs to analyse the data, was developed in co-operation with other members of the department. To calibrate the densitometer traces each gel must contain a set of DNA size standards. The size standards used consisted of a Hpa II digest of pBR322 labelled by a fill-in reaction using the Klenow fragment, dCTP and [α-^{32}P]dGTP. Using radioactively labelled dGTP as a label avoids the presence of labelled, incompletely filled-in fragments. This fill-in labelling method adds two base pairs to the size of the fragment as calculated from the restriction map of pBR322 (Sutcliffe 1978).

Hpa II digestion site

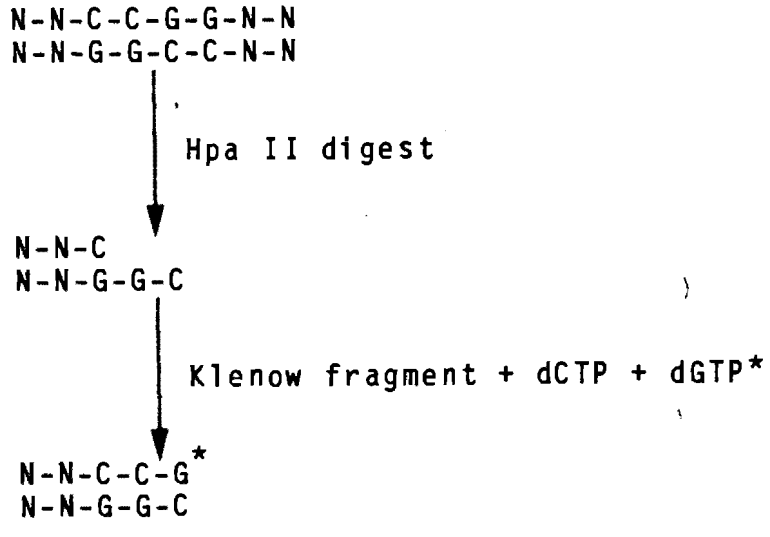


Fig. 56
The labelling of standards by Hpa II digestion and a fill-in reaction using the Klenow fragment of DNA polymerase, dCTP and [α-^{32}P]dGTP.

To calibrate each gel the standards lane was scanned and the distance travelled by each fragment measured.

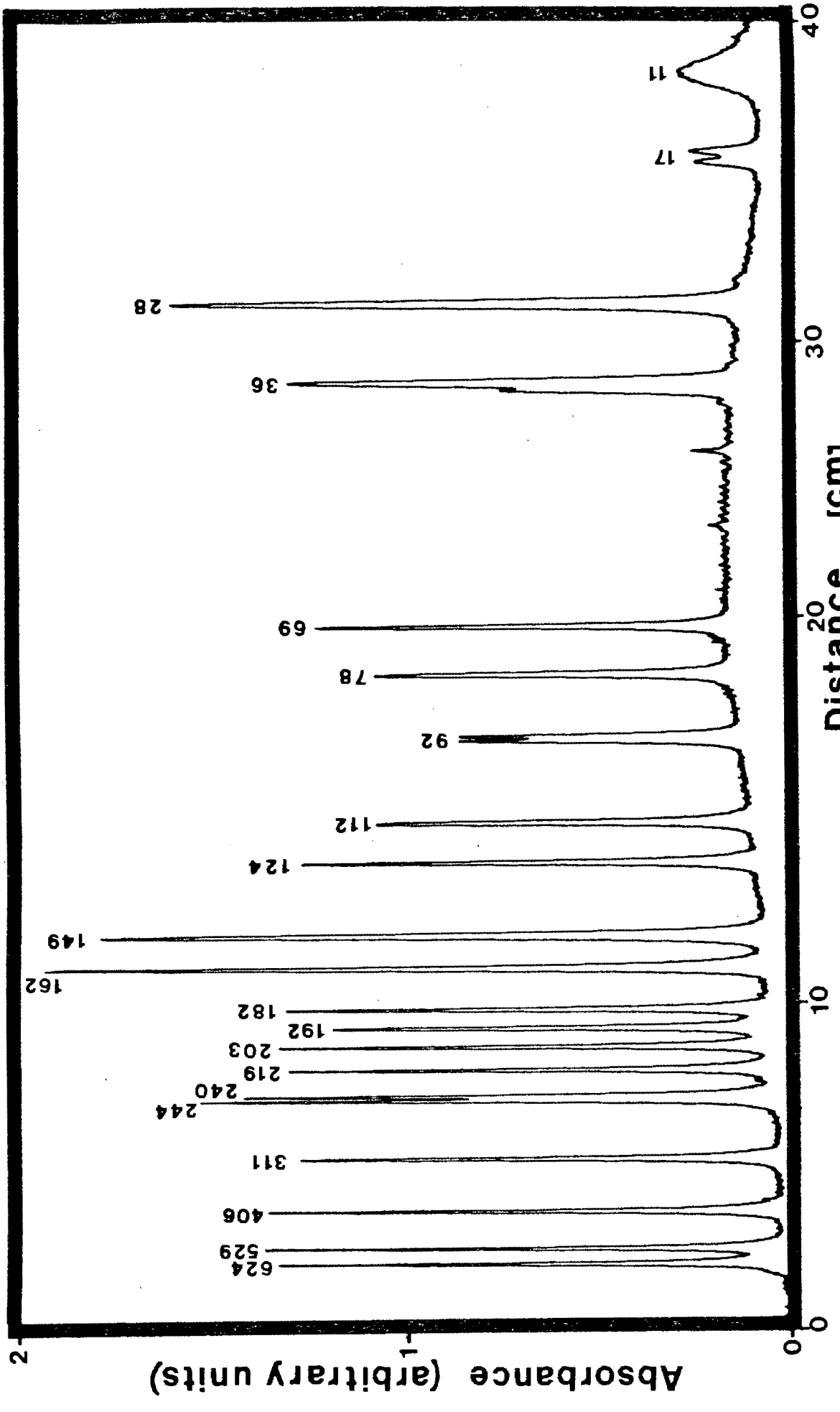


Fig. 57
Densitometer trace of standards lane.
Size markers: Hpa II digest of pBR322 3' fill in end-labelled with [^{32}P]dGTP.
Numbers: The size of each band in base pairs.
Absorbance: Arbitrary units of white light.
The standards lane was scanned and the distance travelled by each band measured to calibrate the gel.

The distance travelled by each fragment was then plotted against the log of the number of bases contained in that fragment. The approximately linear area of these gels is extended due to the varied thickness of the gel (See section 9.3.5). An appropriate polynomial, normally an 8th or 10th order, was chosen to fit the data. This polynomial was then used to calculate the fragment sizes produced by the nuclease digestions.

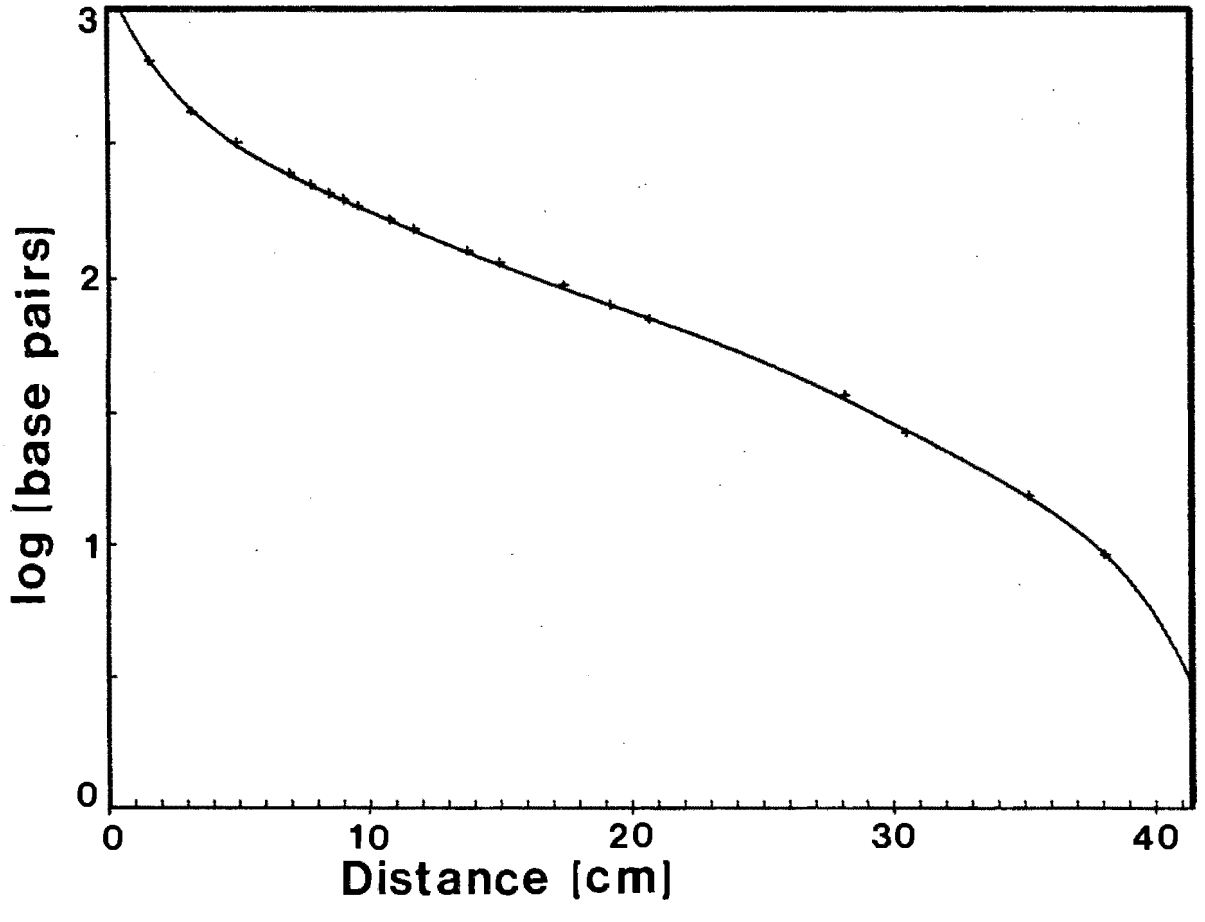


Fig. 58
Curve fit of log(base pairs) versus migration distance.
To calibrate each gel an eighth order polynomial was used to fit the graph of log(size of each standard fragment in base pairs) versus the distance travelled by each fragment.

The calibrations of these gels are only accurate to ±2 bases in the optimal DNA size range. The differences in sequence between the DNA fragments and the size standards cause inaccuracies in the calibration. Such altered mobilities due to sequence differences can be seen in the 92, 36 and 17 base pair size standards (Fig. 57). The inevitable distortions of the gel during washing, fixing and drying also contribute to errors in the 2 bp range. Within each lane the relative distances between peaks can be measured more accurately since the sequences of the fragments that produce the peaks are the same, and their proximity reduces inaccuracies introduced by gel distortions.

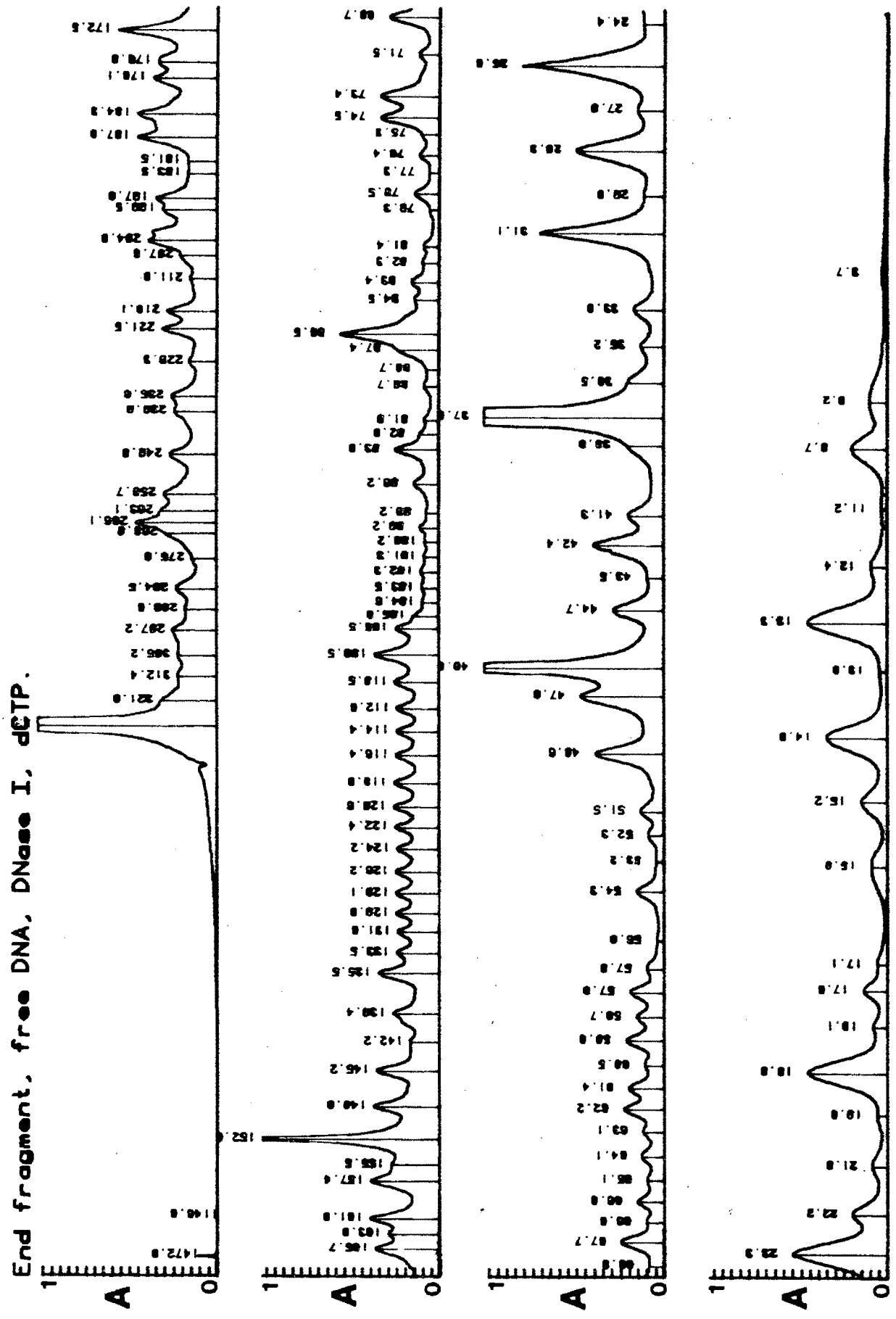
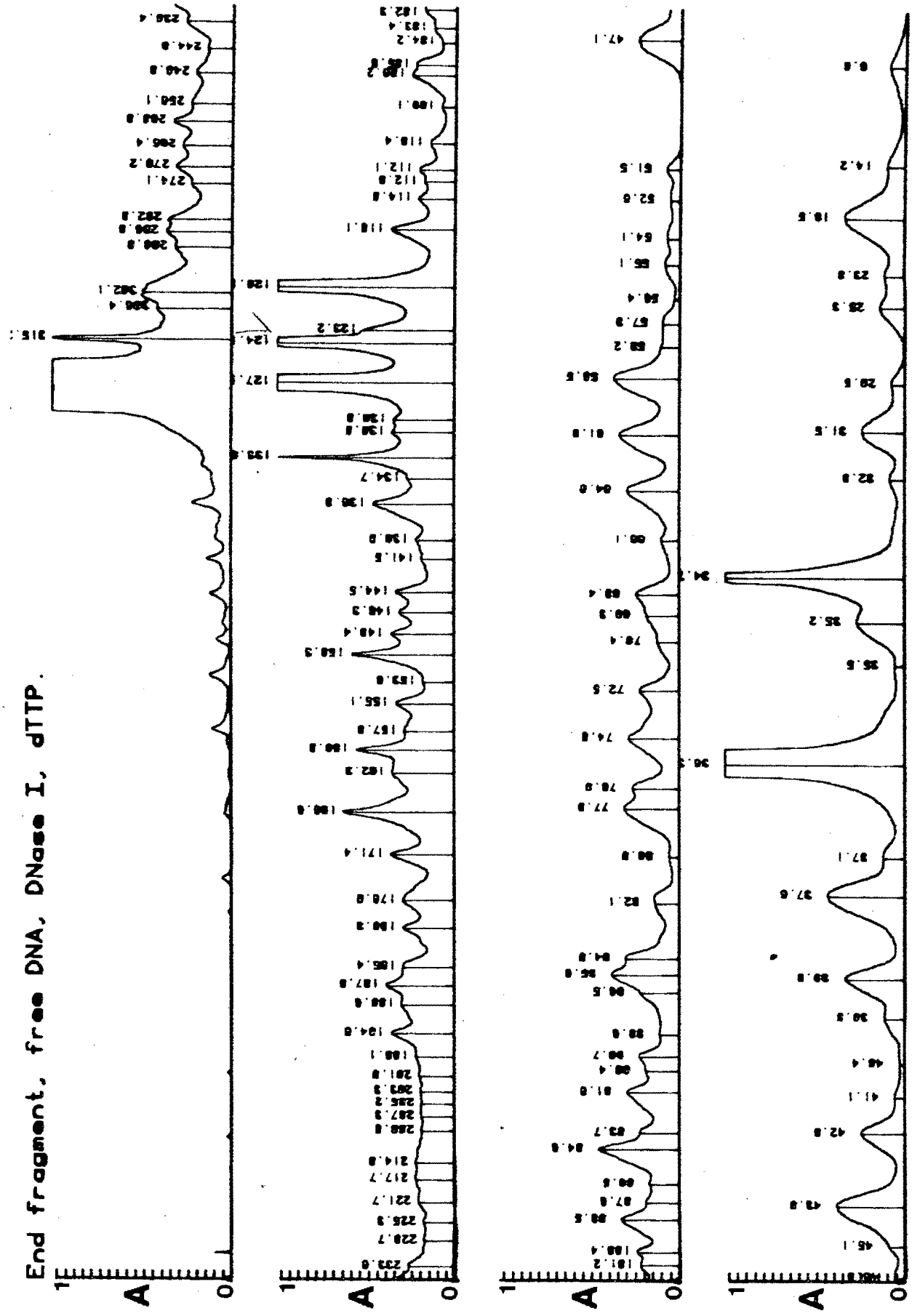
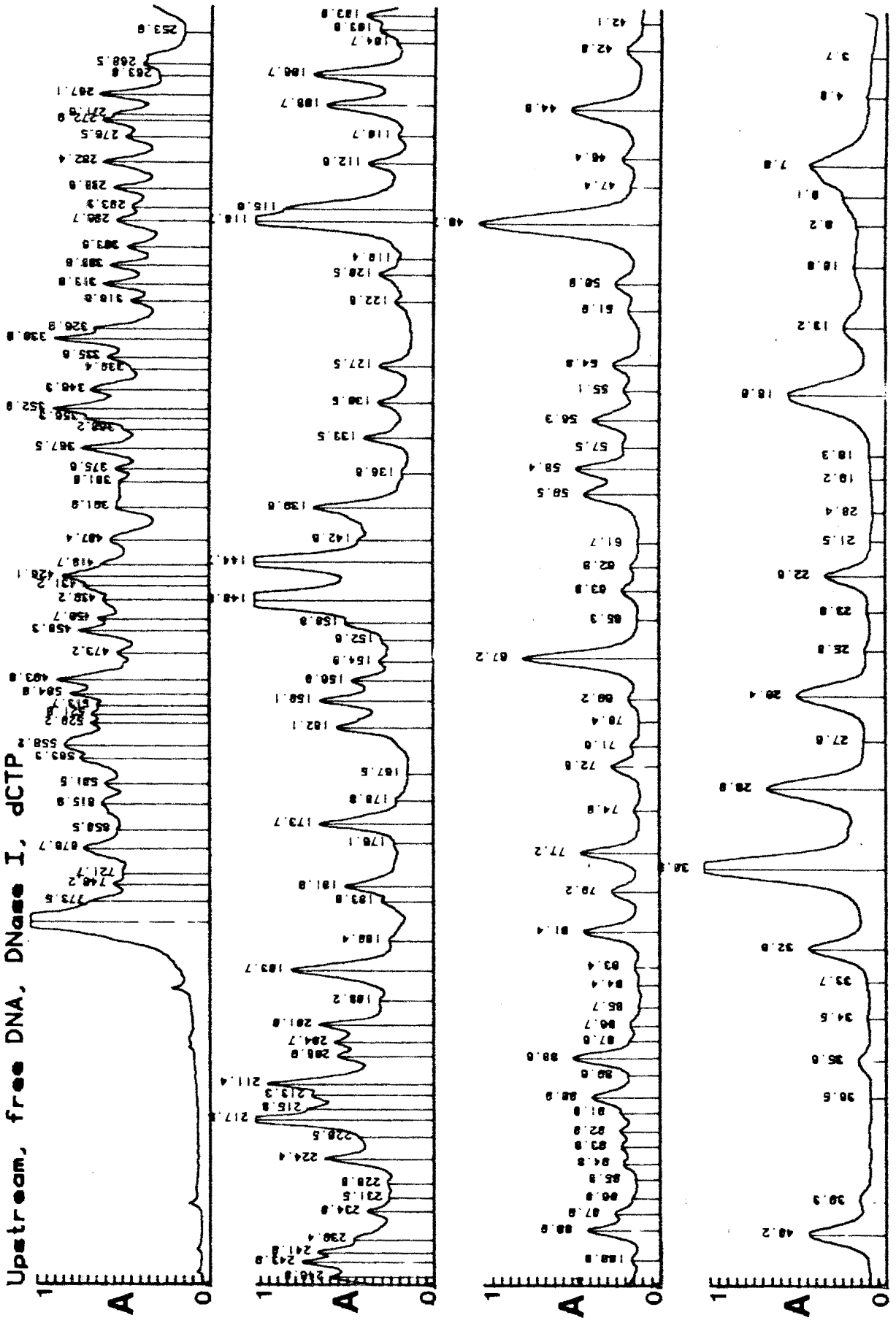


Fig. 59a Densitometer trace of Fig. 54a lane 5.



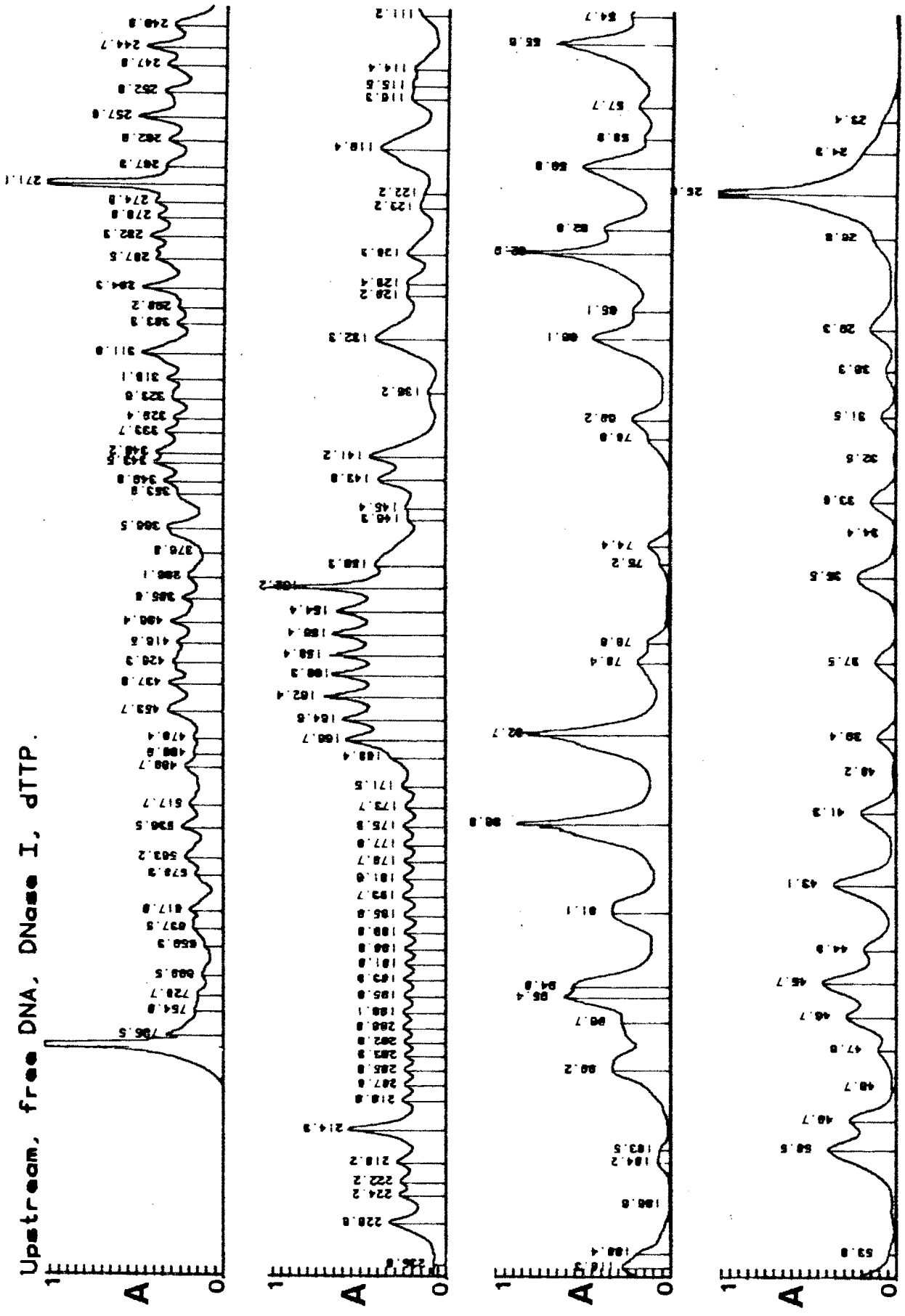
Densitometer trace of Fig.54b lane 5.

Fig. 59b



Densitometer trace of Fig. 55a lane 4.

Fig. 59c



Densitometer trace of Fig. 55b lane 4.

Fig. 59d

A notable feature of the densitometer traces is the transition between the $[d(A\text{-}G)]_{16}$ and $(dG)_{11}$ sequences of the END fragment, where an unexpectedly high peak appears at position 108.5. The simple sequence motifs in the END and UPSTREAM fragments are both flanked by such sites. This may be due to a structural transition or the slippage mechanism (Hentschel 1982). Under the conditions used, the digestion patterns remain the same throughout the time course, indicating that the DNA structure is not changed by the digestion itself. Comparisons made between digestions are therefore not influenced by the degree of digestion. All traces were taken from the 30 second lane. This reduces the contribution of degraded fragments present at zero time. This lane also contains a relatively even yield of fragments over the whole lane, allowing optimal resolution.

Cores were assembled on these excised and labelled DNA fragments. To assess the success of the assembly reaction the assembly mixture was analysed by sucrose gradient centrifugation. The STOP fragment can accomodate one core, the END fragment two cores and the UPSTREAM fragment four cores. Compared to other gradients run under similar conditions (see Chapter 3.3) the radioactive peaks appear in the expected positions on the sucrose gradients. No labelled free DNA appears on the gradients, indicating that all the DNA has been assembled. The lack of aggregates is also indicative of faithful assembly.

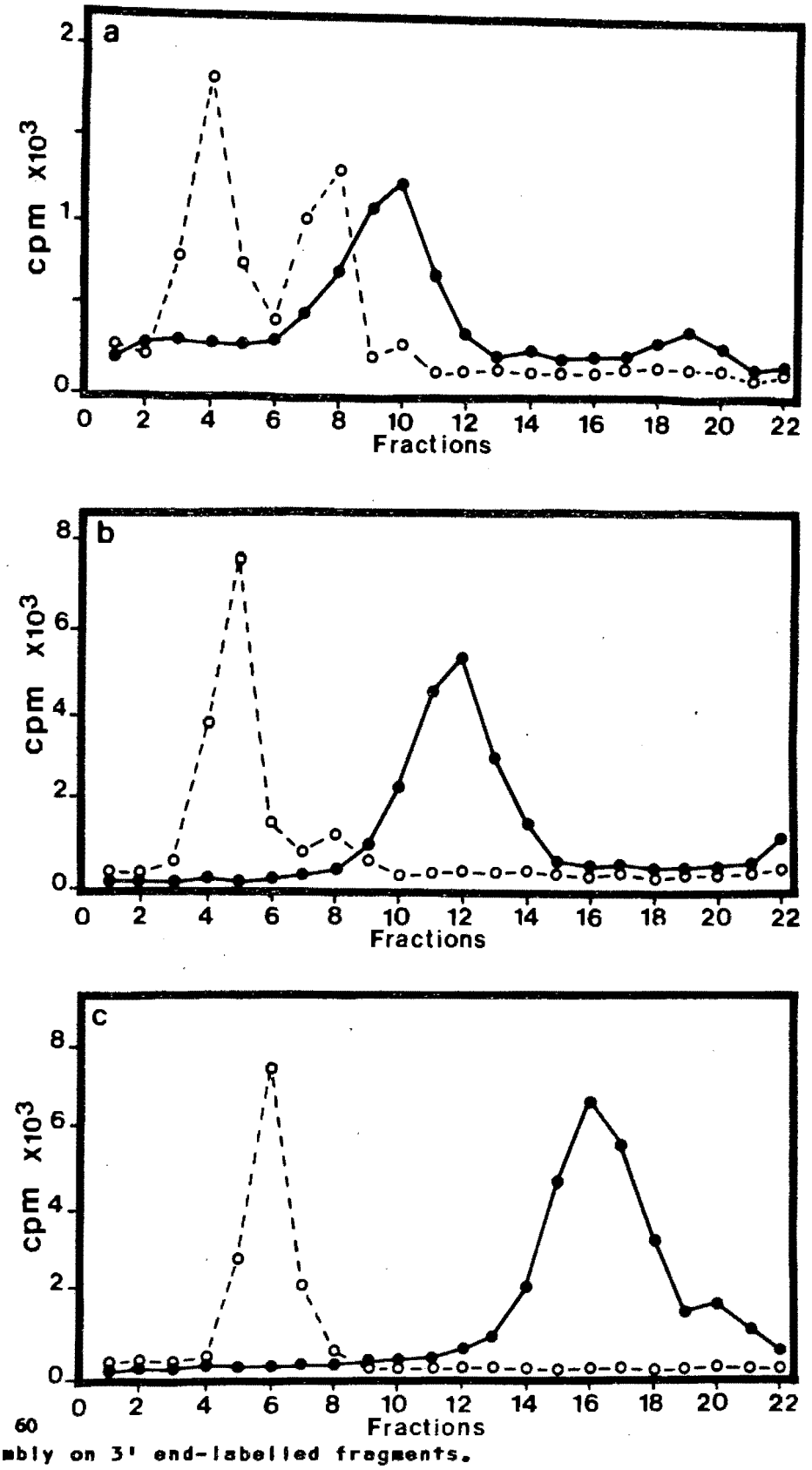


Fig. 60

Assembly on 3' end-labelled fragments.

Gradient: 5 - 20 % (w/v) sucrose. Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.

Assembly conditions:

DNA: (a) Stop fragment
(b) End fragment
(c) Upstream fragment

Histones: Purified by hydroxyapatite and gel exclusion chromatography.
Poly(glutamic acid) size: 50 000 - 100 000 Daltons
Histone to DNA ratio (w/w): 0.64:1
Poly(glutamic acid) to histone ratio (w/w): 2:1
Assembly: 3 hours at 37°C
Free DNA (determined in a separate experiment)
Assembled DNA

7. DISCUSSION OF ASSEMBLY RESULTS

The nuclease digestion protocol for the digestion of free and assembled DNA has been adjusted to produce approximately the same number of cuts per sample, to allow direct lane to lane comparison. This comparison reveals that the digestion patterns of the assembled fragments are different from those of free DNA in two ways:

(1) The rate of DNase I digestion of cores, in all three, fragments is approximately one order of magnitude lower than that of free DNA.

2) There is a periodicity of approximately 10 bp in the cutting preferences in some parts of two of the assembled fragments (For enlargement of Fig. 61 see appendix A; Fig. 1a).

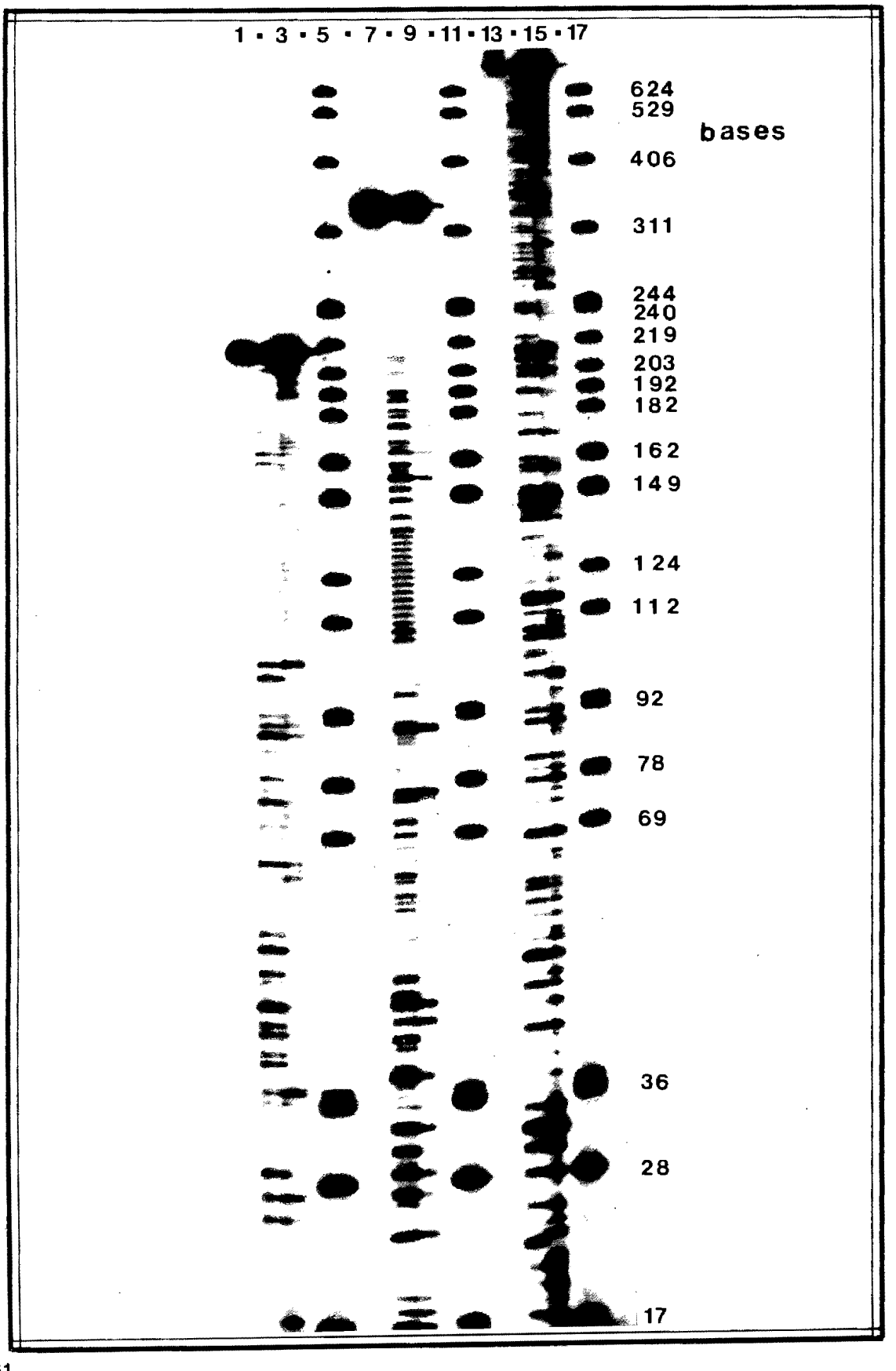


Fig. 61
Denaturing DNA gel of [^{32}P]dCTP labelled fragments.
Lanes 1-3: Stop fragment. Lanes 7-9: End fragment. Lanes 13-15: Upstream fragment. Lanes 1,7 and 13: starting material. Lanes 2,8 and 14: free DNA digested with 1.25 units DNase I/µg DNA for 30 seconds. Lanes 3, 9 and 15: DNA assembled with a histone to DNA ratio of 0.64:1 (w/w) and digested with 12.5u DNase I/µg for 30 seconds. Lanes 5, 11 and 17 size markers. Lanes 4, 6, 10, 12 and 16: sample buffer.

Various controls clearly establish that:

(1) The digestion patterns produced by the free and the assembled DNA are reproducible (Appendix A; Figs. 2 and 3).

(2) In the absence of poly(glutamic acid) the interaction between histones and DNA does not result in the characteristic digestion pattern of the assembled fragments (Appendix A; Fig. 2). It has been shown previously (Fig. 16) that under such conditions assembly does not occur.

(3) The digestion rate of the DNA in such unordered histone-DNA complexes, formed in the absence of poly(glutamic acid), is very low.

(4) The typical DNase I pattern produced after assembly is not due to the presence of poly(glutamic acid) (See appendix A; Fig. 2 and section 9.6.3).

(5) The digestion pattern is not significantly affected by the mobility of the cores during the digestion reaction. The digestion takes place at low ionic strength and temperature, conditions under which mobility is absent or reduced to a minimum (Beard 1978, Weichet 1979, Glotov et al. 1982). This is evident in the digestion kinetics showing a progressive decrease in the undigested DNA with a concomitantly appearing stable pattern of smaller bands. This unchanging pattern indicates that rearrangement of cores (Schlaeger 1981), does not occur (Appendix A; Figs. 2a and 3a).

The fragments were also probed with micrococcal nuclease, though this enzyme is known to be a less sensitive probe for DNA structure than DNase I (Lomonossoff et al. 1981, Cockell et al. 1983, Drew 1984). In accordance with this, the fragments

reveal a relatively featureless digestion pattern. All the DNA appears to be protected to a greater or lesser degree by the histones (see appendix B).

Assembly at a histone:DNA ratio of 0.32:1 (w/w), in order to promote positioning, did not yield significant changes though the overall digestion rate had increased as expected (Appendix A; Fig. 7 and appendix B; Fig. 2).

The periodicity revealed in the densitometric traces (Appendix A) becomes more obvious by displaying the base pair number on the abscissa on a linear scale. This is achieved by taking the polynomial, used for the peak-bp number assignments (Fig. 58), and calculating the positions of these peaks on a linear scale.

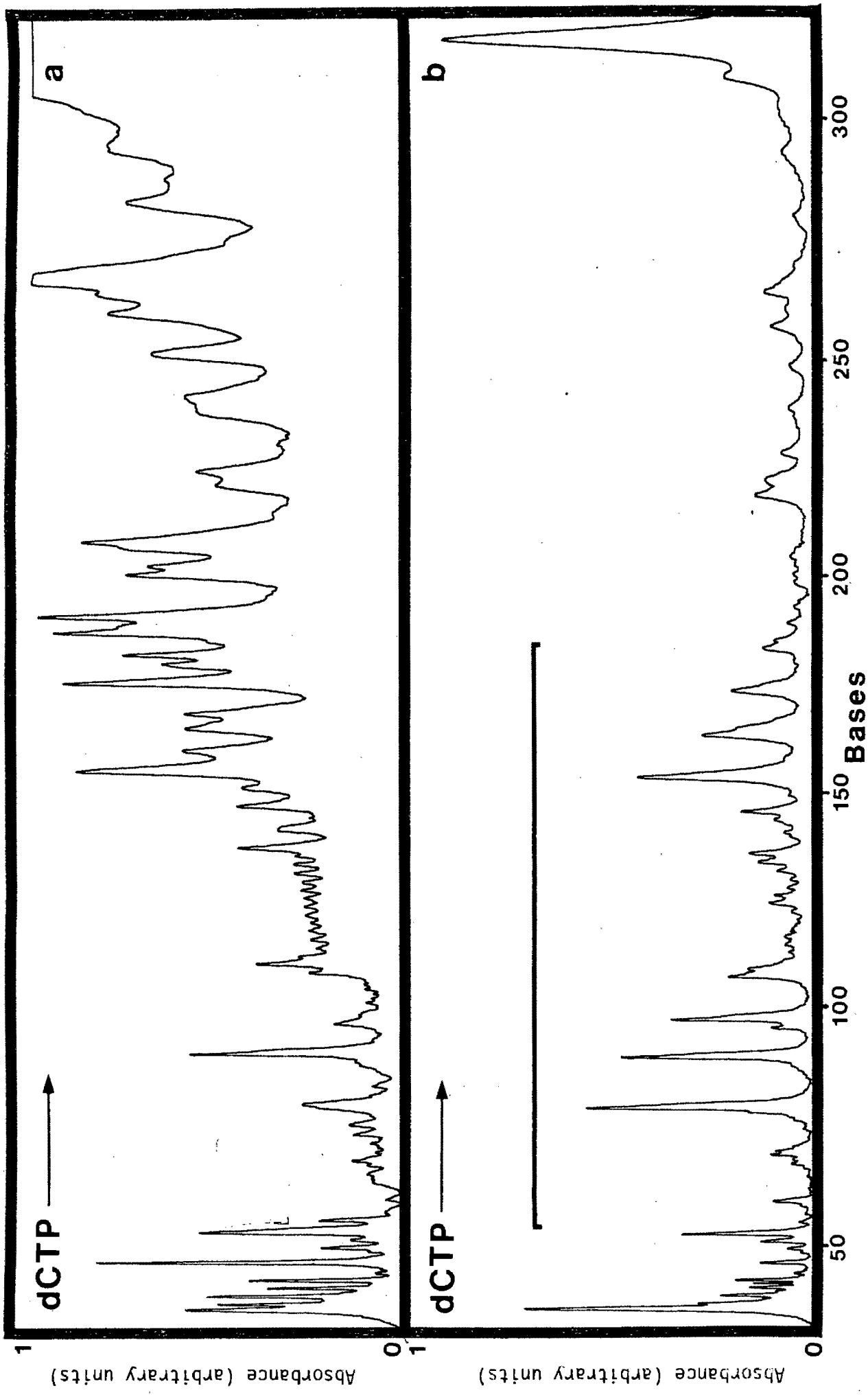


Fig. 62
Linearised densitometer trace of END fragment.
End fragment labelled with (^{32}P)dCTP and digested with DNase I.
Bases: number of bases from the radioactive label. Bracket: approximate position of preferred assembly frame.
(a): free DNA. (b): assembled. The scan was linearised and enlarged.

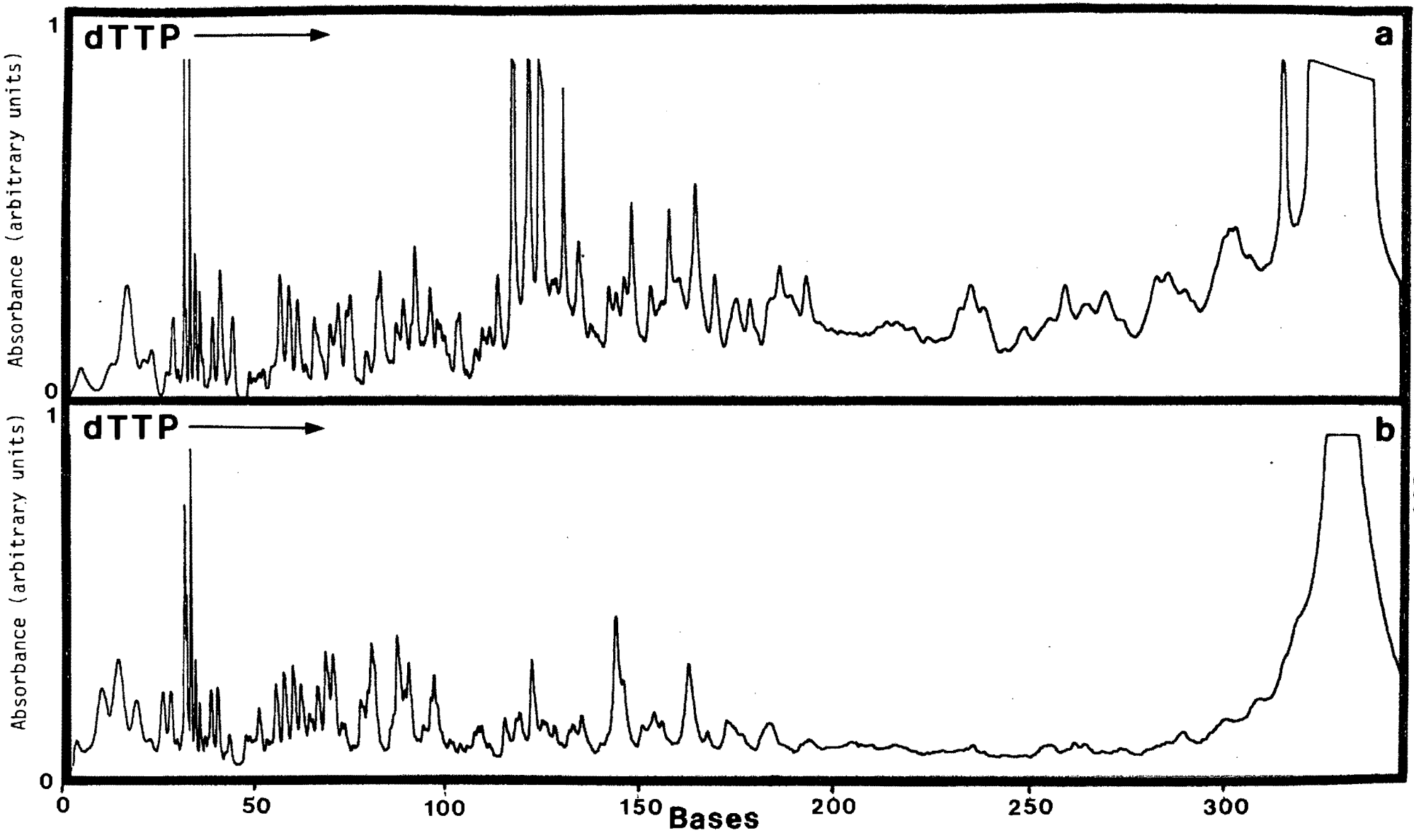


Fig. 63
Linearised densitometer trace of END fragment.
End fragment labelled with α^{32}PdTTP and digested with DNase I.
Bases: number of bases from the radioactive label.
(a): free DNA. (b): assembled. The scan was linearised and enlarged.

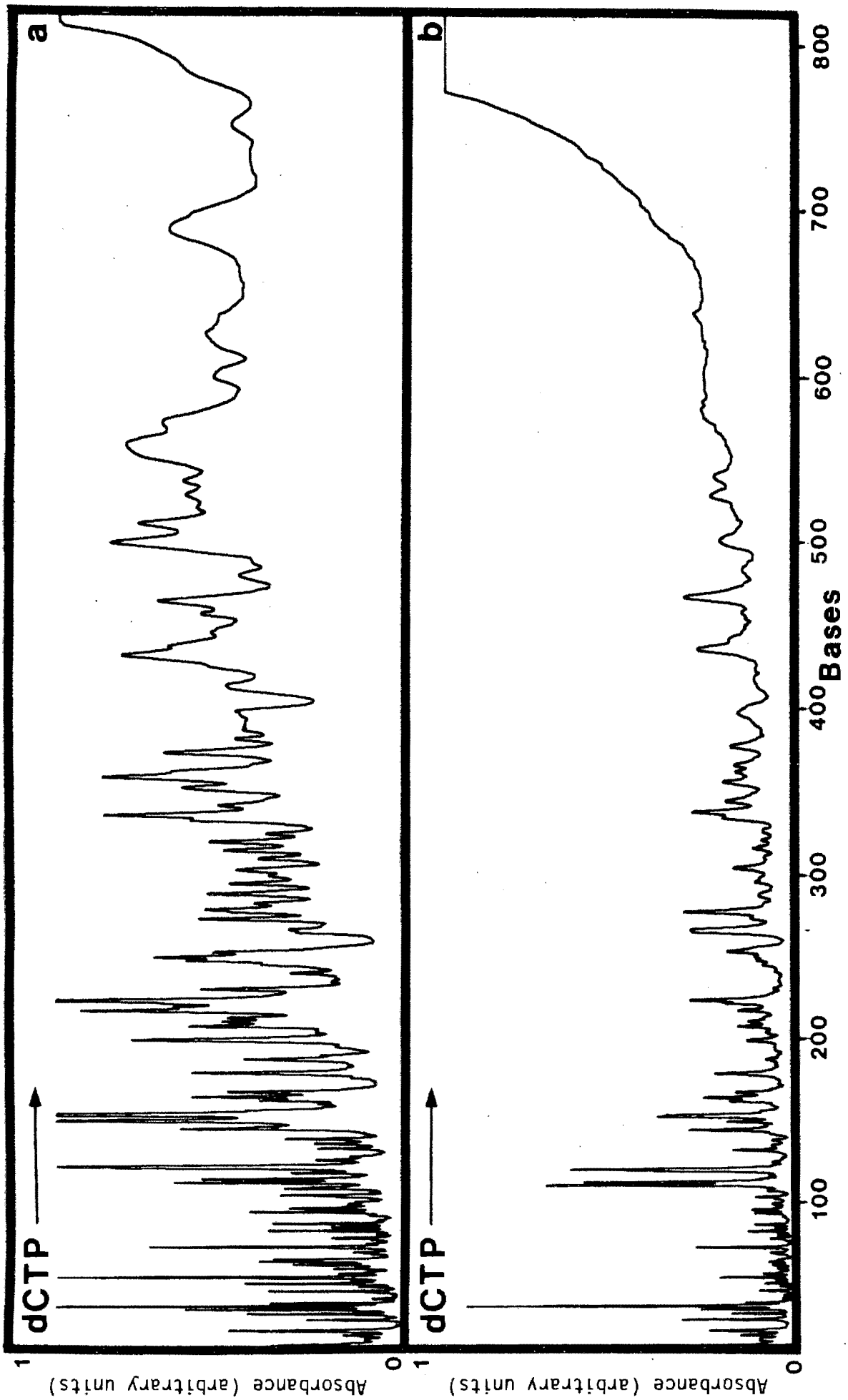


Fig. 64
Linearised densitometer trace of UPSTREAM fragment.
Upstream fragment labelled with [^{32}P]dCTP and digested with DNase I.
Bases: number of bases from the radioactive label.
(a): free DNA. (b): assembled. The scan was linearised and enlarged.

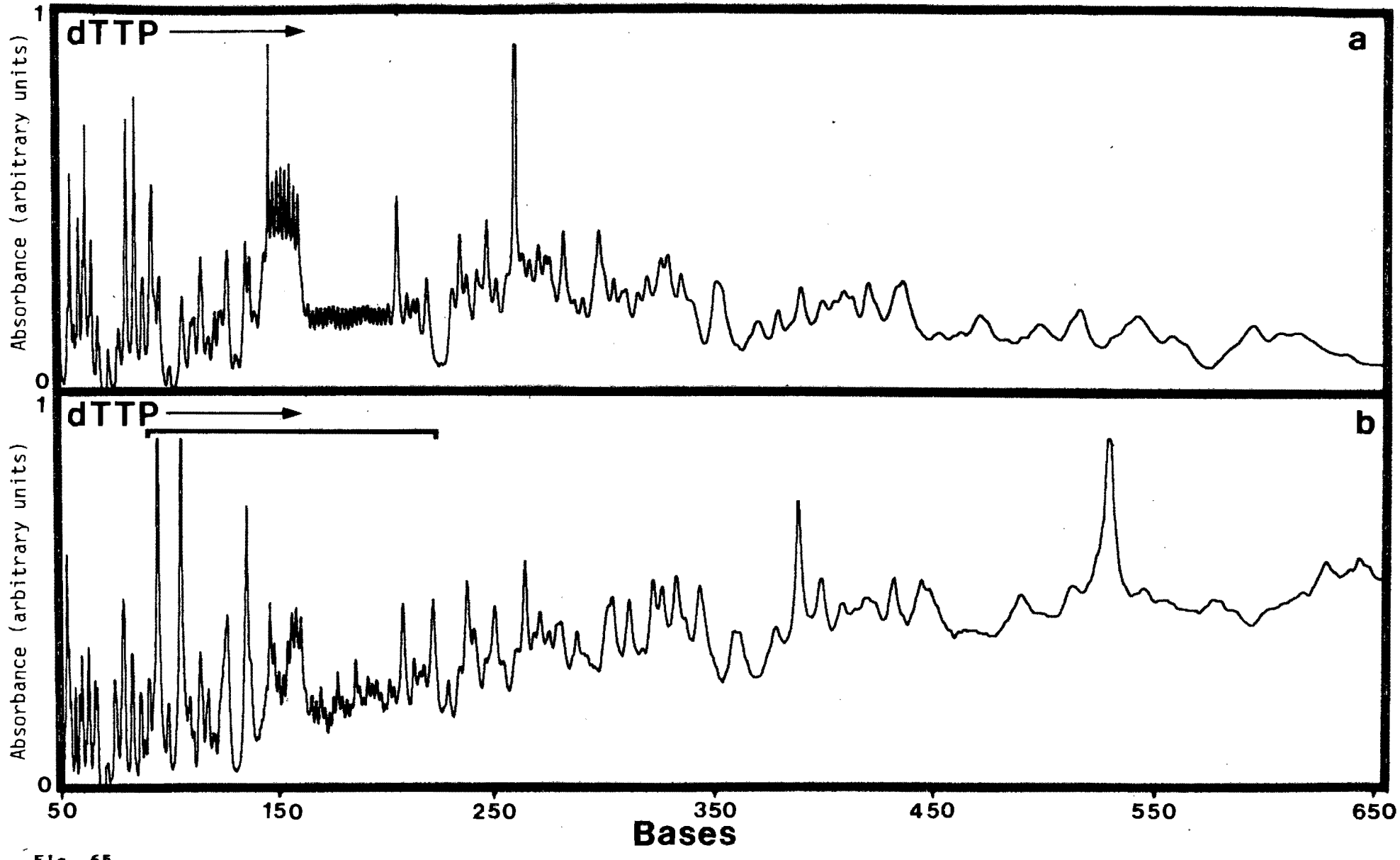


Fig. 65

Linearised densitometer trace of UPSTREAM fragment.

Upstream fragment labelled with [^{32}P]dTTP and digested with DNase I.

Bases: number of bases from the radioactive label. Bracket: approximate position of preferred assembly frame.

(a): free DNA. (b): assembled. The scan was linearised and enlarged.

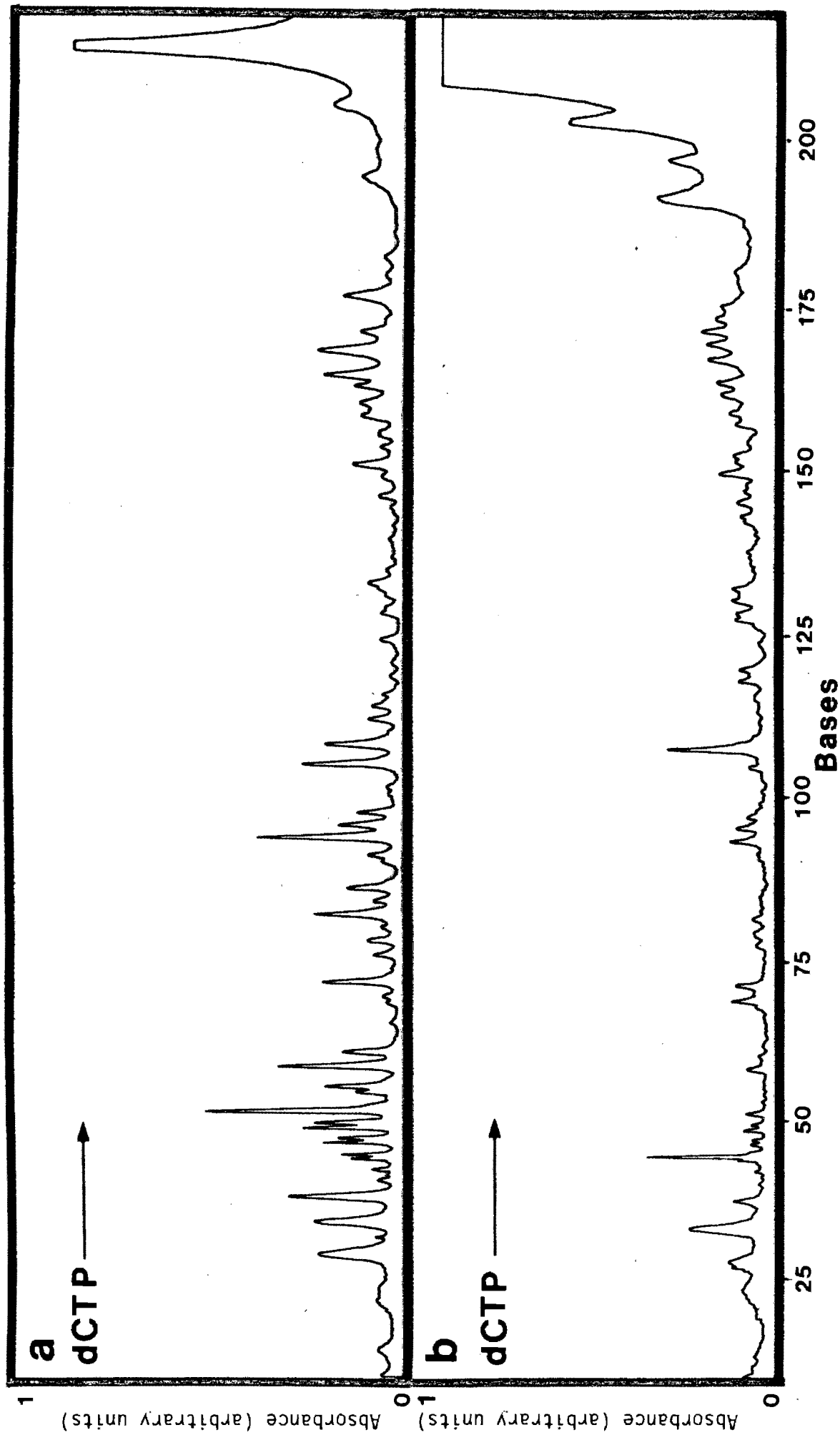


Fig. 66
Linearised densitometer trace of STOP fragment.
STOP fragment labelled with [^{32}P]dCTP and digested with DNase I. Bases: number of bases from the radioactive label.
(a): free DNA (b): assembled The scan was linearised and enlarged.

Once the gels have been linearised periodicity becomes a prominent feature in certain stretches of the assembled DNA. In such areas most of the predominant cuts produced by DNase I digestion appear to be in register. The modulation of the digest of the typical sequence motifs can serve as an orientation point to determine the extent of the repeat both up- and downstream. The linearised presentation in Fig. 62 clearly shows that the DNA of the END fragment has been organised after assembly into a structure exhibiting periodicity in DNase susceptibility. An approximately 10 bp periodicity is particularly distinct in the END fragment between 40-190 bp (Fig. 62), suggesting a higher abundance of cores assembled in this frame in preference to others. For the UPSTREAM fragment (Fig. 65), a periodicity of approximately 10 bp extends from approximately 70-220 bp. The remainder of both fragments, though exhibiting some periodicity does not allow the the size of the period, or the period of a preferential frame to be identified (Figs. 63 and 64). In the STOP fragment (Fig. 66), periodicity in the cutting preference cannot be detected, indicating a random distribution of cores over the length of the fragment.

The monotonous nature of the DNase I digestion pattern of the motifs changes after interaction with histone octamers. This change makes it possible to determine a more accurate value for the repeat. For the END fragment the stretch of alternating bases of the $[d(A\text{-}G)]_{16}$ repeat is used for this purpose. In this sequence repeat, nuclease resistant and sensitive phosphate bonds alternate creating a 2 bp periodicity in the

free DNA. The alternating susceptibility is due to the cutting preferences of the enzyme (Drew and Travers 1984, Drew 1984) (Fig. 67a). For the interpretation of the digestion pattern the following considerations apply: If a susceptible site is placed in an exposed position on the surface of the core, it will result in an optimal rate of digestion at that site. This site is flanked by nuclease resistant phosphate bonds that will remain resistant to nuclease digestion regardless of position. On either side of these resistant bonds, further away from the optimal cutting site, there are two more nuclease sensitive sites that are not placed in optimally exposed positions. Therefore the digestion rate of these bonds will be decreased. The difference in cutting frequency and thus the height of the peaks in the scans will be pronounced (Fig. 67b). Alternatively, if a nuclease resistant phosphate bond is placed in an optimally exposed position on the surface of the nucleosome core, the two flanking nuclease sensitive sites will be equally far away from the optimal site of digestion and the cutting frequency will be low with little difference between them (Fig. 67c). Intermediate positions are possible.

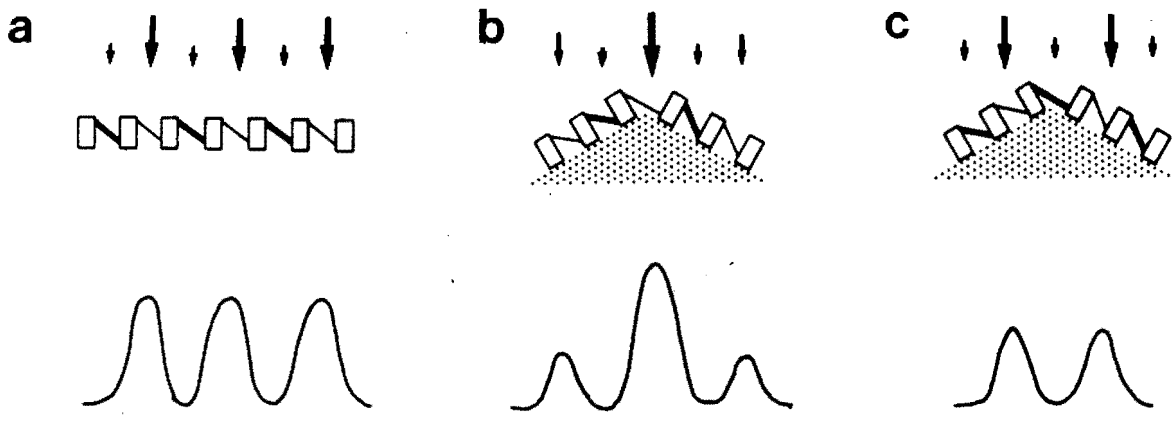


Fig. 67
The effect of position on the core on the digestion pattern of alternating bases.
Squares: Phosphate backbone of an alternating purine sequence.
Thin line: Nuclease sensitive phosphate bond.
Thick line: Nuclease resistant phosphate bond.
Trace: Idealized densitometer trace of nuclease digestion.
Shaded area: Protection provided by the nucleosome core.
(a) Free DNA will produce a featureless alternating pattern.
(b) DNA assembled with the nuclease sensitive site in an optimally exposed position. The difference in hight between peaks produced at that site and adjacent sites will be maximal.
(c) DNA assembled with the nuclease resistant site in an optimally exposed position. The difference in hight between peaks produced at adjacent sites will be minimal.

When a dinucleotide repeat structure comprising at least two turns of the helix associates with an octamer, the DNA can be orientated in two ways with respect to its DNase I susceptible sites. If the DNA repeat is 10 bp, a nuclease sensitive phosphate bond can be placed in an exposed position, then the next exposed position approximately 10 bases further along the helix, will also fall on a nuclease sensitive phosphate bond, resulting in a similar digestion pattern. Alternatively the repeat can be in phase with a nuclease resistant site, again resulting in similar digestion patterns at the beginning and end of the repeat. A number of intermediate positions are also possible. When the repeat is a non-integer, such as 10.5 bp the

appearance of the digestion pattern at the beginning and end of the repeat will differ.

In the $(A\text{-}G)_{16}$ repeat of the END fragment the digestion pattern of the two turns of the DNA can easily be discerned (Fig. 68). The repeat length is approximately 10.5 bp. This repeat has been shown to be typical for the assembly of DNA on the core surface, resulting from the protection of one face of the DNA twisted around the curvature of the nucleosomal core (Prunell et al. 1979, Trifonov and Betteken 1979). Such a situation is clearly present in the motif of the END fragment. The precise sizing of the repeat may depend on the accessability of a particular strand of DNA as seen by the nuclease (Klug and Lutter 1981).

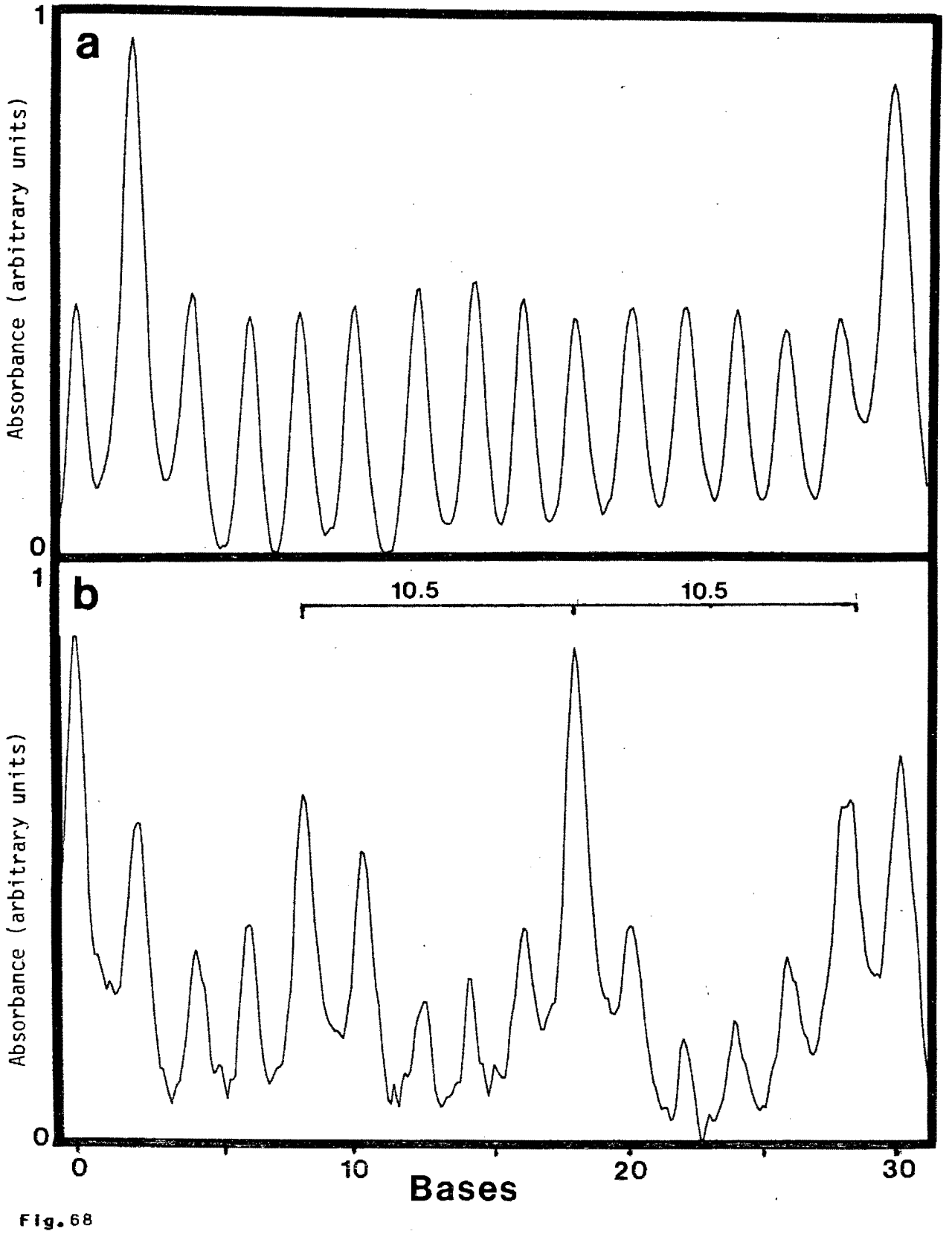


Fig.68
Helix repeat of the End motif:
Linearized densitometer trace of the simple sequence motif of the fragment labelled with dCTP and digested with DNase I.
Bases 1-30 indicate the size of the motif.
(a) Free DNA produce a monotonous pattern.
(b) The assembled fragment produce a 10.5 base pair ladder.
The bracket indicates a nuclease sensitive phosphate bond coinciding with an optimal cutting site with two adjacent 10.5 base pair repeats.

The presence of an apparent periodicity of approximately 10.5 base pairs over the simple sequence motifs does not infer that this periodicity must occur over the whole stretch of putative core positions. Local conformational changes, which are evident in the digestions of free DNA, may influence the repeat. The unequivocal identification of a repeat in a well resolved section of the fragment argues strongly in favour of the presence of a core.

The DNase I digestion patterns of the END and UPSTREAM fragments are best interpreted as the result of two simultaneous core assembly processes competing for positions on the DNA.

The one process is non-specific, the general digestion protection shows that the majority of the DNA has interacted with octamers to result in polycores. The roughly equal preservation of DNA over the entire length argues for the random occupation by cores. However, on this background of what appears to be a random association in the END as well as in the UPSTREAM fragment a second process becomes apparent. Two preferential assembly frames are recognizable, one in END from position 40-180 bp (Fig. 62) and one in UPSTREAM from 80-210 bp (Fig. 65). The area in Fig. 65 from 290-520 bp also appears to show some assembly preference between 250-450 bp judging by an interrupted susceptibility periodicity which gradually fades away towards the 500 bp region. However, in this area the abundance of cores assembled in precise register is

considerably lower than in the other two frames. In the END fragment the transition between random and specific protection occurs on either end of the frame within approximately 10 bp, in the UPSTREAM fragment the transition is less well defined.

In summary then, the "in vitro" assembly of cores on three unique stretches of DNA comprising a total of 1 378 bp has identified two preferred frames of core formation. On the remainder of the DNA the octamers assemble more or less randomly, though in some areas assembly in register appears to occur more frequently than in others.

Since the structure of the octamer is invariant, any selectivity with respect to the interaction between octamers and DNA must rest in the DNA. In an attempt to gain insight into the role the structural diversity of the DNA may play in the specificity or otherwise of the assembly, the potential of the DNA to assume different conformations needs consideration. When a single core is assembled, the bending of the DNA takes place in one plane only, resulting in the protection of one face of the DNA. To allow cores to assemble at equal rates randomly over the whole fragment, on all faces of the DNA, the DNA must be capable of bending in all directions equally well. Constraints in the flexibility of the DNA structure that restrict the possible number of directions in which the DNA will bend may result in a preference as to the face of the DNA and the locus of interaction with the core. This aspect of positioning has been discussed in detail by Trifonov (1980).

The flexibility of the DNA, and thus its conformation, is determined by the base stacking. A considerable amount of information on the influence of neighbouring base pairs on the DNA structure has been obtained from the crystal structures of double stranded synthetic oligo nucleotides (Dickerson and Drew 1981, Wang et al. 1982 and Sakked et al. 1983). From this information, Calladine's rules have been defined in terms of steric hinderance, or clash, between purines on opposite strands of the helix at adjacent base pairs (Calladine 1982). This clash can be relieved by flattening down the propeller twist (Fig 69A), opening up the roll angle (Fig 69B), decreasing the local helix twist angle (Fig. 69C) and sliding the purines out of the base stack, thus increasing the main chain torsion angle on the purine side of the base pair (Fig. 69D) (Dickerson 1983).

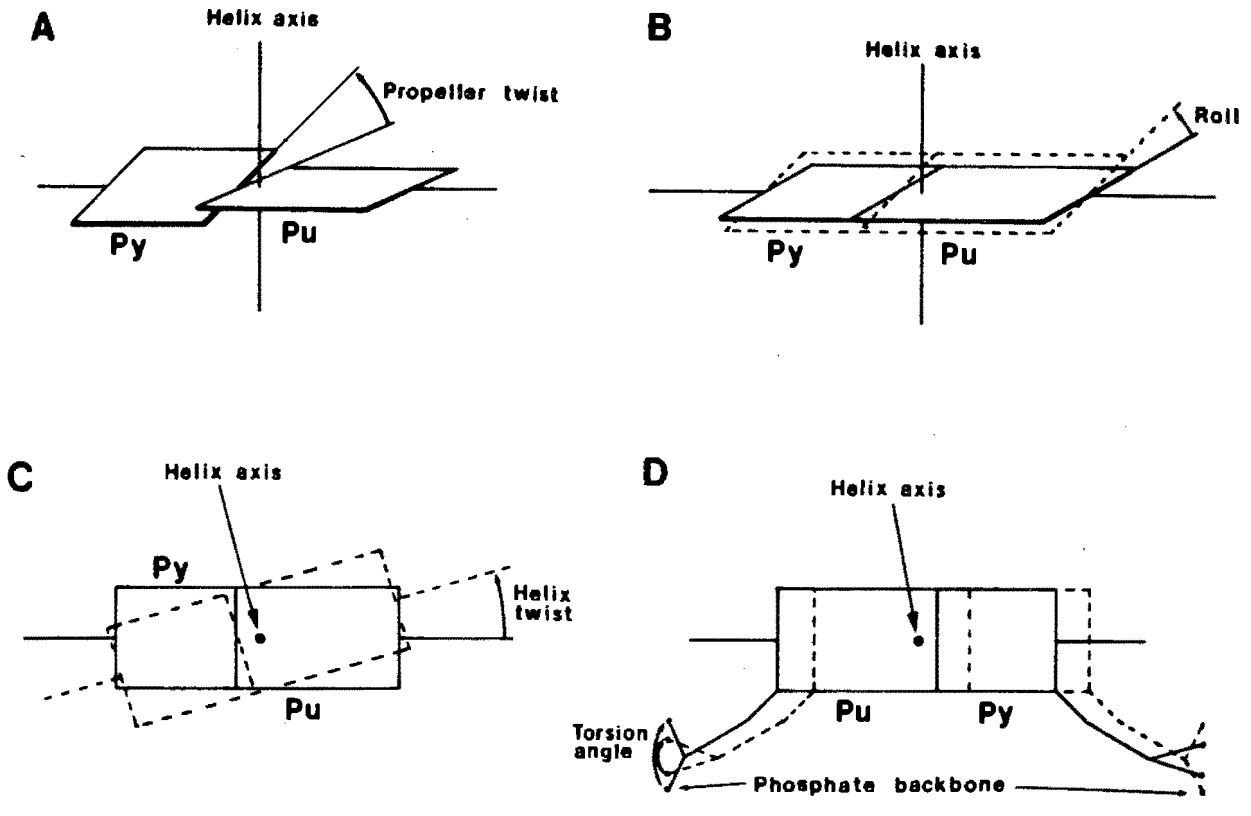


Fig. 69
Schematic drawing of a base pair:
The helix axis runs perpendicularly through each purine.
(A) Perspective view of the propeller twist.
(B) Perspective view of the roll angle.
(C) View along the helix axis of helix twist.
(D) View along the helix axis of the torsion angle.
Py: Pyrimidine
Pu: Purine

It has also been taken into account that clashes of this nature will influence neighbouring bases. Since only pyrimidine-purine or purine-pyrimidine clashes contribute to these angles, they can be simply calculated from the sum functions (Fig. 70).

	R - R - Y - Y - Y - Y - R - R
Σ_1(twist)	+1 -2 +1 +2 -4 +2
Σ_2(roll)	+1 -2 +1 -2 +4 -2
Σ_3(torsion)	+1 -1 -2 +2
Σ_4(propeller twist)	-1 -1 -2 -2

Fig. 70
Elements of sum functions for purine-pyrimidine (R-Y) and pyrimidine-purine (Y-R) base steps (Dickerson 1983).

These predicted values have been related to actual angles determined by single crystal X-ray analysis (Dickerson 1983). I have calculated these sum functions for the UPSTREAM, END and STOP fragments and also for the 5S RNA fragment which has been used by Simpson and Stafford (1983) for "in vitro" assembly (Appendix C).

The roll angles are of particular interest since these reflect possible kinked areas in the DNA (Zhurkin 1983) and are presented in Figs. 71 - 73. (For further structural analysis see appendix C).

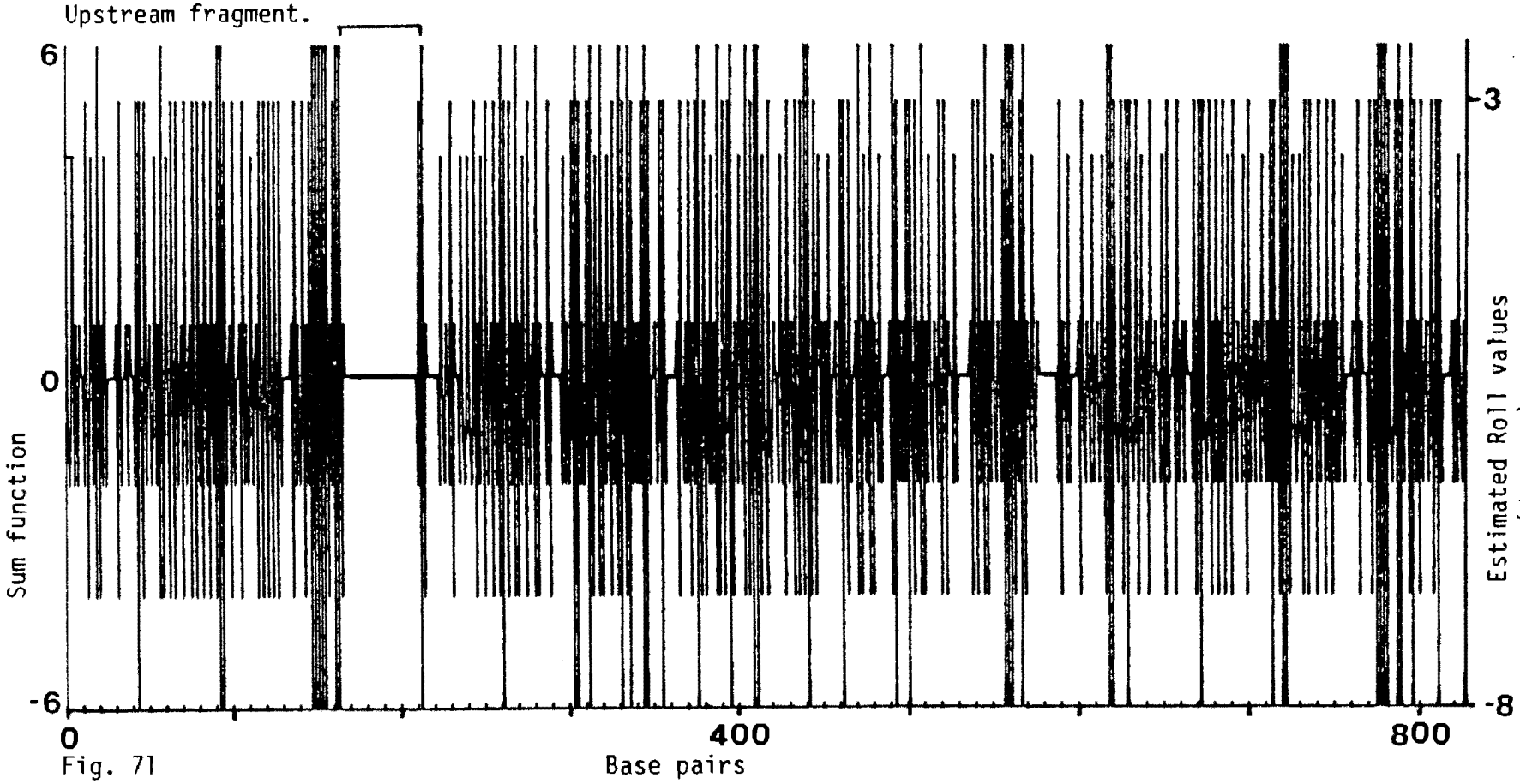


Fig. 71

Sum function for Roll values applied to the UPSTREAM fragment.
Bracket: Sequence motif.

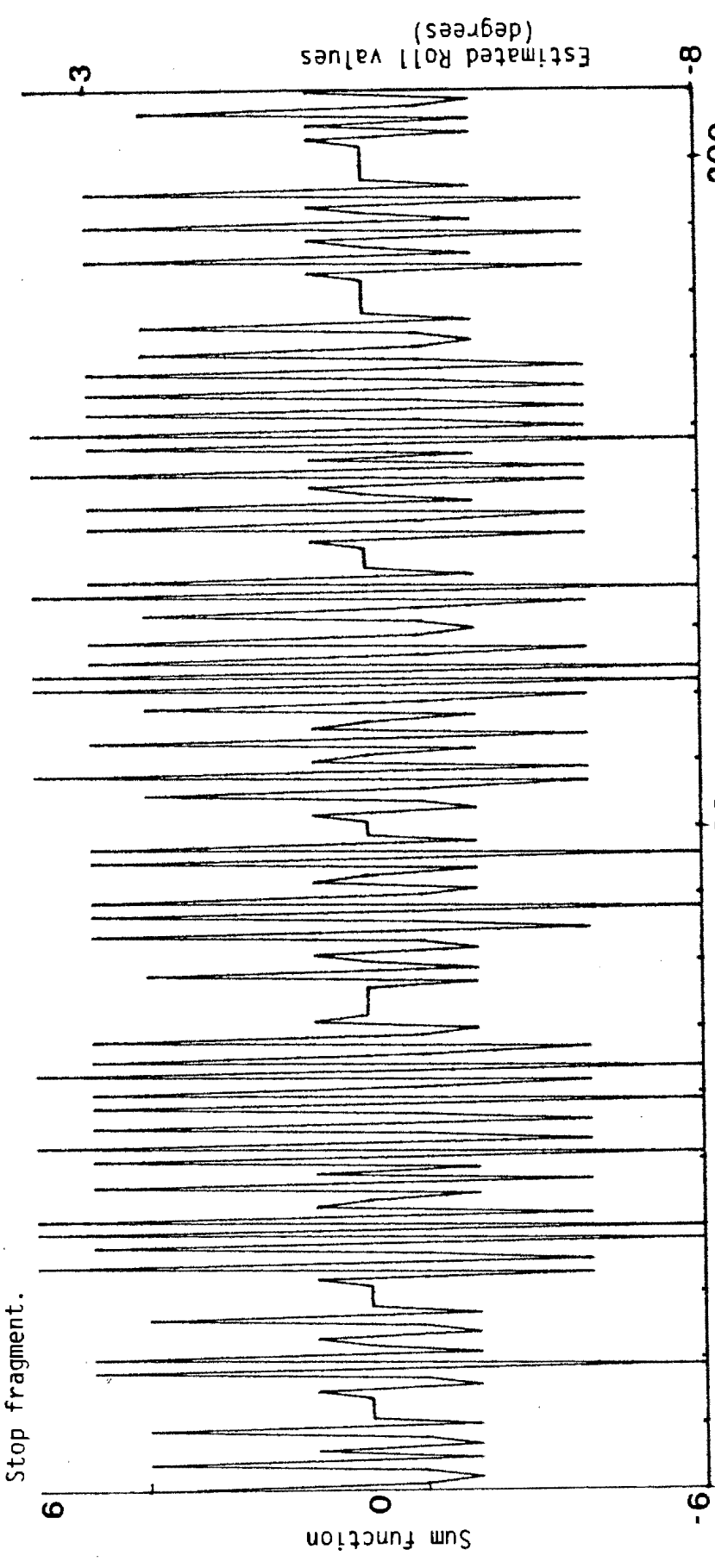


Fig. 72
Sum function for Roll values applied to the STOP fragment.

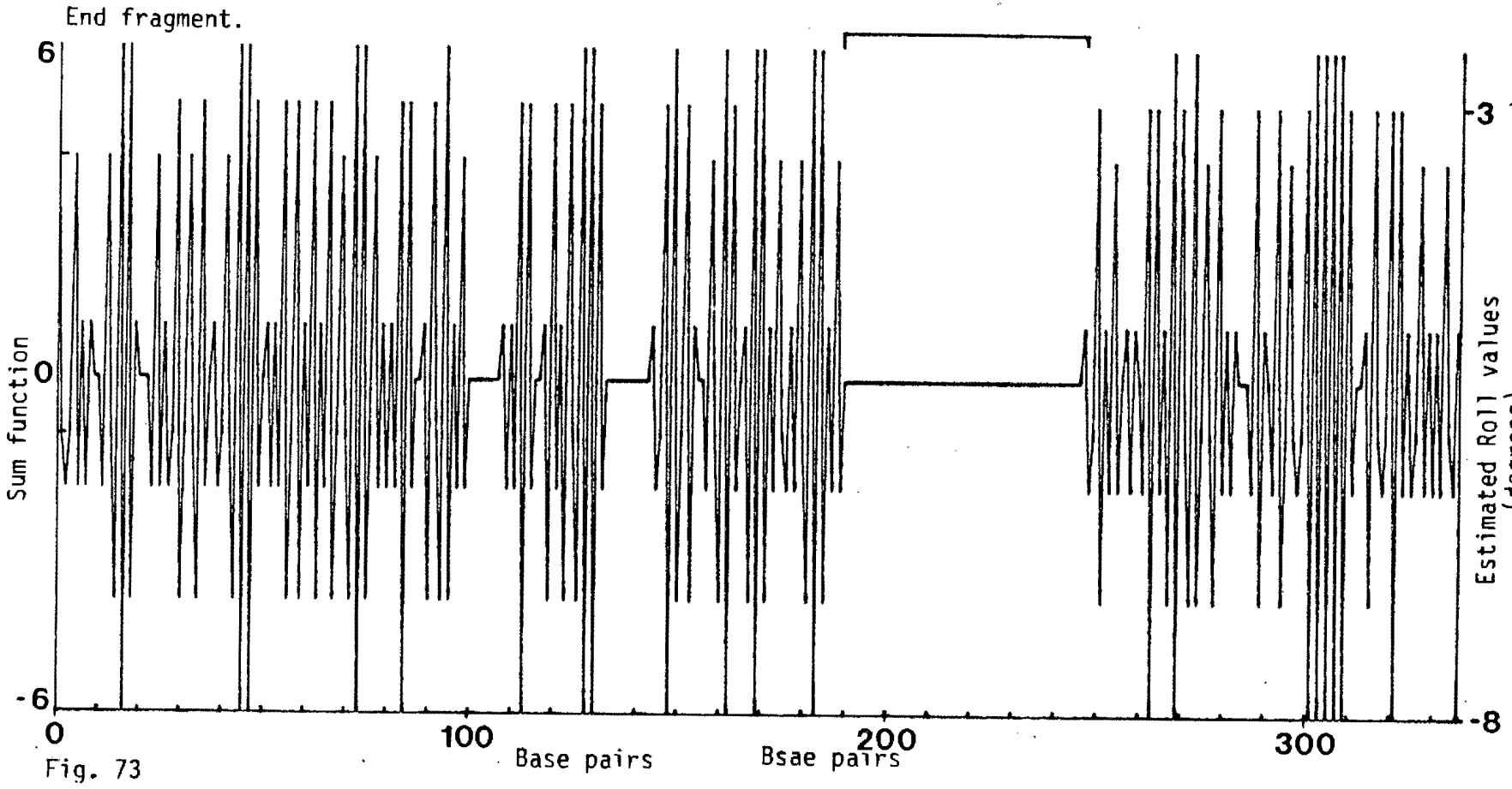


Fig. 73

Sum function for Roll values applied to the END fragment.

Bracket: Sequence motif.

The motifs are striking features of the two fragments. The high level of base stacking and consequently low level of steric hinderence, produced by the poly(d[purine]).poly(d[pyrimidine]) stretch should result in a very rigid structure. These putatively inflexible domains are near highly flexible stretches of DNA, as reflected in the high level of steric interaction and consequently large changes in the roll angles of these predominantly alternating purine-pyrimidine stretches. The UPSTREAM motif is preceded by $(C\text{-}A)_{10}$, also apparent from the nuclease susceptibility (Appendix A; Fig. 6a and b between size markers 149 and 162 bp). In the END fragment a flexible stretch $(pyrimidine\text{-}purine)_8$ is 50 bases removed from the motif. Such sequences with the potential for Z-DNA formation are relatively rare and occur with only 60% of the expected frequency in most DNA sequences (Trifonov et al. 1985). Visual inspection does not reveal any further evidence of ordered structures.

Zhurkin (1984) suggested that a number of strategically located kinks in the DNA, with a 10 or 10.5 bp periodicity over 145 base pairs, may serve to locate the core. The criteria necessary to position the nucleosome core can be calculated as follows:

For an arbitrary sequence [A1], [A2],... [An] the bendability of a 146 base pair fragment starting at [Am] is determined.

For a 10.0 bp period:
f(m)=the sum of Y-R dimers located in positions [m + 3], [m + 13],.. [m + 143] plus the number of R-Y dimers located in [m + 8], [m + 18],.. [m + 138].

For a 10.5 bp period:
f'(m)=the sum of Y-R dimers located in positions [m], [m + 10], [m + 11], [m + 21], [m + 31], [m + 32],.. [m + 147] plus the number of R-Y dimers located in [m + 5], [m + 6], [m + 15], [m + 16],.. [m + 142].

R= purine
Y= pyrimidine

To interpret these results it is necessary to note that: a R-Y dimer is located in position m if dinucleotide [Am]-[Am + 1] is purine-pyrimidine. The maximal possible values of these functions are 29. For a random sequence f(m)= 7.25 and f'(m)= 12.5. The maxima of the functions correspond to the "left" border of the most favourable core positions and only An - 146 base pairs of the fragment can be analysed (For complete analysis see appendix D).

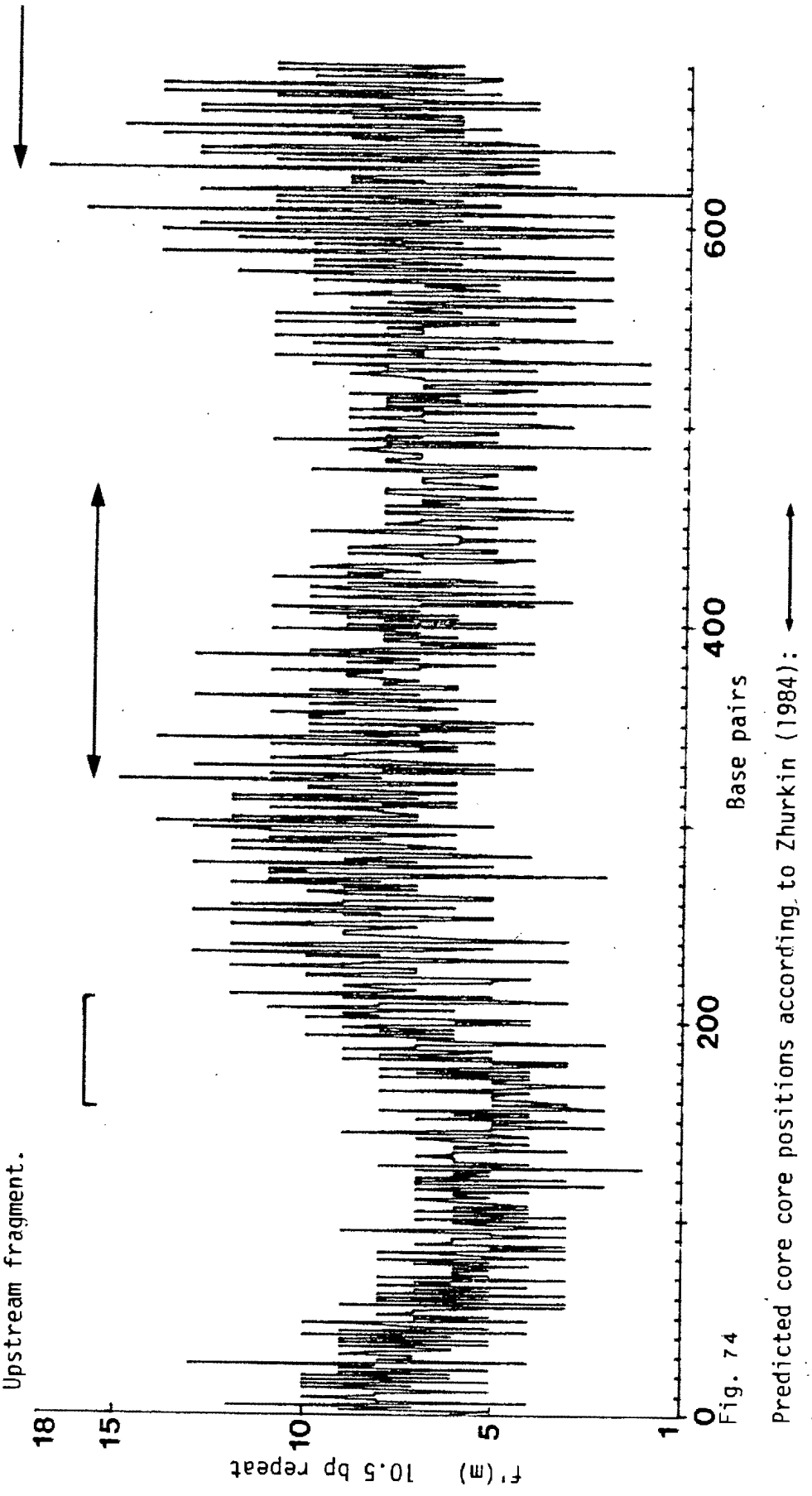


Fig. 74

Predicted core core positions according to Zhurkin (1984): ⟷
Bracket: Sequence motif $(C\text{-}T)_{23}$

By those functions the strongest bends are at approximate positions 320 and 630 of the UPSTREAM fragment (Fig. 74). From Figs. 64 and 65, as well as appendix A; Figs. 5 and 6, it is clear that cores are not positioned preferentially in these positions. It has been reported in the literature that a 260 bp section of the 5S RNA gene of L. variegatus accomodates in a unique position a nucleosome core under assembly conditions similar to those used in these experiments (Simpson and Stafford 1983, Simpson et al. 1985). If this gene sequence (Lu et al. 1980), is subjected to the same analysis, as END and UPSTREAM fragments, it is also not possible to locate regions with strong bending potential (appendix D; Figs. 7 and 8).

The structural predictions considered up to now are based on steric interactions of purine-pyrimidine bases to produce bends in the DNA. The motifs in the END and UPSTREAM fragments are identified by this type of conformational analysis as inflexible (Fig. 71 and 73) and therefore should not be part of a DNA region qualifying for preferential core positions (Fig. 74). However, the digestion patterns clearly identify an approximately 10 bp repeat in the motifs of both fragments. In the END fragment this can be measured to have the value of approximately 10.5 bp. This strongly argues for the existence of cores in these areas. It thus appears that the application of Calladine's rules or Zhurkin's functions, which assume an approximately 10 bp periodicity in the sequence, cannot establish correlations between potential DNA conformations in solution and core positions of "in vitro" assembled cores.

The DNA may contain latent information that is not apparent from our present knowledge of DNA structure. It is well established that the DNA on the surface of the nucleosome core is distorted and the stacking of the base pairs disrupted. Such disruption can be seen in the high resolution crystal structure of the nucleosome core (Richmond et al. 1984) and is also reflected in the NMR spectrum of the core particles (McMurray and van Holde 1985). It is not possible to decide whether these local disturbances are the result of the interaction of the DNA with the octamer, or the primary cause which directs the assembly of the core in that area of the DNA since stresses similar to those present in nucleosome cores can be imposed on the DNA by supercoiling. There is ample evidence that DNA structural transitions can be induced by supercoiling, which in turn may affect nucleosome core placement (Lilley 1980, Panayatatos and Wells 1981, Peck et al. 1982, Weintraub 1983, Villeponteau 1984, Drew et al. 1985, Johnston and Rich 1985, Khowi-Shigematsu 1985). Similarly, the clustering of positive charges on the surface of the octamer may well also have a conformation inducing effect on the DNA. Spermine, for instance, can induce the formation of Z-DNA (Wang et al. 1979 and Russel et al. 1983).

Trifonov and Sussman (1980) have correlated the frequency of dinucleotides in the DNA with chromatin structure. The clustering of neighbouring purines on the one and pyrimidines on the other DNA strand is considered to provide a "wedge" type of conformation of neighbouring base planes which in turn

induces a unidirectional curvature and thus directs the position of the core (Trifonov and Sussman 1980, Trifonov 1980, Mengeritsky and Trifonov 1983, 1984). Wartenfeld et al. (1984) have found a 10.3 bp periodicity of neighbouring purine-purine and A-A bases in the sequences of nucleosomes that have been accurately mapped experimentally in natural chromatin. Both END and UPSTREAM fragments have been monitored by Fourier transforms for such a periodicity in the occurrance of purine-purine doublets. No strong, approximately 10 bp, periodicity has been found.

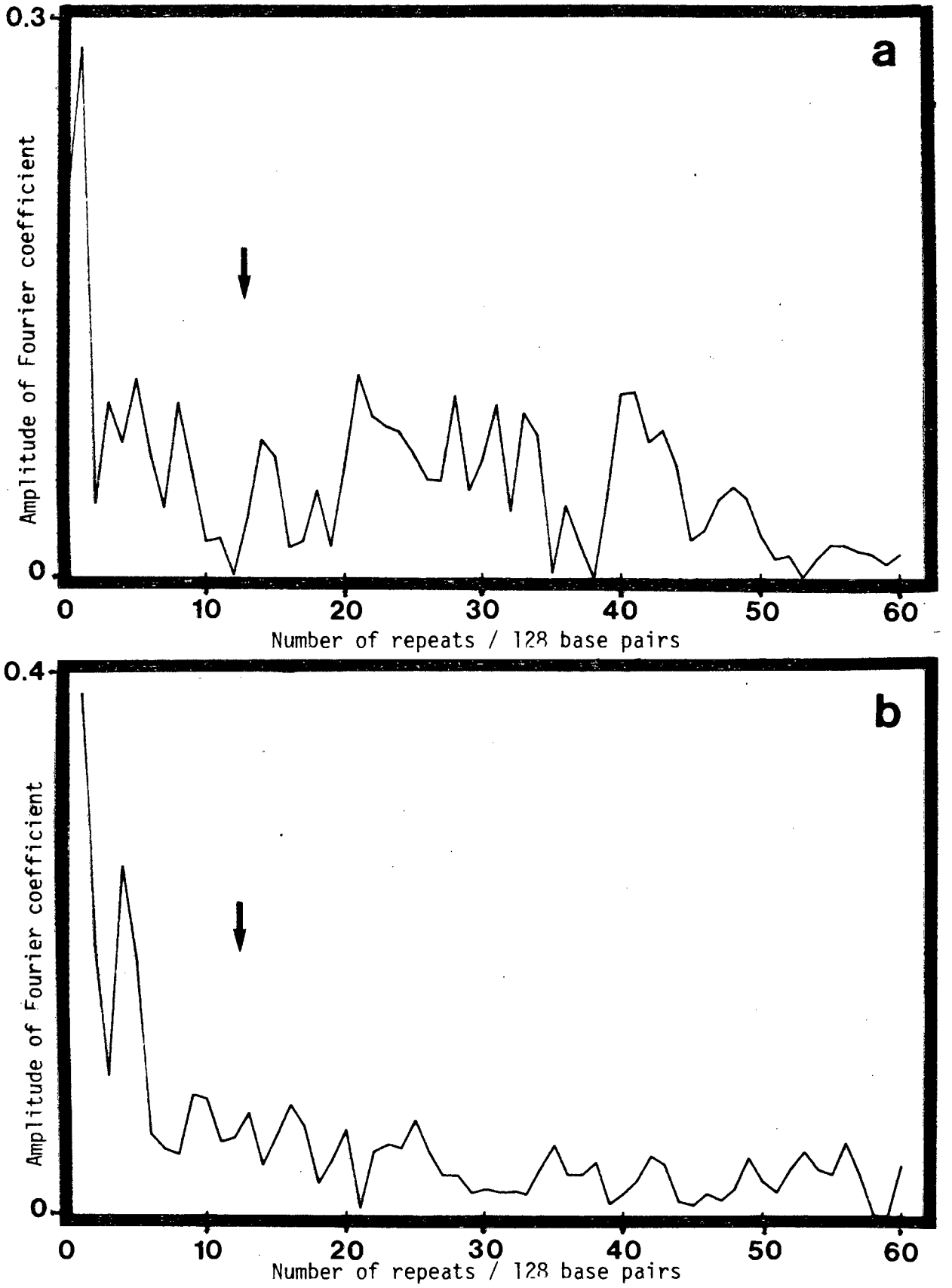


Fig. 75

Fourier transforms of purine-purine dimers in the preferred frames.

(a) UPSTREAM fragment from 80 - 204 base pairs.

(b) END fragment from 160 - 284 base pairs.

A repet structure with a 10 base pair periodicity would have 12.8 repeats/128 base pairs as indicated by the arrow.

The motifs can be regarded as a range of purine dimers and thus wedges. Any nett structural effect that such dimers may have in the centre of the motifs will be cancelled out by the twist of the helix. At the ends of the motifs the wedge effect will not be cancelled out and may promote the unidirectional bending of the DNA.

An isolated strong bend may direct the initiation of core assembly on such a bend. It is known that DNA in solution can contain bends (Wu and Crothers 1984, Hagerman 1984, 1985, Diekman and Wang 1985). (For review see Widom 1984). A bend may, after attracting an octamer to its vertex, force progressively more and more of the DNA to also bend in that plane. A strong bend next to a motif could attract an octamer and thus promote the bending of the motif itself. It is conceivable that such bends lead to the positioning of the octamers in two ways. The core could assemble on any part of the DNA and slide into an optimal position, a "click stop" effect, or the bend can act as a nucleation point for the assembly of the core. In either case the inner face of the bend will have a high negative charge density that will help to stabilize the positively charged octamer. The negative charges on the opposite side of the helix will then facilitate the bending of the DNA molecule around the core (Mirzabekov and Rich 1979).

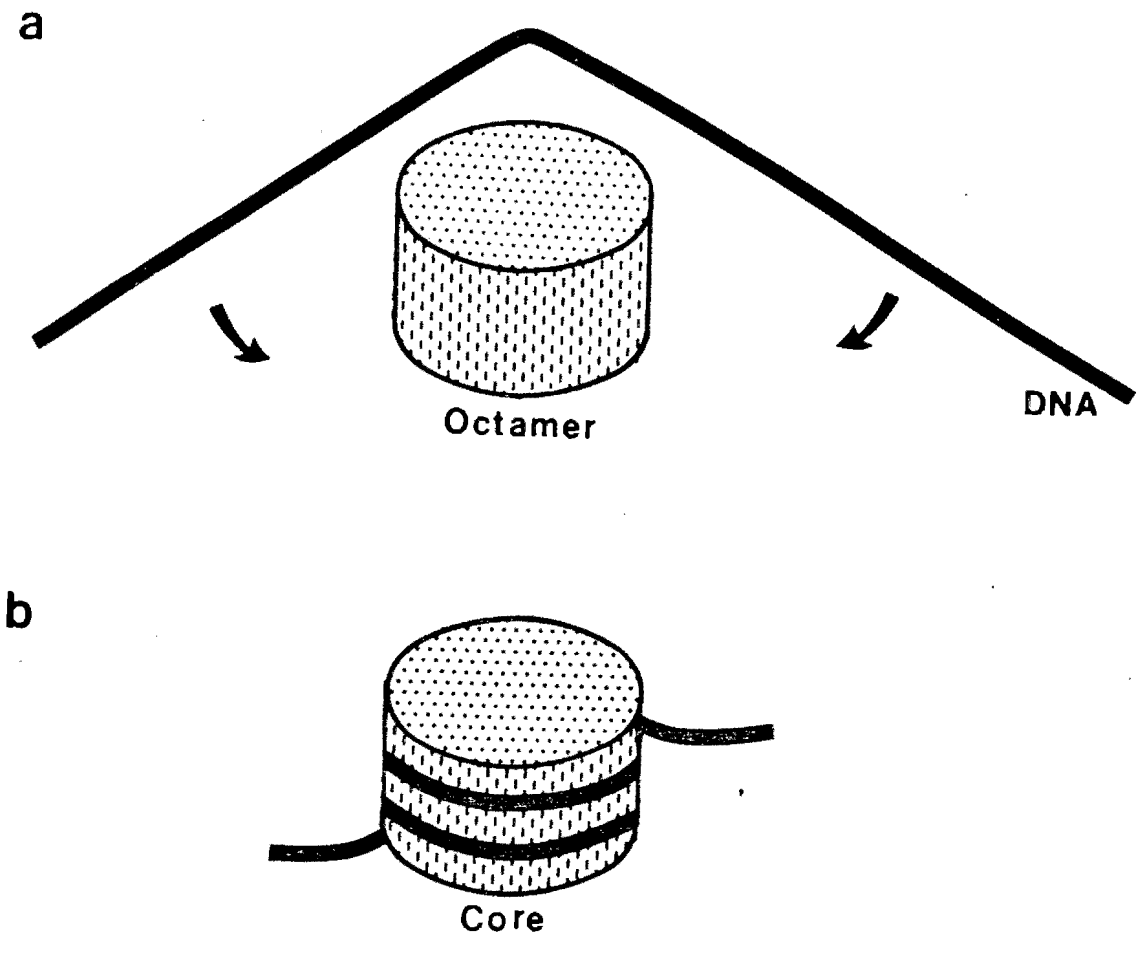


Fig. 76
Diagram to illustrate the influence of a bend in the DNA on core assembly. A bend in the DNA will present a local area of local charge clustering. The octamer will neutralize those charges allowing the DNA to wrap around the octamer. Note that the bending introduced into the DNA by the assembly will be in the same plane as the original bend. b: Assembled core.

These experiments show that the level of positioning varies from essentially random to specific. In the latter case cores are confined to preferential frames. In the absence of tissue factors, this must be an accurate reflection of the level of information contained in the DNA. It can therefore be concluded that the DNA sequence of certain stretches of the fragments investigated contain enough information to influence the positioning of a core, but that in the remainder the strong

interaction between the ionic forces present in both, the octamer and the DNA, result in random association. The results may well reflect the actual "in vivo" situation where a single core may be positioned specifically. The neighbouring nucleosomes, up- and downstream from that nucleation core are then assembled in a phase probably dictated by space requirements, but not DNA sequence specificity.

(8) CONCLUSIONS

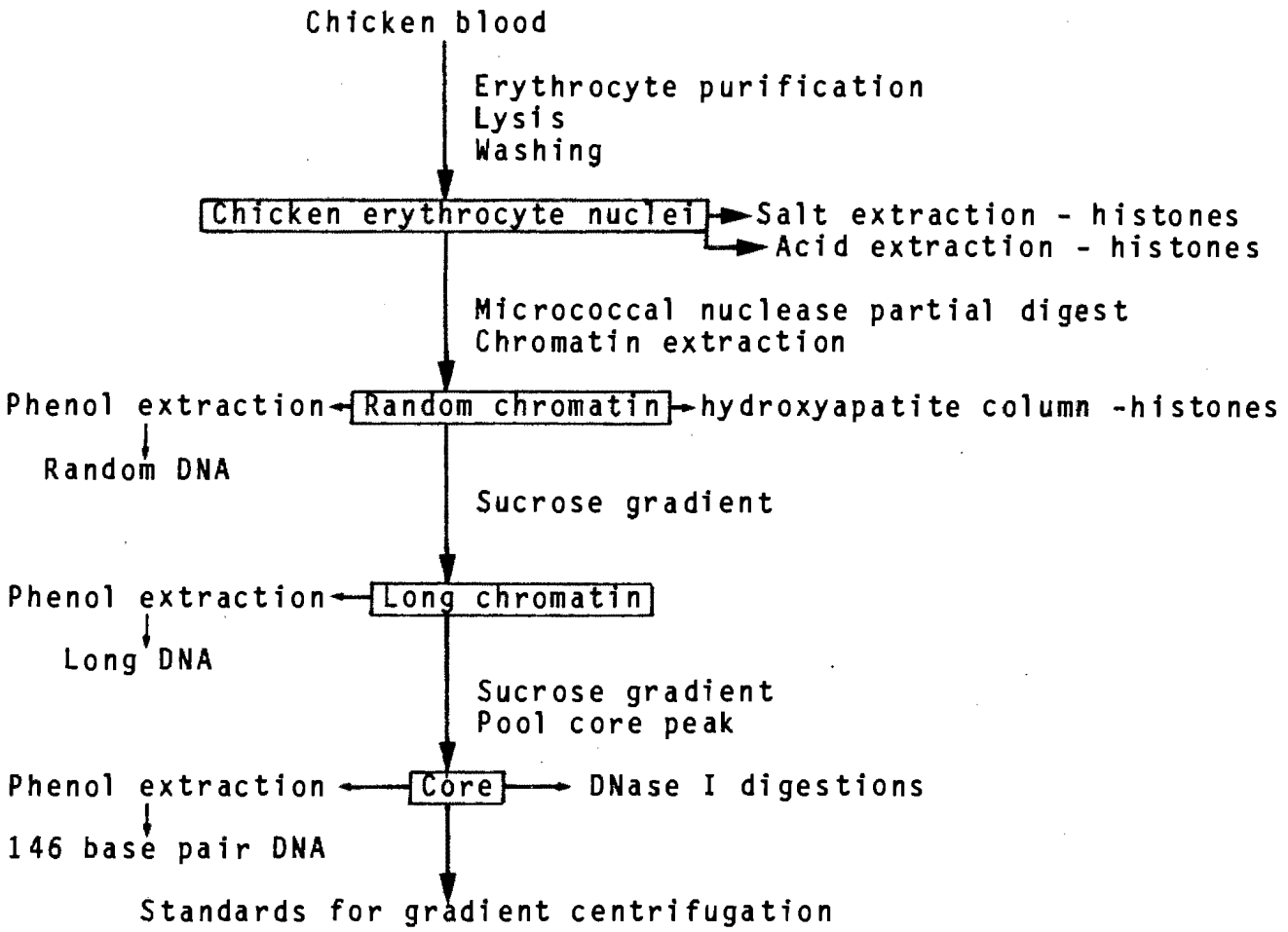
Nucleosome cores can be faithfully assembled, with histone octamers in the presence of poly(glutamic acid), on both random and unique DNA.

Indirect end-labelling of the h22 histone gene quintet does not reveal specific positioning of nucleosome cores.

Direct end-labelling of some selected fragments reveals two preferential frames of assembly in the spacer areas flanking the H1 coding area. In the remainder of those fragments cores assemble in overlapping frames that cannot be resolved, or are randomly positioned.

9. MATERIALS AND METHODS

The preparation of chicken erythrocyte chromatin, histones and DNA was done as follows:



9.1 PREPARATION OF NATURAL CHROMATIN, POLYCORES AND CORES:

Chicken erythrocyte nuclei and chromatin was prepared by modification of some well established methods (Shaw et al. 1978, Ruiz-Carillo and Jorcano 1979).

9.1.1 Preparation of chicken erythrocyte nuclei:

Blood from freshly bled young Leghorn chickens were obtained

from the local abattoir. 250 mℓ of blood was collected in 25 mℓ of a 10% (w/v) solution of trisodium citrate and filtered through 2 layers of cheesecloth. The blood was immediately placed on ice and transported directly to the laboratory. The blood was centrifuged for 5 minutes at 12 000g in glass centrifuge tubes, resulting in approximately 80 mℓ packed cells. The plasma was removed and care was taken to remove the buffy coat completely. The cells were carefully resuspended, with a glass rod, in 150 mℓ SSC (150 mM NaCl, 15mM Trisodium citrate). This washing step was then repeated with 170 mℓ buffer A (65 mM NaCl, 60 mM KCl, 0.15 mM Spermine, 0.5 mM Spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 15 mM Tris/HCl pH 7.5.) (Burgoyne et al. 1974).

The erythrocyte suspension was and made up to 0.1 mM PMSF from a 100 mM stock solution in DMSO and lysed by adding Nonidet LE, from a 10% (w/v) stock solution, to a final concentration of 1% (w/v).

The lysis mixture was centrifuged for 5 minutes at 12 000 g. The supernatant was removed and replaced by 170 mℓ buffer A, 0.1 mM PMSF. The pellets were vigorously suspended by two passes of a loose Dounce homogenizer. The above steps were repeated four times in buffer A and once in buffer A containing 350 mM NaCl, 0.1 mM PMSF.

The nuclei were spun down for 2 minutes at 12 000 g and resuspended in an equal pellet volume of buffer A containing 50% (w/v) glycerol and 0.2 mM PMSF. The nuclei were mixed well

and stored at -20°C. To use stored nuclei the DNA concentration of an aliquot was determined. The amount needed at double the concentration required was washed in buffer A. After determination of the DNA concentration, the suspension was diluted to the required volume.

The DNA concentration of the nuclei was determined by diluting an aliquot 1:10 in buffer A. This was further diluted 1:10 in 4 M NaCl and the absorbance determined at 260 nm. An absorbance of 20 was taken as 1 mg DNA/mℓ.

9.1.2 Preparation of natural random chromatin:

Chicken erythrocyte nuclei were suspended in buffer A and the concentration adjusted to 2 mg DNA/mℓ. 50 mℓ of this suspension was taken and preincubated in a 100 mℓ conical flask at 37°C for 5 minutes. $CaCl_2$, from a 100 mM solution, was added to bring the free calcium concentration up to 1.4 mM. Micrococcal nuclease was added at a ratio of 20 units/mg DNA from a stock solution (20 units micrococcal nuclease/µℓ, 1mM $CaCl_2$, 10 mM Tris pH 8.0). The digestion was carried out for 4 minutes at 37°C. The reaction was terminated by placing the solution on ice and the addition of 0.9 mℓ of a 250 mM EDTA, adjusted to pH 8.0, solution. The digested nuclei were then pelleted at 2000 g for 5 minutes and the pellet resuspended in 10 mℓ 550 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.6 in order to dissociate histones H1 and H5. The resuspended chromatin was homogenized by 4 strokes of a tight Dounce homogenizer and left on ice for 1 hour. To remove any undissolved nuclear debris the chromatin

was centrifuged at 2000 g for 5 minutes.

9.1.3 Preparation of natural long polycores:

To isolate the long polycores from the random chromatin, aliquots of the supernatant, each containing 6 mg DNA, were applied to Beckman SW28 tubes, each containing a 5 to 20% (w/v) sucrose gradient in 500 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 mM Tris/HCl pH 7.6. The gradients were centrifuged for 16 hours at 27 000 rpm at 4°C. The gradients were fractionated and the long polycores pooled.

9.1.4 Preparation of natural cores:

The polycores were dialyzed against 1 mM EDTA, 10 mM TEA/HCl pH 7.6. The fractions were concentrated on an Amicon PM30 membrane to a concentration of 1mg DNA/mℓ buffer. A pilot digestion was carried out for every preparation to determine the optimal time of digestion. An aliquot of long polycores was taken and the calcium concentration adjusted to 1.4 mM free Ca^{++} from a stock solution (100 mM $CaCl_2$, 10 mM Tris/HCl pH 8.0). 1 unit micrococcal nuclease/mg DNA was added and the digestion carried out at 37°C. At the appropriate intervals 40 µℓ aliquots were taken and the reaction stopped by addition of an excess EDTA from a stock solution (250 mM EDTA/NaOH pH 8.0). The DNA from these aliquots was extracted and analysed by denaturing gel electrophoresis. The rest of the polycores were then redigested under the conditions determined by the pilot reaction. After redigestion the digestion mixtures were applied to 6 SW28 centrifuge tubes, each containing a 5 to 20% (w/v) sucrose gradient. The gradients were centrifuged and

fractionated as before and the core peak pooled.

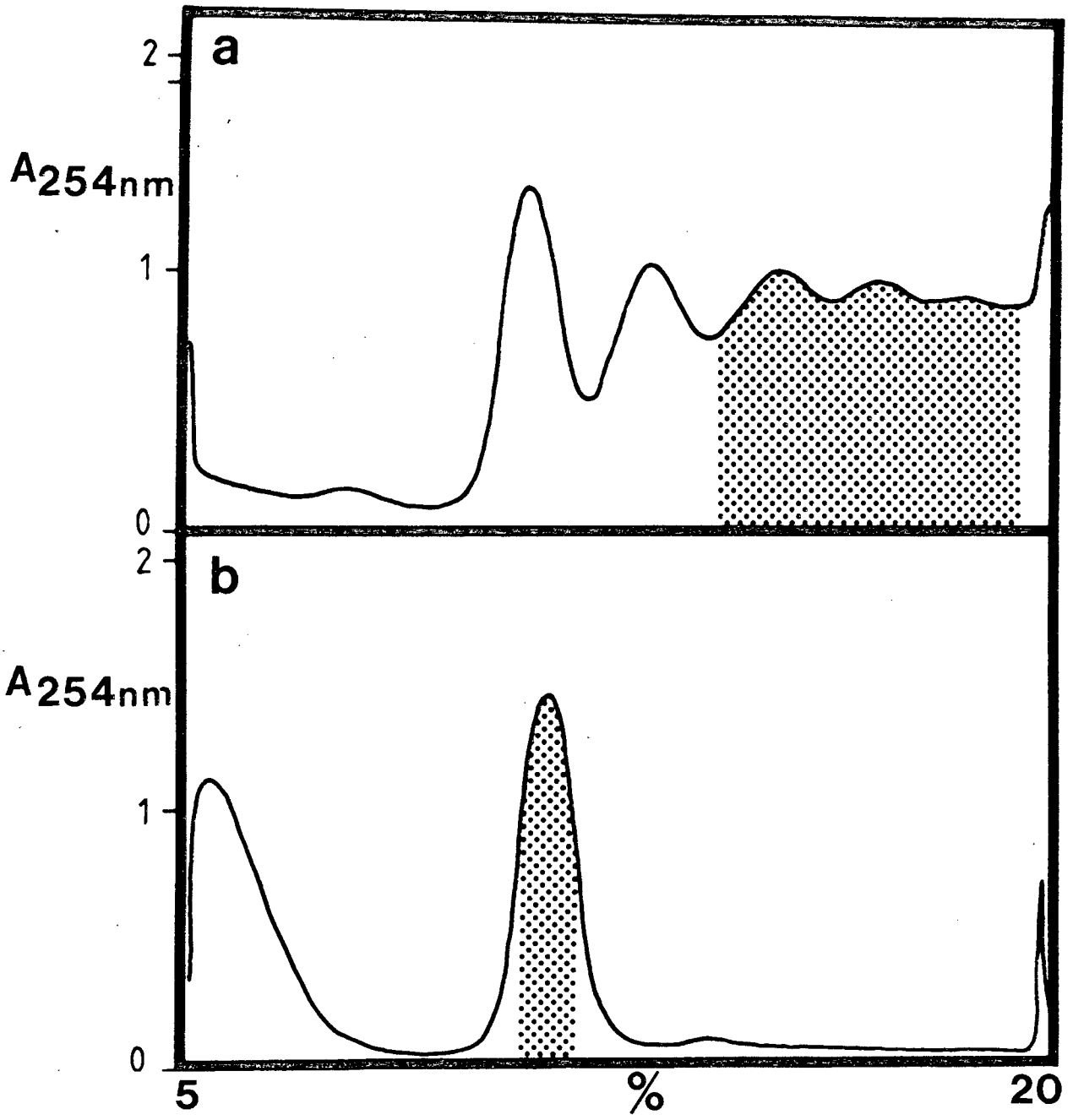


Fig. 77

The purification of cores and polycores on sucrose gradients:

Gradient: 5 - 20% (w/v) sucrose. Rotor: Beckman SW28Ti.
Speed: 27 000 rpm for 16 hours. Temp: 4°C.
Buffer: 500 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and 10 mM Tris/HCl pH 7.6.

(a) Random polycores produced by partial Micrococcal nuclease digest of chicken erythrocyte chromatin.
The shaded area indicates the fractions pooled to produce long polycores and long DNA.

(b) Cores produced by Micrococcal nuclease redigestion of long polycores from (a).
The shaded area indicated the fractions pooled to study DNase I digestion kinetics and produce 146 base pair DNA.

9.2 HISTONE OCTAMERS:

9.2.1 Salt extraction of histones by ultracentrifugation of free chromatin at high ionic strength:

The extraction of histones at high ionic strength was carried out according to Ruiz-Carillo and Jorcano (1979) using the previously prepared chicken erythrocyte nuclei.

9.2.2 Salt extraction of histones from chromatin immobilised on hydroxyapatite:

Hydroxyapatite was prepared according to Bernardi (1971). This method involves first preparing Brushite, followed by conversion to hydroxyapatite in the presence of NaOH and high temperature. During the preparation process great care was taken to promote the formation of large crystals of hydroxyapatite. The addition of $CaCl_2$ and Na_2HPO_4 did not exceed 250 mℓ per hour and the formed crystals were maintained in suspension by gentle stirring with a mechanical paddle stirrer. This method yielded up to 500 mℓ gravity settled hydroxyapatite for every 8 litres of $CaCl_2$ and Na_2HPO_4 used.

The quality of the hydroxyapatite prepared was determined as follows: A solution of 1 mg/mℓ random DNA was prepared in 1 mM EDTA, 10 mM phosphate pH 7.6. 1 mℓ of settled hydroxyapatite was added to 10 mℓ of this DNA solution. The mixture was agitated gently and centrifuged at 2 000 rpm for 2 minutes in

a glass centrifuge tube. The DNA concentration of the supernatant was determined and the amount of DNA bound to the hydroxyapatite calculated. Hydroxyapatite prepared and assayed by these methods normally binds more than 1mg DNA/mℓ of gravity settled hydroxyapatite.

To extract histones from partially digested chromatin bound to hydroxyapatite, chicken erythrocyte nuclei were prepared as before and suspended in buffer A. The DNA concentration of the suspension was adjusted to 6 mg/mℓ. 12 mℓ of this suspension was pelleted and resuspended in 10 mℓ of a 1 mM $CaCl_2$, 4 mM $MgCl_2$, 25 mM KCl, 1 mM PMSF, 50 mM Tris/HCl pH 7.0 solution. To this suspension 2 880 units of micrococcal nuclease was added. The digestion was carried out for 1 hour at 37°C with gentle shaking. The digestion was stopped by centrifuging the nuclei at 2 000 g for 1 minute and resuspension of the pellet in 10 mℓ 0.25 mM EDTA/Tris pH 8.0. This solution was then placed in a 1 cm x 20 cm dialysis bag and dialyzed overnight against 2 ℓ of the same buffer at 4°C. After dialysis the solution was centrifuged in a Sorvall SM24 rotor at 11 000 rpm for 30 minutes at 4°C. The DNA concentration of the solution was determined and adjusted to 5 mg/mℓ.

A hydroxyapatite column measuring 2.5 cm x 20 cm was packed and 5 mℓ of the 5 mg/mℓ extracted chromatin solution applied. The column was eluted at room temperature with the following solutions; 0.45 M NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, followed by 3 M NaCl, 5 mM

Na_2HPO_4, 5 mM NaH_2PO_4 and 0.25 M Na_2HPO_4, 0.25 M NaH_2PO_4, essentially according to Simon and Felsenfeld (1979).

9.2.3 Further octamer purification:

The octamer sample, prepared by salt extraction or the hydroxyapatite method, was concentrated to 5 mg/mℓ using an Amicon PM 10 membrane and then further purified by molecular sieve chromatography on a Sepharose CL 6B column (2.5 cm x 100 cm). The column was eluted with 2 M NaCl, 0.1 mM PMSF and 10 mM Tris/HCl pH 7.6. Fractions were collected and the octamer containing peak pooled. The octamers were concentrated on an Amicon PM 10 membrane and stored in glycerol at -20°C (See section 9.2.6). All reactions were carried out at 4°C.

9.2.4 Acid extraction of histones:

Histones were prepared by acid extraction as follows: Stored or fresh chicken erythrocyte nuclei were adjusted to a DNA concentration of 10 mg/mℓ in buffer A. The nuclei were centrifuged for 1 minutes at 10 000 rpm in a Sorvall SS35 tube at 4°C. The nuclear pellet was extracted with the original volume of 0.25 M HCl. Aggregates were disrupted using 4 strokes of a tight Dounce homogenizer. The solution was then centrifuged at 10 000 rpm for 20 minutes in a Sorvall SS35 rotor at 4°C. The supernatant was retained and the pellet re-extracted as before. The supernatants were pooled and extensively dialyzed against distilled water, followed by freeze drying.

Histone octamers produced by acid extraction and renaturation of core histones, acid extraction of individual histones followed by renaturation and protamine displaced octamers were generous gifts from J.H. Greyling (Greyling et al. 1983).

9.2.5 Radioactive labelling:

The iodine labelling of the histone octamers was carried out by two different methods: (a) Immobilized lactoperoxidase (David and Reisfeld (1974) (b) Pierce iodobeads.

Lactoperoxidase was bound to Sepharose CL 4B beads by the method of David and Reisfeld (1974) and stored at 4°C. 20 µℓ of beads containing immobilized lactoperoxidase was added to 500 µℓ of a 2 mg/mℓ solution of histone octamers, previously dialyzed against 2 M NaCl, 10 mM Phosphate buffer pH 7.0. 10 µℓ 1 mM KI, 10 µℓ 1 mM H_2O_2 and 10 µℓ [^{125}I]NaI were added. The mixture was incubated for 1 hour at 20°C with gentle shaking.

After labelling all unreacted iodine was removed by exclusion chromatography in a Pasteur pipette packed with Sephadex G25, eluted with 1 M NaCl, 10 mM phosphate buffer pH 7.0.

Iodination of histone octamers was carried out on 100 µℓ aliquots of the 5 mg/mℓ histone octamers in storage buffer. All traces of β-mercaptoethanol and glycerol were removed by dialyzing, in a microdialysis chamber, overnight against two changes of 2 M NaCl, 2 mM EDTA, 20 mM Tris/HCl pH 8.0. The volume of the dialyzed octamers was adjusted to 0.2 mℓ with the

same buffer and placed in an Eppendorf vial. To the octamers 0.02 mℓ [^{125}I], and 1 Iodo Bead was added. The reaction mixture was incubated at 20°C for 15 minutes and stopped by removing the Iodo Bead.

The labelled octamers were purified by chromatography using a Pasteur pipette packed with Sephadex G50 and equilibrated in 2 M NaCl, 20 mM Tris/HCl pH 8.0. The appropriate fractions were pooled and stored at -20°C after the addition of glycerol to a final concentration of 50% (w/v).

9.2.6 Storage of octamers:

All octamers prepared by any of the above methods were stored in 1 M NaCl, 0.1 mM β-mercaptoethanol, 10 mM Tris pH 8.0, 0.1 mM PMSF and 50% (w/v) glycerol at -20°C.

9.3 DNA

9.3.1 Preparation of random, long and 145 base pair DNA:

Random chromatin was prepared (Section 9.1.3). After digestion the nuclei were pelleted for 2 minutes in a benchtop centrifuge and resuspended in 500 mM NaCl, 10 mM Tris/HCl pH 8.0, 1mg proteinase K/mℓ. After digestion for 1 hour at 37°C the DNA was phenol extracted until no white protein interface was visible. This was followed by four chloroform/isoamyl alcohol (24:1) extractions and ethanol precipitation in the presence of 30 mM sodium acetate pH 5.0 wih 2 volumes of ethanol at -20°C. The pellet was washed with 80% (v/v) ethanol and dried under vacuum.

Long DNA was extracted from long polycores under the same conditions as used for randon DNA.

145 base pair DNA was extracted from nucleosome cores, produced as for 5' end-labelling reactions (See section 9.1.4). After purification of the cores on a sucrose gradient and dialysis against 20 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.6, 1 mg/mℓ proteinase K was added. After incubation at 20°C for 30 minutes the cores were extracted with phenol and chloroform followed by ethanol precipitation as for random DNA.

9.3.2 Preparation of plasmid and phage DNA:

pBR322 and derivatives in HB101 were prepared as follows: A 1 litre culture was prepared by inoculating 0.3 mℓ glycerol culture into 100 mℓ L broth with the required antibiotic. The culture was incubated at 37°C for 16 hours with vigorous shaking. Of this culture 8 mℓ was inoculated into 800 mℓ L broth and incubated for 3 hours on a shaker at 37°C. To amplify the plasmids, 2 mℓ Chloramphenicol (87.5 mg/mℓ in absolute ethanol) was made up to 200 mℓ with L broth, and added to the culture. The culture was further incubated for 16 hours at 37°C with shaking. The cells were harvested by centrifugation for 30 minutes at 17 000 rpm at 4°C.

The pellets were then resuspended in 8 mℓ 15% (w/v) sucrose 50 mM Tris/HCl pH 7.6. 1 mℓ lysozyme was added from a 10 mg/mℓ freshly made up stock solution. The mixture was incubated for 30 minutes at 4°C with vigorous shaking. To this mixture 1 mℓ of a 500 mM EDTA pH 7.5 solution was added. The mixture was further incubated at 4°C with vigorous shaking. 0.1 mℓ RNase A was added from a 20 mg/mℓ solution in 0.3M sodium acetate pH 5.5 followed directly by 0.2 mℓ of a 10% (w/v) Triton solution. The mixture was incubated at 4°C for 20 minutes with gentle shaking. The lysis mixture was centrifuged for 45 minutes at 17 000 rpm in a Sorvall SM24 rotor at 4°C. The supernatant was extracted twice with phenol/chloroform. The extraction was repeated with chloroform, followed by ethanol precipitation. The DNA was suspended in 1 mℓ 1 mM EDTA 10 mM Tris/HCl and digested with 1 mg proteinase K for 1 hour at 37°C. This solution was phenol extracted and ethanol precipitated as

before.

The pellet was dissolved in a caesium chloride solution containing 0.84 g/mℓ CsCl, 0.2 mg/mℓ ethidium bromide, 1 mM EDTA and 10 mM Tris/HCl pH 7.6. The refractive index was adjusted to 1.3888. The gradient was centrifuged for 16 hours at 55 000 rpm in a 65 VTi rotor at 20°C. The supercoiled fraction was collected by piercing the tube with a hypodermic needle attached to a 1 mℓ syringe. (Maniatis et al. 1982) This fraction was further purified on a second caesium chloride gradient that was run and fractionated as before.

The replicative forms of M13 phages were prepared according to Messing (1983). The inserts were prepared and inserted by standard recombinant techniques (Maniatis et al. 1982) and transfected into JM103 host cells (Messing 1983). The phages were prepared and the single stranded templates extracted according to Messing (1983). The primer was obtained from Boehringer and hybridisation and synthesis of the double stranded DNA carried out according to Hu and Messing (1982).

9.3.3 Extraction of DNA from gels:

DNA restriction fragments were isolated from low melting point agarose gels according to Maniatis et al. 1982.

An alternative method that proved very successful, providing high yields of pure DNA, was the method of Dretzen et al. (1981). This involves electrophoresing the desired DNA fragment into a Whatman DE81 DEAE cellulose filter inserted into the

gel. The filter is then removed from the gel and rinsed in cold water. The DNA is then extracted from the filter in 1.5 M NaCl, 1 mM EDTA and 20 mM Tris/HCl pH 7.5. This method was used in the cloning of pGV403 plasmids and M13 phages.

9.3.4 Radioactive labelling of DNA:

Unless otherwise indicated all radioactive nucleotides used were prepared by the method of Johnson and Walseth (1979). This method was used without modification for the preparation of ribonucleotides. The incorporation of [^{32}Pi] into the gamma labelled ATP was normally better than 99% as assayed by the active charcoal binding assay. This reaction mixture was heated to 65°C for 10 minutes to stop the reactions and used without any further purification.

The preparation of the α-labelled deoxyribonucleotides was modified as follows: The hexokinase cycle was not used to remove any unutilized ATP as the preceeding transfer was normally quantitative as determined by thin layer chromatography (Zweig and Sherman 1964). The pH of the reaction was monitored throughout by applying µl quantities of the reaction mixture to narrow strips of Merck pH paper. Additional cold ATP and kinase was added to the final kinase reaction to ensure a high yield of labelled product.

After the reactions were stopped the labelled nucleotides were further purified by phenol extraction with an equal volume of phenol, followed by extraction with one volume of chloroform. Any remaining chloroform was removed by heating the mixture to

65°C for 10 minutes, followed by drying under vacuum in a centrifugal evaporator.

In our hands the purine labelling reaction was more reliable than the labelling of pyrimidines and [α-^{32}P]dGTP was the nucleotide of choice to use in nick-labelling reactions.

The progress of end- or nick-labelling reactions was routinely monitored by TCA precipitation. During the labelling process a 1 µℓ aliquot was placed on a 25mm diameter Whatman GFC filter. The Cerenkoff radiation of the aliquot was determined in the Tritium channel of a scintillation counter. The filter was then placed on a scintered glass filter and washed with 2 mℓ 10% (w/v) TCA, followed by 2 washes of 15 mℓ 5% (w/v) TCA, and recounted to determine the percentage incorporation.

Nick-labelling reactions were normally carried out by a modified method of Rigby et al. (1977). Each reaction was carried out in a total volume of 45 µℓ containing 1 µg DNA, 0.02 mM dATP, 0.02 mM dCTP, 0.02 mM dTTP, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 ng BSA, 50 mM Tris/HCl pH 7.8, 5 µℓ [α-^{32}P]dGTP (approximately 5 000 Ci/mmole), 4 X 10^{-4} units DNase I (Fresly diluted from stock), 2 units DNA polymerase. The reaction was allowed to proceed at 16°C until the incorporation of the radioactive nucleotides reached a plateau as determined by the TCA precipitation assay. Normally greater than 60% of the radioactive nucleotide is incorporated. All unincorporated nucleotides were removed by spun columns. (Maniatis et al. 1982)

To end-label DNA standards for denaturing DNA gels, the 3' fill in of protruding 5' ends with the Klenow fragment of DNA polymerase was used. The nucleotide chosen for the labelling reaction was normally the last one to be filled in, to prevent the formation of labelled fragments smaller than unit length. A typical labelling reaction contained 0.6 µg of Hpa II digested pBR322 dissolved in 1 µl (1 mM EDTA and 10 mM Tris/HCl pH 8.0), 1 µl 2.5 mM dCTP, 1 µl [α-^{32}P]dGTP, 7 µl (20 mM $MgCl_2$, 20 mM β-mercaptoethanol and 40 mM Tris/HCl pH 7.6) and 1 unit Klenow fragment of DNA polymerase. The reaction was allowed to proceed at 16°C until incorporation of the nucleotides ceased, as monitored by the TCA precipitation assay. This was normally less than 10 minutes. Desalting was carried out on spun columns according to Maniatis et al. (1982).

The 5' end-labelling of DNA standards was carried out according to Maniatis et al. (1982). This involved the dephosphorylation of the DNA with bacterial alkaline phosphatase, followed by phenol extraction, desalting and labelling with T4 polynucleotide kinase and [gamma-^{32}P]ATP. All desalting steps were carried out on spun columns.

9.3.5 DNA electrophoresis:

Denaturing DNA gel electrophoresis of DNA fragments produced after DNase I digestion of 5'-labelled cores was carried out according to Maxam and Gilbert (1977 and 1980). The gel consisted of 8% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide in TBE buffer (90 mM Tris/Borate pH 8.3 and 2.5 mM EDTA according to Peacock and Dingman 1967) and 8 M urea. TBE buffer was used in the reservoir of the electrophoresis chamber. The size of the gels was 20 cm x 23 cm x 1.8 mm. Samples were dissolved in sample buffer (8 M urea, 0.01% (w/v) bromophenol blue and TBE buffer diluted 1/10), incubated for 1 minutes in a boiling waterbath and rapidly cooled on ice. The gels were run at 1 200 V at 50-60°C. After electrophoresis the gels were wrapped in plastic cling film and exposed overnight, or longer, to Cronex 4 X-ray film at -70°C.

DNA fragments produced by nuclease digestion of pGV403 plasmids were analysed on high resolution denaturing gels. These gels contained 8 M urea and a 20:1 acrylamide, bis-acrylamide ratio as before. The optimal acrylamide concentration was used for every fragment analysed, namely 6%, 7% and 8% (w/v) for the UPSTREAM, END and STOP fragments respectively. The gels measured 30 cm x 45 cm and varied in thickness from 0.4 mm at the top to 0.8 mm at the bottom.

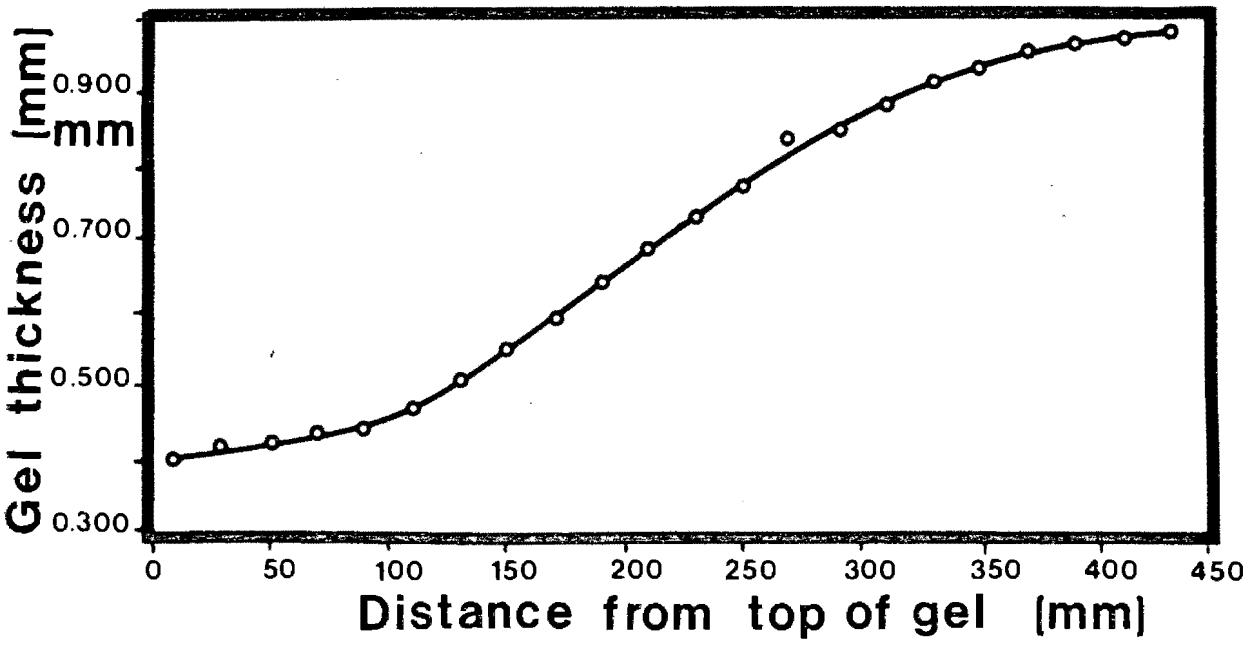


Fig. 78
The thickness of sequencing gels:
The thickness of a gel cast between two glass plates was calculated by measuring the thickness of the gel and the plates, at various distances from the top of the gel, with a micrometer and subtracting the thickness of the plates.

The wells were formed by a "sharks tooth" comb (Bethesda Research Laboratories) and an even running temperature ensured by a thermostatically controlled waterjacket.

The gel solutions were prepared as follows: A 40% (w/v) acrylamide (Merck) and 2% (w/v) bis-acrylamide (Sigma) solution was deionized by mixing gently with 1 g Amberlite/ 20 mℓ solution and filtered. The urea used for these gels was recrystallized and filtered and dissolved just before use. The gel was prepared by making 100 mℓ gel solution containing, the required acrylamide concentration, 8 M urea, 1% (w/v) AMPS (from a freshly made up 10% (w/v) solution in water) and 50 µℓ TEMED. The gel was then poured horizontally (Garoff and Ansorge 1981) and left to set for an hour. After the gel had set the gel plates were assembled in the electrophoresis

chamber and the gels pre-electrophoresed for 15 minutes at 1 000 V with the waterjacket at 55°C. The DNA samples were dissolved in formamide, previously deionized with Amberlite, and heated to 90°C on a heating block for 10 minutes. The samples were applied directly from the heating block to the gel. The gel was run at 900 V and 50°C. After electrophoresis the gel was fixed in a mixture of 10% (v/v) methanol and 10% (v/v) acetic acid in water for 15 minutes, followed by washing in water for 30 minutes and drying under high vacuum on a heating plate. The gels were then exposed to the X-ray film at -80°C without intensifying screens.

Double stranded DNA fragments were analysed on horizontal agarose gels in TBE buffer. The agarose was dissolved in boiling TBE. The gel mixture was then cooled to 50°C and ethidium bromide was added from a stock solution (10 mg/mℓ in water) to a final concentration of 1 µg/mℓ. The gel chambers were constructed essentially according to Maniatis et al. (1982). All agarose was type IV, obtained from Sigma. To monitor restriction enzyme digestions miniature gels (85 mm x 125 mm) containing 60 mℓ agarose solution were run at 80 mA constant current in the presence of 1 µg/mℓ ethidium bromide. The samples were applied in sample application buffer (50% (w/v) glycerol, 0.1 mM EDTA and 0.01% (w/v) bromophenol blue). The progress of the electrophoresis was followed by using a hand-held UV light source.

High resolution agarose gels (20 cm x 25 cm) were prepared in the same way as miniature gels, but using 200 mℓ gel solution.

These gels were run overnight at 38 mA constant current until the bromophenol blue reached a position 2 cm from the bottom. The gels were visualized on an ultraviolet light box and photographed through a 2X red filter on Kodak Panatomic X film. The film was processed using D76 developer according to the manufacturers instructions.

Southern transfers were carried out according to Maniatis et al. 1982). Gels were denatured for 1 hour in 500 mℓ denaturing solution, (1.5 M NaCl and 0.5 M NaOH) followed by 1 hour in 500 mℓ renaturing solution (1 M Tris/HCl pH 8.0 and 1.5 M NaCl). The DNA was transferred onto Nitrocellulose obtained from Schleicher and Schuell (Southern 1975). The filter was then washed in 2X SSC and baked for 1 hour at 80°C.

Hybridizations were carried out in sealed plastic bags according to Maniatis et al. (1982). Prehybridizations and hybridizations were carried out at 65°C.

9.3.6 Restriction enzyme digestions:

Unless otherwise indicated, restriction enzymes were obtained from Amersham and digestions carried out according to the manufacturer's instructions. Cla I was obtained from Boehringer.

9.3.7 Ligation reactions:

The insert to vector ratio of the ligation reactions was calculated according to Dugaiczyk (1975). Ligation reactions were carried out routinely overnight at 4°C. For vectors with blunt, or complementary cohesive ends the vectors were first treated with bacterial alkaline phosphatase, followed by phenol extraction as for 5' end-labeling.

To produce blunt ends from the protruding 5' ends remaining after restriction enzyme digestion, the recessed 3' ends were filled in using the same reaction conditions as for 3' fill in labelling. An excess of all the nucleotides necessary for the reaction was provided and the reaction allowed to proceed for at least 30 minutes at 16°C.

9.3.8 Propagation of plasmids:

HB101 (F^-, hsdS20 (r^{-B}, m^{-B}), recA13,ara-14, proA2, lacY1, galK2, rpsL20 (SM^r), xyl-5, mtl-1, supE44, λ^-) competent cells were prepared by inoculating 5 mℓ of L broth with a loop from a stock glycerol culture and incubating overnight at 37°C with shaking. 5 mℓ of this grown culture was inoculated into 500 mℓ L broth (Maniatis et al. 1982) and incubated for 90 minutes at 37°C. This was followed by chilling the cells on ice for 10 minutes. The cells were pelleted in a Sorvall GSA rotor at 4 000 rpm and 4°C for 10 minutes. The pellet was resuspended in ice cold CT buffer (50 mM $CaCl_2$, 1 mM Tris/HCl pH 8.0) and kept on ice for 15 minutes. These cells were centrifuged down as before

and the pellet resuspended in 7 mℓ ice cold CT buffer containing 20% (w/v) glycerol. The resuspended cells were aliquotted and stored at -80°C.

Each ligation reaction was carried out with suitable controls. (Maniatis et al. 1982). The transformation mixture was made up by addition of 10 ng plasmid DNA suspended in 10 µℓ (1 mM EDTA, 10 mM Tris/HCl pH 7.6) to 200 µℓ competent cells. The mixture was mixed gently and incubated on ice for 30 minutes, at 42°C for 1.5 minutes and room temperature 10 minutes. 1 mℓ L broth containing a suitable antibiotic was added with gentle agitation and the cells incubated at 37°C for 1 hour without shaking. 100 µℓ of the transformed cells were plated out on Petri dishes containing L broth with 1.5% (w/v) Bacto-agar and a suitable antibiotic (Maniatis et al. 1982).

The insertion of DNA fragments into the pBR322 plasmid was determined by the inactivation of the relevant antibiotic site. Antibiotic sensitive colonies were grown up and screened for the correct sized insert. The identity of the insert was confirmed by restriction enzyme digestion of the insert.

The presence of inserts in the pGV403 plasmids was determined by colony hybridizations on Hybond N (Amersham) using nick-labelled h22 as a probe. The hybridisations were carried out according to the product literature. The positive colonies were picked and screened for the correct sized insert.

Bacterial strains were maintained as glycerol cultures. A

colony was picked from a streaked plate and inoculated into a plastic tube containing 2 mℓ L broth and a suitable antibiotic. This culture was incubated overnight at 37°C with shaking and 2.4 mℓ 80% (w/v) glycerol was added. The cells were well mixed and maintained at -20°C for storage up to 6 months or -80°C for long term storage.

9.4 POLY(GLUTAMIC ACID):

Poly(glutamic acid) of various molecular weights were obtained from Sigma.

Acetylation was carried out under anhydrous conditions by a combination of various methods (Miller and Great 1972, Wegrzynowicz et al. 1975, Montelaro and Rueckert 1975). To prepare the anhydrous solvent, analytical grade dioxane was obtained from Merck. All water was removed from the dioxane by refluxing over sodium metal under nitrogen for 1 hour, followed by distillation under nitrogen. The distilled dioxane was collected and aliquotted into glass ampoules under nitrogen. Due its flamability the dioxane was frozen in liquid nitrogen before sealing the ampoule with an acetylene flame. The prepared dioxane aliquots were stored under liquid nitrogen.

Acetic anhydride was obtained from Amersham in a gaseous form, sealed in a glass ampoule. The andydride vapours were frozen by placing the bottom of the ampoule in liquid nitrogen and gently warming the top of the ampoule with hot air. After 1 hour the top of the ampoule was swiftly removed and anhydrous dioxane introduced. The top of the ampoule was resealed and the whole

ampoule gently warmed, starting from the top, until the whole ampoule was at room temperature. The ampoule was opened and the acetic anhydride rapidly aliquotted under nitrogen into 1 mℓ glass ampoules. The ampoules were frozen and sealed with an acetylene flame and stored under liquid nitrogen. All manipulations were carried out under an extraction hood. When ampoules are removed from the liquid nitrogen, they are rapidly wrapped in absorbant tissues and placed in an extraction hood, as they can explode.

To label poly(glutamic acid) a sealed glass ampoule containing 2.5 mCi [^{3}H]acetic anhydride was opened and 0.6 mg poly(glutamic acid) (10 mg/mℓ in 10 mM phosphate buffer pH 8.0) was swiftly added. The reaction mixture was well mixed and incubated at 20°C for 30 min.

The labelled poly(glutamic acid) was purified on a small molecular sieve column. A Pasteur pipette was stopped with siliconized glass wool and packed with swollen degassed Sephadex G50. The column was eluted with 10 mM phosphate buffer pH 8.0 and 250 µℓ fractions collected.

9.5 ASSEMBLED NUCLEOSOME CORES:

9.5.1 Preparation of assembled nucleosome cores:

Cores were assembled for sucrose gradient analysis by the addition of 720 µg poly(glutamic acid) from a stock solution containing 10 mg/mℓ in 2 mM EDTA and 50 mM Tris/HCl pH 7.6 to

360 µg of a 10 mg /mℓ stock solution of histone octamers in 50% (w/v) glycerol, 1 M NaCl, 1 mM β-mercaptoethanol, 10 mM phosphate buffer pH 7.0. 436 µℓ 350 mM NaCl, 1mM EDTA, 10 mM Tris/HCl pH 7.6 was added followed by 1.7 mℓ (1 mM EDTA, 70 mM Tris/HCl pH 7.6). The random or long DNA was added as 102.8 µℓ of a 3.5 mg/mℓ solution. The mixture was incubated at 37°C for 3 hours.

The assembled polycores were dialyzed against 20 mM ammonium acetate, 0.2 mM EDTA, 2 mM β-mercaptoethanol, 5 mM Tris/HCl pH7.5 at 4°C for 2 hours. 32 µℓ of a 100 mM $CaCl_2$, 10 mM Tris/HCl pH 8.0 stock solution and 1.5 µℓ micrococcal nuclease stock solution (20 units micrococcal nuclease (Worthington)/µℓ 10 mM $CaCl_2$ and 10 mM Tris/HCl pH7.0), was added to 2 mℓ of the assembly mixture. The digestions were stopped at the appropriate times by taking out 330 µℓ aliquots and adding it to Eppendorf tubes containing, 10 µℓ of 250 mM EDTA. The redigested cores were applied to 5 to 20% (w/v) sucrose gradients containing 20 mM NaCl, 2mM EDTA and 20 mM Tris/HCl and centrifuged for 16 hours in a Beckman SW40 Ti rotor at 30 000 rpm.

To assemble nucleosome cores on 145 base pair DNA the same protocol was used as for polycores, but with a histone to DNA ratio of 1.4:1 (w/w), to compensate for the relatively smaller amount of free DNA in the spacer areas that is normally lost during the redigestion process. The products of assembly were applied to a 5 to 20% (w/v) sucrose gradient as for redigested polycores and centrifuged under the same conditions and the

core peak pooled.

9.5.2 Sucrose gradient centrifugation:

Sucrose gradients from 5-25% (w/v) sucrose in 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF were poured in Beckman SW40 tubes. Gradients were centrifuged overnight at 35 000 rpm at 4°C and then fractionated on an Isco gradient fractionater.

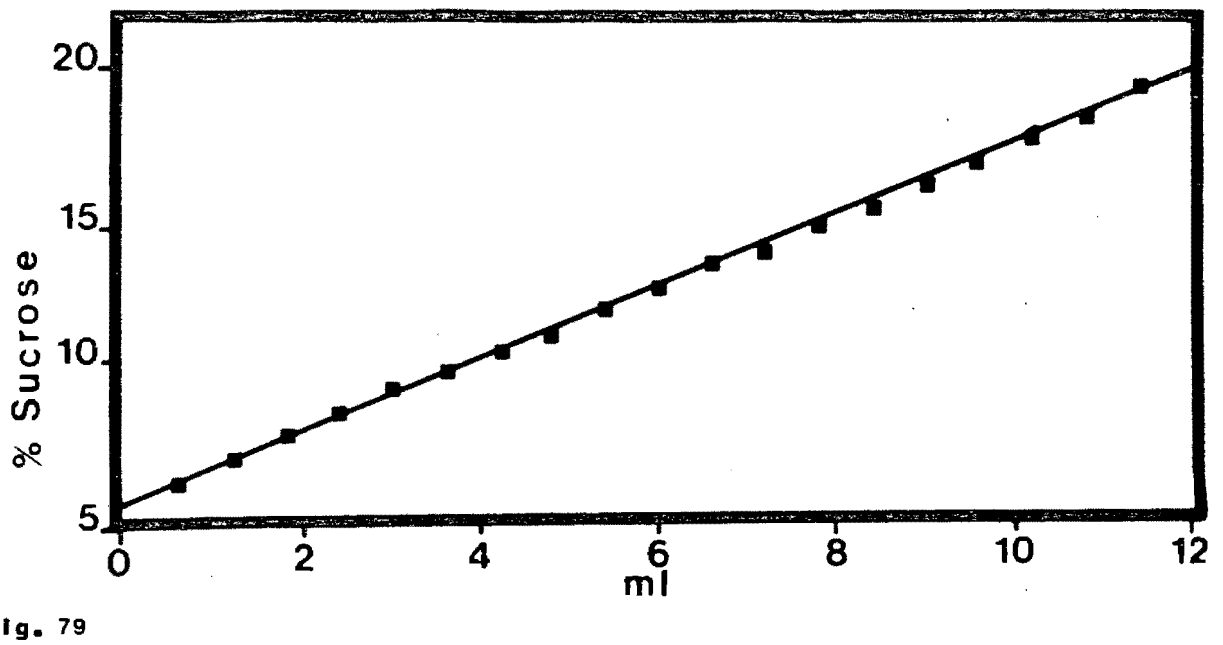


Fig. 79
The sucrose concentration of sucrose gradients after centrifugation:
Rotor: Beckman SW40 Ti.
Speed: 35 000 rpm for 16 hours. Temp: 4°C.
Buffer: 20 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 20 mM Tris/HCl pH 7.6.
After sentrifugation the gradient was fractionated and the sucrose concentration determined with a refractometer.

9.5.3 DNase I digestion:

Natural and assembled nucleosome cores were prepared, redigested and purified by ultracentrifugation on 5 to 20% (w/v) sucrose gradients as described in section 9.1.4. After centrifugation the gradient was fractionated and the core peak pooled. The cores were then diluted to a concentration of 0.1

mg/mℓ DNA in kinase buffer (10 mM $MgCl_2$, 3.6 mM DTT, 0.1 mM PMSF, 70 mM Tris/HCl pH 8.0). Of the diluted cores 200 µℓ was then placed in a microdialysis chamber and dialized for 2 hours at 4°C against kinase buffer.

After dialysis the cores were transferred to an Eppendorf tube and 4 units of polynucleotide kinase (Amersham) added, followed by 4 µℓ [gamma-^{32}P]ATP (10 mCi/mℓ approx. 5 000 Ci/mmol, Amersham). The mixture was mixed well and incubated at 37°C for for 1 hour.

The [^{32}P]-labelled cores were digested with 1 unit of DNase I from a stock solution (100 units DNase I/µℓ in 10 mM $CaCl_2$, 50% (w/v) glycerol and 10 mM Tris/HCl pH 8.0). After appropriate digestion times 20 µℓ aliquots of the digestion mixture were removed and transferred to a tube with 10 µℓ of a solution containing 1% (w/v) SDS, 25 mM EDTA, and 600 mM sodium acetate to stop the reaction. The reaction kinetics are only linear up to 100 seconds under the reaction conditions used.

The samples were prepared for electrophoresis by two phenol and two chloroform extractions, followed by ethanol precipitation. After ethanol precipitation the dried samples were made up to 20 µℓ in sample buffer, boiled for 5 min followed by rapid cooling on ice. 10 µℓ of the samples were applied to an 8% (w/v) polyacrylamide gel and electrophoresed until the bromophenol blue reached the bottom.

After electrophoresis the gel was enclosed in a plastic envelope and autoradiographed on a Dupont Cronex 4 X-ray plate at -20°C.

To quantitate the radioactivity at each cutting site, the gel was placed on its autoradiogram. The gel was then cut into pieces, each containing a 10 base pair band from one lane. The radioactivity contained in these slices was determined by liqui scintillation counting (Lutter 1978).

The rate constants for the reaction were determined according to Lutter (1978) as described in Retief et al. (1984).

9.5.4 Electron microscopy:

The sample preparation has been described previously (Retief et al. 1984).

9.5.5 Analytical hydroxyapatite chromatography:

Hydroxyapatite was prepared as before (See section 9.2.2). 0.2 mℓ of settled hydroxyapatite was packed in a Bio Rad Dispo column. The column was equilibrated in 10 mM phosphate buffer pH 6.8. 0.05 mℓ of a 5 mg/mℓ solution of BSA was applied followed by 10 µℓ of the assembly mixture. The assembled polycores were flushed with 3 aliquots of 0.2 mℓ 10 mM phosphate buffer. The column was then rinsed with 2 M NaCl, 10 mM Phospate buffer pH 6.8, followed by 0.5 M Phosphate buffer pH 6.8.

9.5.6 Analytical exclusion chromatography:

A Pasteur pipette was plugged with silinated glass wool and packed with Sepharose CL 6B. The column was equilibrated in 2 M NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.6. 0.2 mℓ of the assembly mixture was applied and the column was eluted with the above buffer. 0.2 mℓ aliquots were collected.

9.5.7 Low ionic strength gel electrophoresis:

Electrophoresis of histone DNA complexes was carried out at low ionic strength. A gel (20 cm x 20 cm x 2 mm) containing 8% (w/v) acrylamide and 0.11% (w/v) bis-acrylamide in a buffer containing 10% (w/v) glycerol, 2 µg/mℓ riboflavin, 2 mM EDTA and 10 mM triethanolamine/HCl pH 7.6 was poured and set in the presence of strong light. On top of this gel the wells were cast in a stacker gel 20 mm high containing, 5.3% (w/v) acrylamide and 0.07% (w/v) bis-acrylamide, 7% (w/v) glycerol, 2 µg/mℓ riboflavin, 1.3 mM EDTA and 6.7 mM triethanolamine/HCl pH 7.6. The samples were diluted in a sample buffer containing 80% (w/v) glycerol, 10 mM EDTA/HCl pH7.6 and bromophenol blue as a tracer dye and applied to the gel. The gel was run for 16 hours at 170 volts and 20°C with recirculating electrode buffer (2 mM EDTA and 10 mM triethanolamine pH 7.6)

9.6 INDIRECT AND DIRECT END-LABELLING:

For indirect end-labelling the electrophoretically purified vector and insert were mixed for ligation with a 100 fold excess of the vector. The preparation of the probes for indirect end-labelling is described in section 6.3.1.

To label pGV403 plasmid inserts, a typical digestion reaction contained; 18 µℓ insert containing pGV403 plasmids (6 mg/mℓ in 1 mM EDTA and 10 mM Tris/HCl pH 8.0), 2 µℓ Hinc II buffer (70 mM $MgCl_2$, 600 mM NaCl, 70 mM β-mercaptoethanol and 100 mM Tris/HCl pH 8.0), 21 units Tth 111 I. The digestion was allowed to proceed overnight at 65°C.

10 µℓ of the digestion mixture was added to 10 µℓ (20 mM $MgCl_2$, 20 mM β-mercaptoetanol and 40 mM Tris/HCl pH 7.6), 2 µℓ [α-^{32}P]dCTP (Amersham) and 1 unit Klenow fragment of DNA polymerase. The reaction was allowed to proceed at 16°C and followed by the TCA precipitation assay. The incorporation normally stopped after 10 minutes, at which time the reaction was stopped by the addition of 3 µℓ 250 mM EDTA.

A typical direct end-labelling assembly reaction was carried out as follows: 2 µℓ poly(glutamic acid) from a stock solution (10 mg poly(glutamic acid)/mℓ in 10 mM phosphate buffer pH 6.8) was placed in the bottom of an Eppendorf tube. To this 1 µℓ chicken erythrocyte octamers (6.2 mg/mℓ in 1 M NaCl, 50% (w/v) glycerol, 0.1 mM PMSF and 10 mM Tris/HCl pH

7.6) and 1 µl 1 M NaCl was added. This droplet was mixed well by repeated pipetting. Of this mixture 2 µl was transferred to the bottom af another Eppendorf tube containing 5 µl of Tth 111 I digested pGV403 in stopped labelling mixture. The volume of the assembly mixture was made up to 10 µl with 1 mM EDTA and 10 mM Tris HCl pH 7.6. This mixture was incubated for three hours at 37°C. After assembly the mixture was allowed to equilibrate at 20°C for 60 minutes, followed by 30 minutes at 16°C.

DNase I digestions were carried out by adding 3 µl 100 mM $MgCl_2$ and 10 unit DNase I (freshly diluted from a stock solution containing 100 units/µl in 10 mM $CaCl_2$ and 10 mM Tris/HCl pH 6.0). After an appropriate time at 16°C aliquots were removed from the digestion mixture and placed in 20 µl of stop solution containing: 1% (w/v) SDS, 300 mM sodium acetate, 250 mM EDTA and 1 mg/ml proteinase K. The solution was incubated at 20°C for 20 minutes, followed by 2 phenol extractions with 20 µl phenol and 3 chloroform extractions. The DNA was further purified by ethanol precipitation and washing with 80% (v/v) ethanol. The dried pellets were suspended in sequencing gel sample buffer and run on sequencing gels.

10 BIBLIOGRAPHY

Arceci,R.J.; Gross,P.R. (1980).
Developmental Biology 80 : 186-207.
Histone variants and chromatin structure during sea urchin development.

Aulehla-Scholz,Ch.; Jacob,E. (1980).
Z. Naturforsch. 35: 733-740.
Subcloning of histone DNA sequences of phage Lambda Sam 7 h 22 in plasmid pBR 322.

Ausio,J.; Seger,D.; Eisenberg,H. (1984).
J. Molec. Biol. 176: 77-104.
Nucleosome core particle stability and conformational change. - Effect of temperature, particle and NaCl concentrations, and crosslinking of histone H3 sulfhydryl groups.

Axel,R.; Melchior,W.; Soliner-Webb,JR.B.; Felsenfeld,G. (1974).
Proc. Natl. Acad. Sci. USA 71: 4101-4105.
Specific sites of interaction between histones and DNA in chromatin.

Baer,B.; Rhodes,D. (1983).
Nature 301: 482-488.
Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes.

Beard,P. (1978).
Cell 15: 955-967.
Mobility of histones on the chromosome of Simian Virus 40.

Beaudette,N.V.; Okabayashi,H.; Fasman,G.D. (1982).
Biochemistry 21: 1765-1772.
Conformational effects of organic solvents on histone complexes.

Bentley,G.A.; Lewit-Bentley,A.; Finch,J.T.; Podjarny,A.D.; Roth,M. (1984).
J. Molec. Biol. 176: 55-75.
Crystal structure of the nucleosome core particle at 16 angström.

Bernardi,G. (1971).
Methods in Enzymology 21: 95-111.
Chromatography of nucleic acids on Hydroxyapatite columns.

Birnstiel,M.; Telford,J.; Weinberg,E.; Stafford,D. (1974).
Proc. Natl. Acad. Sci. USA 71: 2900-2904.
Isolation and some properties of the genes coding for histone proteins.

Birnstiel,M.L.; Schaffner,W.; Smith,H.O. (1977).
Nature 266: 603-607.
DNA sequences coding for the H2B histone of Psammechinus miliaris.

Bloom,K.S.; Anderson,J.N. (1978).
J. Biological Chemistry. 253: 4446-4450.
Fractionation and characterization of chromosomal protein by the Hydroxyapatite dissociation method.

Böck,H.; Abler,S.; Zhang,X-Y.; Fritton,H.; Igo-Kemenes,T. (1984).
J. Molec. Biol. 176: 131-154.
Positioning of nucleosomes in satellite I-containing chromatin of rat liver.

Bonne-Andrea,C.; Harper,F.; Sobczak,J. (1984).
The EMBO Journal 3: 1193-1199.
Rat liver HMGI: a physiological nucleosome assembly factor.

Brandt,W.J.; Strickland,W.N.; Strickland,M.; Carlisle,L.; Woods,D.; Von Holt,C. (1979).
Eur. J. Biochem. 94: 1-10.
A histone programme during the life cycle of the sea urchin.

Brown,F.L.; Musich,P.R.; Maio,J.J. (1979).
J. Molec. Biol. 131: 777-799.
The repetitative sequence structure of component alpha DNA and its relationship to the nucleosomes of the African Green Monkey.

Bryan,P.N.; Hofstetter,H.; Birnstell,M.L. (1981).
Cell 27: 459-466.
Nucleosome arrangement on tRNA genes of Xenopus laevis.

Bryan,P.N.; Olah,J.; Birnstiel,M.L. (1983).
Cell 33: 843-848.
Major changes in the 5' and 3' chromatin structure of sea urchin histone genes accompany their activation and inactivation in development.

Burgoyne,L.A.; Hewish,D.R.; Mobbs,J. (1974).
Biochem. J. 143: 67-72.
Mammalian chromatin substructure studies with the Calcium-Magnesium endonuclease and two-dimensional polyacrylamide-gel electrophoresis.

Burlinghame,R.W.; Love,W.E.; Wang,B-C.; Hamlin,R.; Xuong,N-H.; Moudrianakis,E.N. (1985).
Science 228: 546-553.
Crystallographic structure of the octameric histone core of the nucleosome at a resolution of 3.3 Å.

Busslinger,M.; Portmann,R.; Birnstiel,M.L. (1979).
Nucleic Acids Research 6: 2997-3008.
A regulatory sequence near the 3' end of the sea urchin histone genes.

Busslinger,M.; Portmann,R.; Irminger,J.C.; Birnstiel,M.L. (1980).
Nucleic Acids Research 8: 957-977.
Ubiquitous and gene-specific regulatory 5' sequences in a sea urchin histone DNA clone coding for histone protein variants.

Calladine,C.R. (1982).
J. Molec. Biol. 161: 343-352.
Mechanics of sequence-dependent stacking of bases in B-DNA.

Camerini-Otero,R.D.; Sollner-Webb,B.; Felsenfeld,G. (1976).
Cell 8: 333-347.
The organization of histones and DNA in chromatin: Evidence for an arginine-rich histone kernel.

Camerini-Otero,R.D.; Felsenfeld,G. (1977).
Nucleic Acids Research 4: 1159-1181.
Supercoiling energy and nucleosome formation: the role of the arginine-rich histone kernel.

Cartwright,I.L.; Abmayer,S.M.; Fleischmann,G.; Lowenhaupt,K.; Elgin,S.C.R.; Keene,M.A.; Howard,G.C. (1982).
CRC Critical Reviews in Biochem. 13: 1-85.
Chromatin structure and gene activity: The role of nonhistone chromosomal proteins.

Chau,M.V.; Gralla,J.; Martinson,H.G. (1979).
Biochemistry 18: 1068-1074.
DNA sequence directs placement of histone cores on restriction fragments during nucleosome formation.

Childs,G.; Maxson,R.; Kedes,L.H. (1979).
Dev. Biol. 73: 153-173.
Histone gene expression during sea urchin embryogenesis: isolation and characterisation of early and late messenger RNAs of Strongylocentrotus purpuratus by gene-specific hybridisation and template activity.

Cockell,M.; Rhodes,D.; Klug,A. (1983).
J. Molec. Biol. 170: 423-446.
Location of the primary sites of micrococcal nuclease cleavage on the nucleosome core.

Cohen,L.H.; Newrock,K.M.; Zweidler,A. (1975).
Science 190: 994-997.
Stage specific switches in histone synthesis during embryogenesis of the sea urchin.

Compton,J.L.; Bellard,M.; Chambon,P. (1976).
Proc. Natl. Acad. Sci. USA 73: 4382-4386.
Biochemical evidence of variability in the DNA repeat length in the chromatin of higher eukaryotes.

Cotten,M.; Chalkley,R. (1985).
Nucleic acids research 13: 401-414.
Hyperacetylated histones facilitate chromatin assembly "in vitro".

Daban,J-R.; Cantor,C.R. (1982).
J. Molec. Biol. 156: 749-769.
Structural and kinetic study of the self-assembly of nucleosome core particles.

David,G.S.; Reisfeld,R.A. (1974).
Biochemistry 13: 1014-1021.
Protein iodination with solid state Lactoperoxidase..

Dickerson,R.E.; Drew,H.R. (1981).
Proc. Natl. Acad. Sci. USA 78: 7318-7322.
Kinematic model for B-DNA.

Dickerson,R.E. (1981).
Scientific American 249: 87-102.
The DNA helix and how it is read.

Dickerson,R.E.; Drew,H.R. (1981).
J. Molec. Biol. 149: 761-786.
Structure of a B-DNA dodecamer II: influence of base sequence on helix structure.

Dickerson,R.E. (1983).
J. Mol. Biol. 166: 419-441.
Base sequence and helix structure variation in B and A DNA..

Diekmann,S.; Wang,J.C. (1985).
J. Molec. Biol. 186: 1-11.
On the sequence determinants and flexibility of the kinetoplast DNA fragment with abnormal gel electrophoretic mobilities.

Dingwall,C.; Lomonossoff,G.P.; Laskey,R.A. (1981).
Nucleic Acids Research 9: 2659-2673.
High sequence specificity of micrococcal nuclease.

Dretzen,G.; Bellard,M.; Sassone-Corsi,P.; Chambon,P. (1981).
Analyt. Biochem. 112: 295-298.
A reliable method for the recovery of DNA fragments from agarose and acrylamide gels.

Drew,H.R. (1984).
J. Molec. Biol. 176: 535-557.
Structural specificities of five commonly used nucleases.

Drew,H.R.; Travers,A.A. (1984).
Cell 37: 491-502.
DNA structural variations in the E. coli tyrT promoter.

Drew,H.R.; Weeks,J.R.; Travers,A.A. (1985).
The EMBO Journal 4: 1025-1032.
Negative supercoiling induces spontaneous unwinding of a bacterial promoter.

Dugaiczyk,A.; Boyer,H.W.; Goodman,H.M. (1975).
J. Molec. Biol. 96: 171-184.
Ligation of Eco R1 endonuclease-generated DNA fragments into linear and circular structures.

Duhamel,R.C.; Meezan,E.; Brendel,K. (1980).
Biochemica et Biophysica Acta 626: 432-442.
Metachromatic staining with Coomassie Brilliant blue R-250 of the proline rich calf histone, H1.

Earnshaw,W.C.; Honda,B.M.; Laskey,R.A. (1980).
Cell 21: 373-383.
Assembly of nucleosomes: the reaction involving X. laevis nucleoplasmin.

Ellison,M.J.; Pulleyblank,D.E. (1983).
J. Biological Chemistry 258: 13321-13327.
Pathways of assembly of nucleohistone complexes formed in vitro under physiological conditions.

Ellison,M.J.; Pulleyblank,D.E. (1983).
J. Biological Chemistry 258: 13307-13313.
The assembly of an H2A2,H2B2,H3,H4 hexamer onto DNA under conditions of physiological ionic strength.

Ellison,M.J.; Pulleyblank,D.E. (1983).
J. Biological Chemistry 258: 13314-13320.
Internal structure of discrete nucleohistone complexes which form in vitro under conditions of physiological ionic strength.

Faulhaber,I.; Bernardi,G. (1967).
Biochim. Biophys. Acta 140: 561-564.
Chromatography of calf-thymus nucleoprotein on hydroxyapatite columns.

Fedor,M.J.; Daniell,E. (1983).
Nucleic Acids Research 11: 4417-4434.
DNase I cleavage of adenoviral nucleoprotein.

Felsenfeld,G. (1978).
Nature 271: 115-122.
Chromatin.

Finch,J.T.; Brown,R.S.; Rhodes,D.; Richmond,T.; Rushton,B.; Lutter,L.C.; Klug,A. (1981).
J. Molec. Biol. 145: 757-769.
X-ray diffraction study of a new crystal form of the nucleosome core showing higher resolution.

Fittler,F.; Zachau,H.G. (1979).
Nucelc Acids Research 7: 1-13.
Subunit structure of &-satellite DNA containing chromatin from African green monkey cells.

Gargiulo,G.; Worcel,A. (1983).
J. Molec. Biol. 170: 699-722.
Analysis of the chromatin assembled in germinal vesicles of Xenopus oocytes.

Garoff,H.; Ansörge,W. (1981).
Analytical Biochemistry 115: 450-457.
Improvements of DNA sequencing gels.

Germond,J.E.; Hirt,B.; Oudet,P.; Gross-Bellard,M.; Chambon,P. (1975).
Proc. Nat. Acad. Sci. USA 72: 1843-1847.
Folding of the DNA double helix in chromatin-like structures from Simian Virus 40.

Germond,J-E.; Bellard,M.; Oudet,P.; Chambon,P. (1976).
Nucleic Acids Research 11: 3173-3192.
Stability of nucleosomes in native and reconstituted chromatins.

Germond,J.-E.; Ronviere-Yaniv,J.; Yaniv,M.; Brutlag,D. (1979).
Proc. Natl. Acad. Sci. USA 76: 3779-3783.
Nicking-closing enzyme assembles nucleosome-like structures "in vitro".

Glikin,G.C.; Ruberti,I.; Worcel,A. (1984).
Cell 37: 33-41.
Chromatin assembly in Xenopus oocytes: in vitro studies.

Glotov,B.O.; Rudin,A.V.; Severin,E.S. (1982).
Biochimica et Biophysica Acta. 696: 275-284.
Conditions for sliding of nucleosomes along DNA: SV 40 minichromosomes.

Gottesfeld,J.M. (1980).
Nucleic Acids Research 8: 905-923.
Organisation of 5S genes in chromatin of Xenopus laevis.

Gottesfeld,J.M.; Bloomer,L.S. (1980).
Cell 21: 751-760.
Nonrandom alignment of nucleosomes on 5s RNA genes of X. laevis.

Gottesfeld,J.; Bloomer,L.S. (1982).
Cell 28: 781-791.
Assembly of transcriptionally active 5S RNA gene chromatin in vitro.

Greulich,K.O.; Ausio,J.; Eisenberg,H. (1985).
J. Molec. Biol. 186: 167-173.
Nucleosome core particle structure and structural changes in solution.

Greyling,H.J.; Schwager,S.; Sewell,B.T.; von Holt,C. (1983).
Eur. J. Biochem. 137: 221-226.
The identity of conformational states in reconstituted and native histone octamers.

Haeutle,M.T.; Martinson,H.G. (1983).
J. Cell Biol. 97: 387-387.
A nuclease-hypersensitive DNA sequence reconstitutes efficiently into nucleosomes and the degree of sequence specificity of reconstitution depends on the method of assembly.

Hagerman,P.J. (1984).
Proc. Natl. Acad. Sci. USA 81: 4632-4636.
Evidence for the existence of stable curvature of DNA in solution.

Hagerman,P.J. (1985).
Biochemistry 24: 7033-7036.
Sequence dependent curvature of DNA: a test of the phasing hypothesis.

Hentschel,C.; Irminger,J-C.; Bucher,P.; Birnstiel,M.L. (1980).
Nature 285: 147-151.
Sea urchin histone mRNA termini are located in gene regions downstream from putative regulatory sequences.

Hentschel,C.C.; Birnstiel,M.L. (1981).
Cell 25: 301-313.
The organisation and expression of histone gene families.

Hentschel,C.C. (1982).
Nature 295: 714-716.
Homocopolymer sequences in the spacer of a sea urchin histone gene repeat are sensitive to S1 nuclease.

Hiwasa,T.; Segawa,M.; Yamaguchi,N.; Oda,K. (1981).
J. Biochem. 89: 1375-1389.
Phasing of nucleosomes in SV40 chromatin reconstituted in vitro..

Hogan,M.; LeGrange,J.; Austin,B. (1983).
Nature 304: 752-754.
Dependence of DNA helix flexibility on base composition.

Holt,C.A.; Childs,G. (1984).
Nucleic Acids Research. 12: 6455-6471.
A new family of tandem repetitive early histone genes in the sea urchin Lytechinus pictus: evidence for concerted evolution within tandem arrays..

Hörz,W.; Altenburger,W. (1981).
Nucleic Acids Research 9: 2643-2658.
Seq.ence specific cleavage of DNA by micrococcal nuclease.

Hu,N.; Messing,J. (1982).
Gene 17: 271-277.
The making of strand-specific M13 probes.

Igo-Kemenes,T.; Omori,A.; Zachau,H.G. (1980).
Nucleic Acids Research 8: 5377-5391.
Non-random arrangement of nucleosomes in satellite I containing chromatin from rat liver.

Igo-Kemenes,T.; Hörz,W.; Zachau,H.G. (1982).
Ann. Rev. Biochem. 51: 89-121.
Chromatin.

Jakobovits,E.B.; Bratosin,S.; Aloni,Y. (1982).
Virology 120: 340-348.
Formation of a nucleosome-free region in SV40 minichromosomes is dependent upon a restricted segment of DNA.

Johnson,R.A.; Walseth,T.F edited by Brooker,G.; Greengard,P.; Robison,G.A. (1979).
Advances in Cyclic Nucleotide Res. 19: 135-167.
The enzymatic preparation of [α-^{32}P]ATP, [α-^{32}P]GTP, [^{32}P]cAMP, and [^{32}P]cGMP, anf their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases.

Johnston,B.H.; Rich,A. (1985).
Cell 42: 713-724.
Chemical probes of DNA conformation: Detection of Z-DNA at nucleotide resolution.

Kedes,L.H.; Birnstiel,M.L. (1971).
Nature New Biology 230: 165-169.
Reiteration and clustering of DNA sequences complementary to histone messenger RNA.

Kedes,L.H. (1979).
Ann. Rew. Biochem. 48: 837-70.
Histone genes and histone messengers.

Khowi-Shigematsu,T.; Khowi,Y. (1985).
Cell 43: 199-206.
Poly(dG)-poly(dC) sequences, under tortional stress, induces altered DNA conformation upon neighbouring DNA sequences.

Kievan,L.; Dattagupta,N.; Hogan,M.; Crothers,D.M. (1978).
Biochemistry 17: 4533-4540.
Physical studies of nucleosome assembly.

Klug,A.; Lutter,L.C. (1981).
Nucelc Acids Research. 9: 4267-4283.
The helical periodicity of the DNA on the nucleosome.

Klug,A.; Finch,J.T.; Richmond,J.T. (1985).
Science 229: 1109-1110.
Crystallographic structure of the octamer histone core of the nucleosome.

Kornberg,R.D. (1974).
Science 184: 868-871.
Chromatin structure: a repeating unit of histones and DNA.

Kornberg,R.D. (1977).
Ann. Rev. Biochem. 46: 931-954.
Structure of chromatin.

Kornberg,R.D.; Klug,A. (1981).
Scientific American 244: 48-60.
The nucleosome.

Kornberg,R. (1981).
Nature 292: 579-580.
The location of nucleosomes in chromatin: specific or statistical?

Krohne,G.; Franke,W.W. (1980).
Proc. Natl. Acad. Sci. USA 77: 1034-1038.
Immunological identification and localization of the predominant nuclear protein of the amphibian oocyte nucleus..

Krone,G.; Franke,W.W. (1980).
Experimental Cell Research 129: 167-189.
A major soluable acidic protein located in nuclei of diverse vertebrate species..

Kunkel,G.R.; Martinson,H.G. (1981).
Nucleic Acids Research 9: 6869-6888.
Nucleosomes will not form on double stranded RNA or over poly(dA)-poly(dT) tracts in recombinant DNA.

Laskey,R.A.; Mills,A.D.; Morris,N.R. (1977).
Cell 10: 237-243.
Assembly of SV40 chromatin in a cell-free system from Xenopus eggs.

Laskey,R.A.; Honda,B.M.; Mills,A.D.; Finch,J.T. (1978).
Nature 275: 416-420.
Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA.

Laskey,R.A.; Earnshaw,W.C. (1980).
Nature 286: 763-767.
Nucleosome assembly.

Levy,A.; Noll,M. (1980).
Nucleic Acids Research 8: 6059-6068.
Multiple phases of nucleosomes in the hsp 70 genes of Drosophila melanogaster.

Lilley,D.M.J. (1980).
Proc. Natl. Acad. Sci. USA 77: 6468-6472.
The inverted repeat as a recognizable structural feature in supercoiled DNA molecules.

Linxweiler,W.; Hörz,W. (1984).
Nucleic Acids Rsearch 12: 9395-9413.
Reconstitution of mononucleosomes: characterisation of distinct particles that differ in the position of the core.

Linxweiler,W.; Hörz,W. (1985).
Cell 42: 281-290.
Reconstitution experiments show that sequence specific histone-DNA interactions are the basis for nucleosome phasing on mouse satellite DNA.

Lomonossoff,G.P.; Butler,P.J.G.; Klug,A. (1981).
J. Molec. Biol. 149: 745-760.
Sequence-dependent variation in the conformation of DNA.

Louis,C.; Schedl,P. (1980).
Cell 22: 387-392.
Chromatin structure of the 5S RNA genes of D. melanogaster.

Lu,A-L.; Steege,D.A.; Stafford,D.W. (1980).
Nucleic Acids Research 8: 1839-1853.
Nucleotide sequence of a 5S ribosomal RNA gene in the sea urchin Lytechinus variegatus.

Lutter,L.C. (1978).
J. Molec. Biol. 124: 391-420.
Kinetic analysis of Deoxyribonuclease I cleavages in the nucleosome core: Evidence for a DNA superhelix.

Maniatis,T.; Fritsch,E.F.; Sambrook,J. (1982).

Molecular cloning: a laboratory manual.

Maxam,A.M.; Gilbert,W. (1977).
Proc. Natl. Acad. Sci. USA 74: 560-564.
A new method for sequencing DNA.

Maxam,A.M.; Gilbert,W. (1980).
Methods in Enzymology 65: 499-530.
Sequencing end-labeled DNA with base-specific chemical cleavages.

McGhee,J.D.; Felsenfeld,G.; Eisenberg,H. (1980).
Biophys. J. : 261-270.
Nucleosome structure and conformational changes.

McGhee,J.D.; Felsenfeld,G. (1980).
Ann. Rev. Biochem. 49: 1115-1156.
Nucleosome structure.

McGhee,J.D.; Felsenfeld,G. (1983).
Cell 32: 1205-1215.
Another potential artifact in the study of nucleosome phasing by chromatin digestion with Micrococcal nuclease.

McMurray,C.T.; van Holde,K.E. (1985).
Biochemistry 24: 7037-7044.
Proton NMR investigation of the nucleosome core particle: Evidence for regions of altered hydrogen bonding.

Mengeritsky,G.; Trifonov,E.N. (1983).
Nucleic Acids Research 11: 3833-3851.
Nucleotide sequence directed mapping of the nucleosomes..

Mengeritsky,G.; Trifonov,E.N. (1984).
Cell Biophysics 6: 1-8.
Nucleotide sequence-directed mapping of the nucleosomes of SV40 chromatin.

Messing,J.; Gronenborn,B.; Muller-Hill,B.; Hofschneider,P.H. (1977).
Proc. Natl. Acad. Sci. USA 74: 3642-3546.
Filamentous coliphage M13 as a cloning vehicle: insertion of a hind II fragment of the lac regulatory region in M13 replicative form in vitro.

Messing,J. (1983).
Methods in Enzymology 111: 20-35.
New M13 vectors for cloning.

Michalski-scrive,C.; Aubert,J-P.; Couppez,M.; Biserte,G. (1982).
Biochimie 64: 347-355.
UV differential study of the histones H2A-H2B-H3-H4 octamer.

Miller,I.R.; Great,H. (1972).
Biopolymers. 11: 2533-2536.
Protein labeling by Acetylation.

Miller,F.D.; Rattner,J.b.; and Van de Sande,J.H. (1982).
Coldspring Harbour Symposia 47: 571-575.
Nucleosome-core assembly on B and Z forms of poly[d(G-m5C)].

Miller,F.D.; Dixon,G.H.; Rattner,J.B.; van de Sande,J.H. (1985).
Biochemistry 24: 102-109.
Assembly and characterization of nucleosomal cores on B- vs. Z-form DNA.

Mills,A.D.; Laskey,R.A.; Black,P.; DeRobertis,E.M. (1980).
J. Molec. Biol. 139: 561-568.
An acidic protein which assembles nucleosomes "in vitro" is the most abundant protein in Xenopus oocyte nuclei.

Mirzabekov,A.D.; Rich,A. (1979).
Proc. Natl. Acad. Sci. USA 76: 1118-1121.
Asymmetric lateral distribution of unshielded phosphate groups in nucleosomal DNA and its role in DNA bending.

Mirzabekov,A.D. (1980).
Quatrerly Review of Biophysics 13: 255-295.
Nucleosomes structure and its dynamic transitions.

Montelaro,R.C.; Rueckert,R.R. (1975).
J. Biol. Chem. 250: 1413-1421.
Radiolabeling of proteins and viruses "in vitro" by acetylation with radioactive acetic anhydride.

Moudrianakis,E.N.; Love,W.E.; Wang,B.C.; Young,N.G.; Burlinghame,R.W. (1985).

Science 229: 1110-1112.
Reply to Klug et al. (1985).

Muller,M.T. (1983).
Biochem. Biophys. Res. Comm. 114: 99-106.
Nucleosomes contain DNA binding proteins that resist dissociation by Sodium Dodecyl Sulphate.

Musich,P.R.; Malo,J.J.; Brown,F.L. (1977).
J. Molec. Biol. 117: 657-677.
Subunit structure of chromatin and the organisation of eukaryotic highly repetitave DNA: indications of a phase relation between restriction sites and chromatin subunits in African Green Monkey and calf nuclei.

Musich,P.R.; Brown,F.L.; Malo,J.J. (1982).
Proc. Natl. Acad. Sci. USA 79: 118-122.
Nucleosome phasing and the micrococcal nuclease cleavage of African green monkey component alpha DNA.

Nedospasov,S.A.; Georgiev,G.P. (1980).
Biochem. Biophys. Ress. Comm. 92: 532-539.
Non-random cleavage of SV40 in the compact minichromosome and free in solution by micrococcal nuclease.

Nelson,T.; Hsieh,T-S.; Brutlag,D. (1979).
Proc. Natl. Acad. Sci. USA 76: 5510-5514.
Extracts of Drosophila embiyos mediate chromatin-assembly in vitro.

Nelson,T.; Wiegand,R.; Brutlag,D. (1981).
Biochemistry 20: 2594-2601.
Ribonucleic acid and other polyanions facilitate chromatin assembly in Vitro.

Neuhoff,V.; Stamm,R.; Eibl,H. (1985).
Electrophoresis 6: 427-448.
Clear background and highly sensitive protein staining with Coomassie blue dyes in polyacrylamide gels: A systematic analysis.

Nickol,J.; Martin,R.G. (1983).
Proc. Natl. Acad. Sci. USA 80: 4669-4673.
DNA stem-loop structures bind poorly to histone octamer cores.

Oohara,I.; Suyama,A.; Wada,A. (1983).
Biochimica et Biophysica Acta 741: 322-332.
Reconstitution mechanism of nucleosome core particles mediated by poly(L-Glutamic acid).

Oudet,P.; Gross-Bellard,M.; Chambon,P. (1975).
Cell 4: 281-300.
Electron microscopic and biochemical evidence that chromatin is a repeating structure.

Oudet,P.; GermondJ.E.; Sures,M.; Gallwitz,D.; Bellard,M.; Chambon,P. (1977).
Cold Spring Harbour 42: 287-300.
Nucleosome structure I: All four histones, H2A,H2B,H3 and H4, are required to form a nucleosome, but an H3-H4 subnucleosomal particle is formed with H3-H4 alone.

Panayotatos,N.; Wells,R.D. (1981).
Nature 289: 466-470.
Cruciform structures in supercoiled DNA.

Paull,U.H.; Seebeck,T.; Braun,R. (1982).
Nucleic acids research 10: 4121-4133.
Sequence-specific cleavage of chromatin by staphylococcal nuclease can generate an atypical nucleosome pattern.

Peacock,A.C.; Dingman,C.W. (1967).
Biochemistry 6: 1818-1827.
Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis.

Peck,L.J.; Nordheim,A.; Rich,A.; Wang,J.C. (1982).
Proc. Natl. Acad. Sci. USA 79: 4560-4564.
Flipping of cloned d(pCpG)n.d(pCpG)n DNA sequences from right- to left-handed helical structure by salt, Co(III), or negative supercoiling.

Pfeiffer,W.; Zachau,H.G. (1980).
Nucleic Acids Research 8: 4621-4638.
Accessability of expressed and non-expressed genes to a restriction nuclease.

Ponder,B.A.J.; Crawford,L.V. (1977).
Cell 11: 35-49.
The arrangement of nucleosomes in nucleoprotein complexes from polyoma virus and SV40.

Portmann,R.; Schaffner,W.; Birnstiel,M.L. (1976).
Nature 264: 31-34.
Partial denaturation mapping of cloned histone DNA from the sea urchin Psammechinus miliaris.

Portmann,R.; Bussinger,M. (1979).
Experimentia 35: 974-974.
Is the histone-H1 gene a transposable element?

Prunell,A.; Kornberg,R.D. (1977).
Cold Spring Harbour Symposia 42: 103-108.
Relation of nucleosomes to DNA sequences.

Prunell,A.; Kornberg,R.D.; Lutter,L.; Klug,A.; Levitt,M.; Crick,F.H.C. (1979).
Science 204: 855-858.
Periodicity of deoxyribonuclease I digestion of chromatin.

Prunell,A. (1982).
The EMBO Journal 1: 173-179.
Nucleosome reconstitution on plasmid-inserted poly(dA).poly(dT)..

Ramm,E.I.; Zalenskaya,I.A; Zalensky,A.O.; Vorobyev,V.I. (1983).
Biophys. 28: 743-744.

Ramm,E.I.; Zalenskaya,I.A; Zalensky,A.O.; Vorobyev,V.I. (1983).
Biophys. 28: 743-744.
Investigation of reconstitution and association processes of nucleosomes with various histone contents.

Ramsay,N.; Felsenfeld,G.; Rushton,B.M.; McGhee,J.D. (1984).
The EMBO Journal 3: 2605-2611.
A 145-base pair sequence that positions itself precisely and asymmetrically on the nucleosome core.

Retief,J.D.; Sewell,B.T.; Greyling,H.J.; Schwager,S.; von Holt,C. (1984).
FEBS Letters 167: 170-175.
Probing with DNase I of nucleosomal core particles assembled in vitro in the presence of polyglutamic acid.

Richmond,T.J.; Finch,J.T.; Rushton,B.; Rhodes,B.; Klug,A. (1984).
Nature 311: 532-537.
Structure of the nucleosome core particle at 7 A resolution.

Rigby,P.W.J.; Dieckmann,M.; Rhodes,C.; Berg,P. (1977).
J. Molec. Biol. 113: 237-251.
Labeling deoxyribonucleic acid to high specific activity "in vitro" by nick translation with DNA polymerase I.

Rindt,K.-P.; Nover,L. (1980).
Biologisches Zentralblatt 99: 641-673.
Chromatin structure and function.

Ruiz-Carillo,A.; Jorcano,J.L. (1979).
Biochemistry 18: 768-774.
H3.H4 Tetramer directs DNA and core histone octamer assembly in the nucleosome core particle.

Ruiz-Carillo,A.; Jorcano,J.L.; Eder,G.; Lurz,R. (1979).
Proc. Natl. Acad. Sci. USA 76: 3284-3288.
In vitro particle and nucleosome assembly at physiological ionic strength.

Russel,W.C.; Precious,B.; Martin,S.R.; Bayley,P.M. (1983).
The EMBO Journal 2: 1647-1653.
Differential promotion and supression of Z - B transitions in polydG-Cl by histone subclasses, polyamino acids and polyamines.

Samal,B.; Worcel,A. (1981).
Cell 23: 401-409.
Chromatin structure of the histone genes of D. melanogaster.

Sanger,F.; Coulson,A.R. (1975).
J. Molec. Biol. 94: 441-448.
A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase.

Saragosti,S.; Moyne,G.; Yaniv,M. (1980).
Cell 20: 65-73.
Absence of nucleosomes in a Fraction of SV40 chromatin between the origen of replication and the region coding for the late leader RNA.

Schaffner,W.; Gross,K.; Telford,J.; Birnstell,M. (1976).
Cell 8: 471-478.
Molecular analysis of the histone gene cluster of Psammechinus miliaris : II. The arrangement of the five histone-coding and spacer sequences.

Schaffner,W.; Kunz,G.; Daetwyler,H.; Telford,J.; Smith,H.O.; Birnstiel,M.L. (1978).
Cell 14: 655-671.
Genes and spacers of cloned sea urchin histone DNA analysed by sequencing.

Schlaeger,E-J. (1981).
Biochem. Biophys. Res. Comm. 103: 674-681.
Nucleosome assembly capacity of nuclear lysate after nuclease digestion.

Schwager,S.; Retief,J.D.; de Groot,P.; von Holt,C. (1985).
FEBS Letters 189: 305-309.
Rapid exchange of histones H2A and H2B in sea urchin embryo chromatin.

Shakket,Z.; Rabinovich,D. (1983).
J. Molec. Biol. 166: 183-201.
Sequence-dependent conformation of an A-DNA double helix: The crystal structure of the octamer d(G-G-T-A-T-A-C-C).

Shaw,B.R.; Richards,R.G.; and Gross,P.M. (1978).
Chromatin structure and function : 125-136.
Preparation and analysis of core particles and nucleosomes: A convenient method for studying the protein composition of nucleosomes using protamine-release into triton-acid-urea gels.

Simon,R.H.; Camerini-Otero,R.D.; Felsenfeld,G. (1978).
Nucleic Acids Research 5: 4805-4818.
An octamer of histones H3 and H4 forms a compact complex with DNA of nucleosome size.

Simon,R.H.; Felsenfeld,G. (1979).
Nucleic Acids Research 6: 689-696.
A new procedure for purifying histone pairs H2A+H2B and H3+H4 from chromatin using hydroxylapatite.

Simpson,R.T.; Kunzler,P. (1979).
Nucleic Acids Research 6: 1387-1415.
Chromatin and core particles formed from the inner histones and synthetic polydeoxyribonucleotides of defined sequence..

Simpson,R.T.; Stafford,D.W. (1983).
Proc. Patl. Acad. Sci. USA 80: 51-55.
Structureal features of a phased nucleosome core particle.

Simpson,R.T.; Thoma,F.; Brubaker,J.M. (1985).
Cell 42: 799-808.
Chromatin reconstituted from tandemly repeated cloned DNA fragments and core histones: A model system for study of higher order structure.

Singer,D.S. (1979).
J. Biol. Chem. 254: 5506-5514.
Arrangement of a highly repeated DNA sequence in the genome and chromatin of the African Green Monkey.

Smith,H.O. (1980).
Methods in Enzymology 65: 371-380.
Recovery of DNA from gels.

Soliner-Webb,B.; Camarini-Otero,R.D.; Felsenfeld,G. (1976).
Cell 9: 179-193.
Chromatin structure as probed by nucleases and proteases: evidence for the central role of histones H3 and H4.

Southern,E.M. (1975).
J. Molec. Biol. 98: 503-517.
Detection of specific sequences among DNA fragments separated by gel electrophoresis.

Stein,A.; Bina-Stein,M.; Simpson,R.T. (1977).
Proc. Natl. Acad. Sci. USA 74: 2780-2784.
Crosslinked histone octamer as a model of the nucleosome core.

Steinmetz,M.; Streeck,R.E.; Zachau,H.G. (1978).
Eur. J. Biochem. 83: 615-628.
Histone-DNA complexes and histone H1-depleted chromatin.

Stein,A. (1979).
J. Molec. Biol. 130: 103-134.
DNA folding by histones: The kinetics of chromatin core particle reassembly and the interaction of nucleosomes with histones.

Stein,A.; Whitlock Jr,J.P.; Bina,M. (1979).
Proc. Natl. Acad. Sci. USA 76: 5000-5004.
Acidic polypeptides can assemble both histones and chromatin in vitro at physiological ionic strength.

Stein,A.; Holley,K.; Zelliff,J.; Townsend,T. (1985).
Biochemistry 24: 1783-1790.
Interactions between core histones and chromatin at physiological ionic strength.

Stockley,P.G.; Thomas,J.O. (1979).
FEBS Letters 99: 129-135.
A nucleosome-like particle containing an octamer of the argenine-rich histones H3 and H4.

Sutcliffe,J.G. (1978).
Nucleic Acids Research 5: 2721-2728.
pBR322 restriction map derived from DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long.

Tatchell,K.; Van Holde,K.E. (1977).
Biochemistry 16: 5295-5303.
Reconstitution of chromatin Core particles.

Tautz,D.; Renz,M. (1984).
Nucleic acids research 12: 4127-4138.
Simple sequences are ubiquitous repetitive components of eukaryotic genomes.

Thomas,J.O.; Kornberg,R.D. (1975).
Proc. Natl. Acad. Sci. USA 72: 2626-2630.
An octamer of histones in chromatin and free in solution.

Thomas,T.O.; Thompson,R.J. (1977).
Cell 10: 633-640.
Variation in chromatin structure in two cell types from the same tissue: a Short DNA repeat length in cerebral cortex neurons.

Thomas,J.O.; Oudet,P. (1979).
Nucleic Acids Research 7: 611-623.
Complexes of the arginine rich histone tetramer (H3)2(H4)2 with negatively supercoiled DNA: electron microscopy and chemical cross-linking.

Trifonov,E. (1978).
Nucleic Acids Research 5: 1371-1380.
The helical model of the nucleosome core.

Trifonov,E.N.; Bettecken,T. (1979).
Biochemistry 18: 454-456.
Noninteger pitch and nuclease sensitivity of chromatin DNA.

Trifonov,E.N. (1980).
Nucleic Acids Research 8: 4041-4053.
Sequence-dependent deformational anisotrophy of chromatin DNA.

Trifonov,E.N.; Sussman,J.L. (1980).
Proc. Natl. Acad. Sci. USA 77: 3816-3820.
The pitch of chromatin DNA is reflected in its nucleotide sequence.

Trifonov,E.N. (1983).
Cold Spring Harbour Sym on Quant B 47: 271-277.
Sequence-dependent variations of B-DNA structure and protein-DNA recognition.

Trifonov,E.N. (1983).
Nucleic Acids:The vectors of life : 373-385.
Nucleosomal DNA structure.

Trifonov,E.N.; Konopka,A.K.; Jovin,T.M. (1985).
FEBS Letters 185: 197-202.
Unusual frequencies of certain alternating purine-pyrimidine runs in natural DNA sequences: relation to Z-DNA.

Varshavsky,A.J.; Nedospasov,S.A.; Schmatchenko,V.V.; Bakayev,P.M.; Chumackov,P.M.; Georgiev,G.P. (1977).
Nucleic Acids Research 4: 3303-3325.
Compact form of SV40 viral minichromosome is resistant to nuclease: possible implications for chromatin structure.

Varshafsky,A.J.; Sundin,O.; Bohn,M. (1979).
Cell 16: 453-466.
A strech of "Late" SV40 viral DNA about 400 bp long which includes the origen of replication is specifically exposed in SV40 minichromosomes.

Villeponteau,B.; Lundell,M.; Martinson,H. (1984).
Cell 39: 469-478.
Torsional stress promotes the DNase I sensitivity of active genes.

Volkaert,G.; De Vleeschouwer,E.; Blocker,H.; Frank,R. (1984).
Gene Anal. Techn. 1: 52-59.
A novel type of cloning vectors for ultrarapid chemical degradation sequencing of DNA.

Von Holt,C.; De Groot,P.; Schwager,S.; Brant,W.F. (1984).
Histone genes. : 65-105.
The structure of sea urchin histones and considerations on their function.

Von Holt,C. (1985).
Bio Essays 3: 120-124.
Histones in perspective.

Wang,A.H.-J.; Quigley,G.J.; Kolpak,F.J.; Crawford,J.L.; van Boom,J.H.; van der Marel,G.; Rich,A. (1979).
Nature 282: 680-686.
Molecular structure of a left-handed double helical DNA fragment at atomic resolution.

Wang,J.C. (1979).
Proc. Natl. Acad. Sci. USA 76: 200-203.
Helical repeat of DNA in solution.

Wang,A.H.-J.; Fujii,S.; van Boom,J.H.; Rich,A. (1982).
Proc. Natl. Acad. Sci. USA 79: 3968-3972.
Molecular structure of the octamer d(G-G-C-C-G-G-C-C): modified DNA.

Wang,A.H.-J.; Fujii,S.; van Boom,J.H.; van der Marel,G.A.; van Boeckel,S.A.A.; Rich,A. (1982).
Nature 299: 601-604.
Molecular structure of r(GCG)d(TATACGC): a DNA-RNA hybrid helix joined to double helical DNA.

Wartenfeld,R.; Mengeritsky,G.; Trifonov,E. (1984).
CODATA Bull. 56: 14-17.
Periodicity in nucleosomal DNA sequences.

Wasylyk,B.; Oudet,P.; Chambon,P. (1979).
Nucleic Acids Research 7: 705-713.
Preferential in vitro assembly of nucleosome cores on some AT-rich regions of SV40 DNA.

Wegrzynowicz,Z.; Kloczewiak,M.; Kopec,M. (1975).
J. Lab. Clin. Med. 86: 360-368.
Bovine fibrinogen modified with 3H-acetic anhydride.

Weichet,W.O. (1979).
Nucleic Acids Research 7: 291-304.
On the de novo formation of compact oligosomes at high ionic strength. Evidence for nucleosome sliding in high salt.

Weintraub,H. (1983).
Cell 32: 1191-1203.
A dominant role for DNA secondary structure in forming hypersensitive structures in chromatin.

Widom,J. (1984).
BioEssays 2: 11-14.
Bent DNA for gene regulation and DNA packaging.

Widom,J. (1984).
Nature 309: 312-313.
DNA bending and kinking.

Wilhelm,F.X.; Wilhelm,M.L.; Erard,M.; Daune,M.P. (1978).
Nucleic Acids Research 5: 505-521.
Reconstitution of chromatin: Assembly of the nucleosome.

Wilhelm,M.L.; Wilhelm,F.X. (1980).
Biochemistry 19: 4327-4331.
Conformation of nucleosome core particles and chromatin in high salt concentration.

Wittig,B.; Wittig,S. (1979).
Cell 18: 1173-1183.
A phase relationship associates tRNA structural gene sequences with nucleosome cores.

Wittig,S.; Wittig,B. (1981).
Hoppe Seylers Z. 362: 386-386.
In vitro reconstitution of nucleosomes on tRNA genes and transcription of the reconstituted chromatin.

Wittig,S.; Wittig,B. (1982).
Nature 297: 31-38.
Function of a tRNA gene promotor depends on nucleosome position.

Wittig,S.; Wittig,B. (1982).
Nucelc Acids Research 10: 3647-3665.
Site directed in vitro assembly of nucleosomes.

Worcel,A.; Gargiulo,G.; Jessee,B.; Udvardy,A.; Louis,C.; Schedl,P. (1983).
Nucleic Acids Research 11: 421-439.
Chromatin fine structure of the histone gene complex of Drosophila melanogaster.

Wu,C. (1980).
Nature 286: 854-859.
The 5' ends of the Drosophila heat shock genes in chromatin are hypersensitive to DNase I.

Wu,H-M.; Crothers,D.M. (1984).
Nature 308: 509-513.
The locus of sequence-directed and protein-induced DNA bending.

Yoshida,M. (1983).
Biochem. Biophys. Res. Comm. 116: 217-221.
Mg^{2+} , Ca^{2+} dependent unwinding of DNA by poly-L-glutamic acid.

Zachau,H.G.; Igo-Kemenes,T. (1981).
Cell 24: 597-598.
Face to phase with nucleosomes.

Zhang,X-Y.; Fittler,F.; Hörz,W. (1983).
Nuclei Acids Research 11 : 4287-4306.
Eight different highly specific nucleosome phases on alpha-sattelite DNA in the African green monkey.

Zhang,X-Y.; Hörz,W. (1984).
J. Molec. Biol. 176: 105-129.
Nucleosomes are positioned on mouse satellite DNA in multiple highly specific frames that are correlated with a divergent subrepeat of nine base pairs.

Zhurkin,V.B. (1983).
FEBS Letters 158: 293-297.
Specific alignment of nucleosomes on DNA correlates with periodic distribution of purine-pyrimidine and pyrimidine-purine dimers.

Zweig,G.; Sherma,J. (Eds) (1964).
Handbook of Chromatography 1: 504-506.
Nucleotides.

APPENDIX A

Additional autoradiograms and densitometer traces:

Fig.	1a	Enlargement of Fig. 61
	1b	Densitometer trace, STOP fragment free DNA (Fig. 61).
	1c	Densitometer trace, STOP fragment assembled (Fig. 61).
Fig.	2a	END fragment, dCTP labelled.
	2b	Densitometer trace, END fragment free DNA (Fig. 62).
	2c	Densitometer trace, END fragment assembled (Fig. 62).
	2d	Densitometer trace, END fragment and histones (Fig. 62).
Fig.	3a	END fragment, dCTP labelled.
	3b	END fragment, dCTP labelled, enlarged.
	3c	Densitometer trace, END fragment free DNA.
	3d	Densitometer trace, END fragment assembled.
Fig.	4a	END fragment, dTTP labelled.
	4b	END fragment, dTTP labelled, enlarged.
	4c	Densitometer trace, END fragment free DNA.
	4d	Densitometer trace, END fragment assembled.
Fig.	5a	UPSTREAM fragment, dCTP labelled.
	5b	UPSTREAM fragment, dCTP labelled, enlarged.
	5c	Densitometer trace, UPSTREAM fragment free DNA.
	5d	Densitometer trace, UPSTREAM fragment assembled.
Fig.	6a	UPSTREAM fragment, dTTP labelled.
	6b	UPSTREAM fragment, dTTP labelled, enlarged.
	6c	Densitometer trace, UPSTREAM fragment free DNA.
	6d	Densitometer trace, UPSTREAM fragment assembled.
Fig.	7	UPSTREAM fragment, dCTP labelled, low protein ratio.

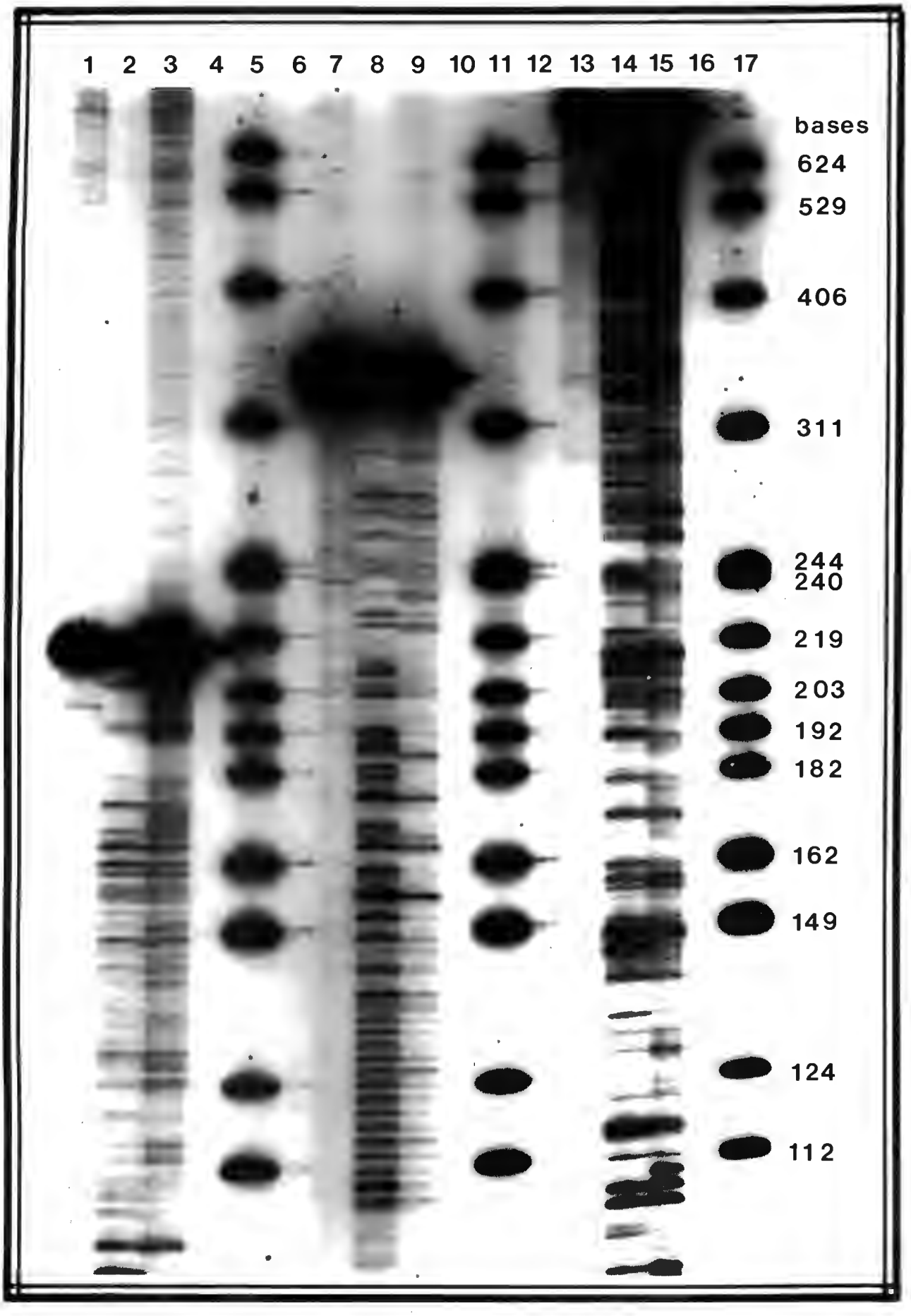
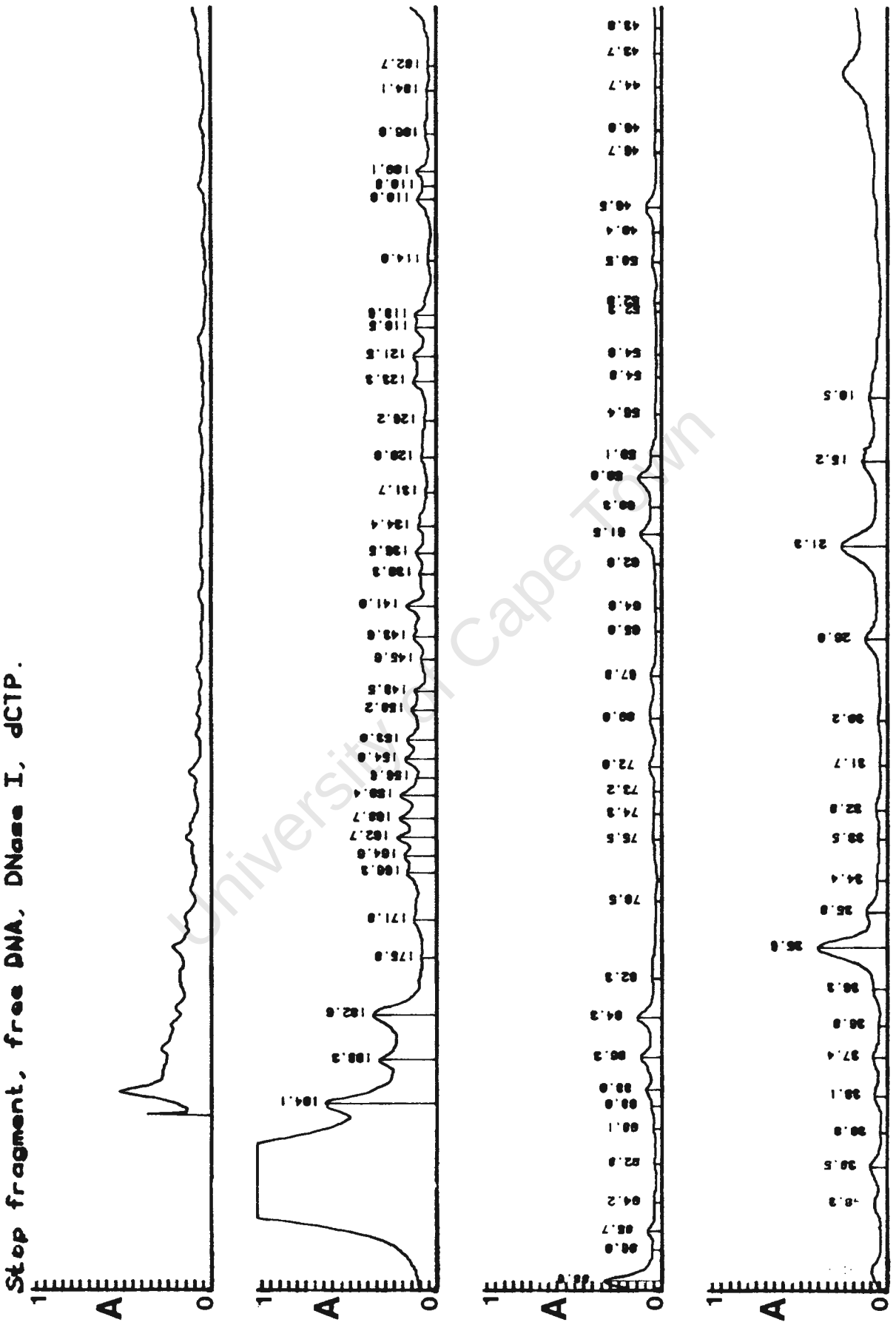
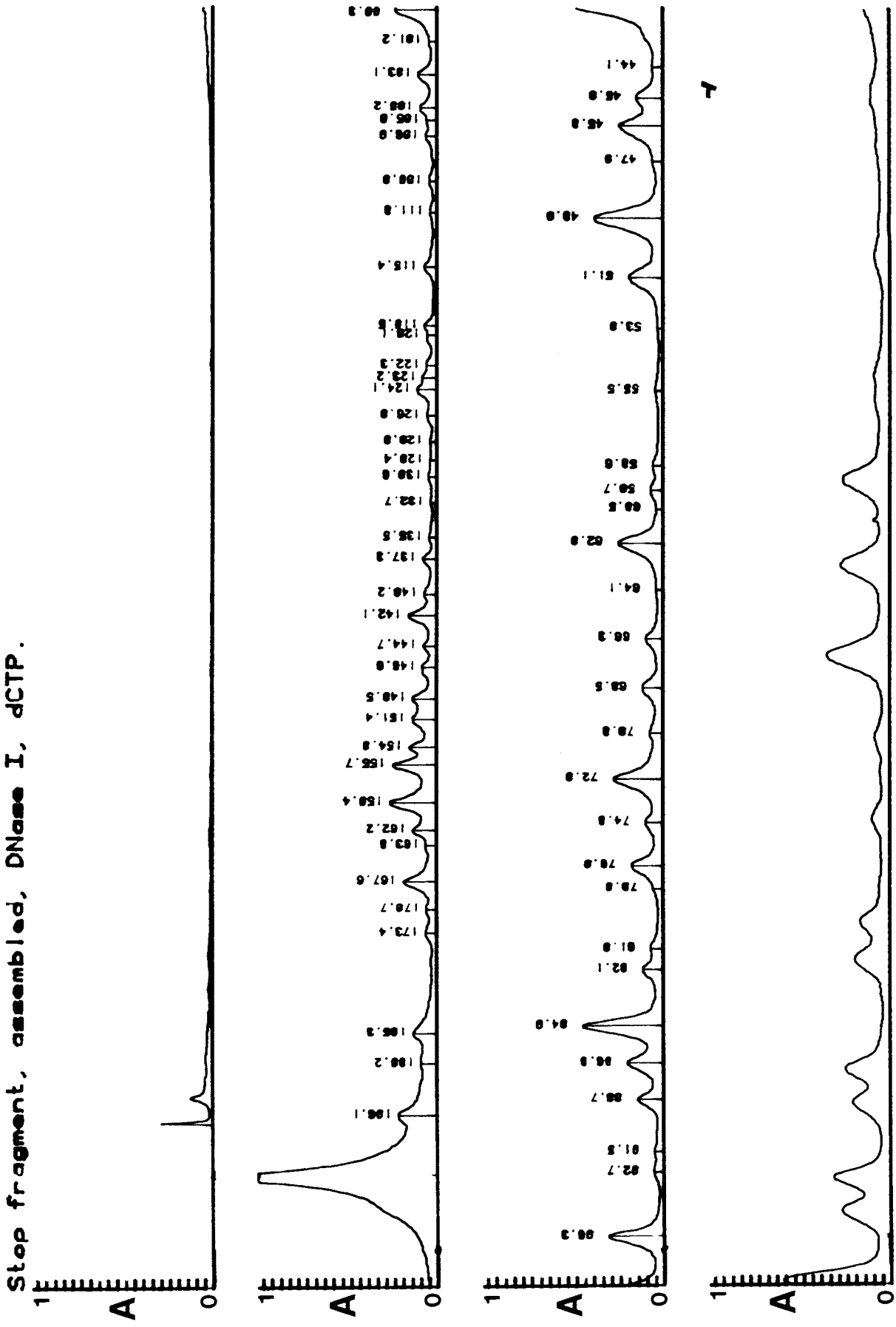


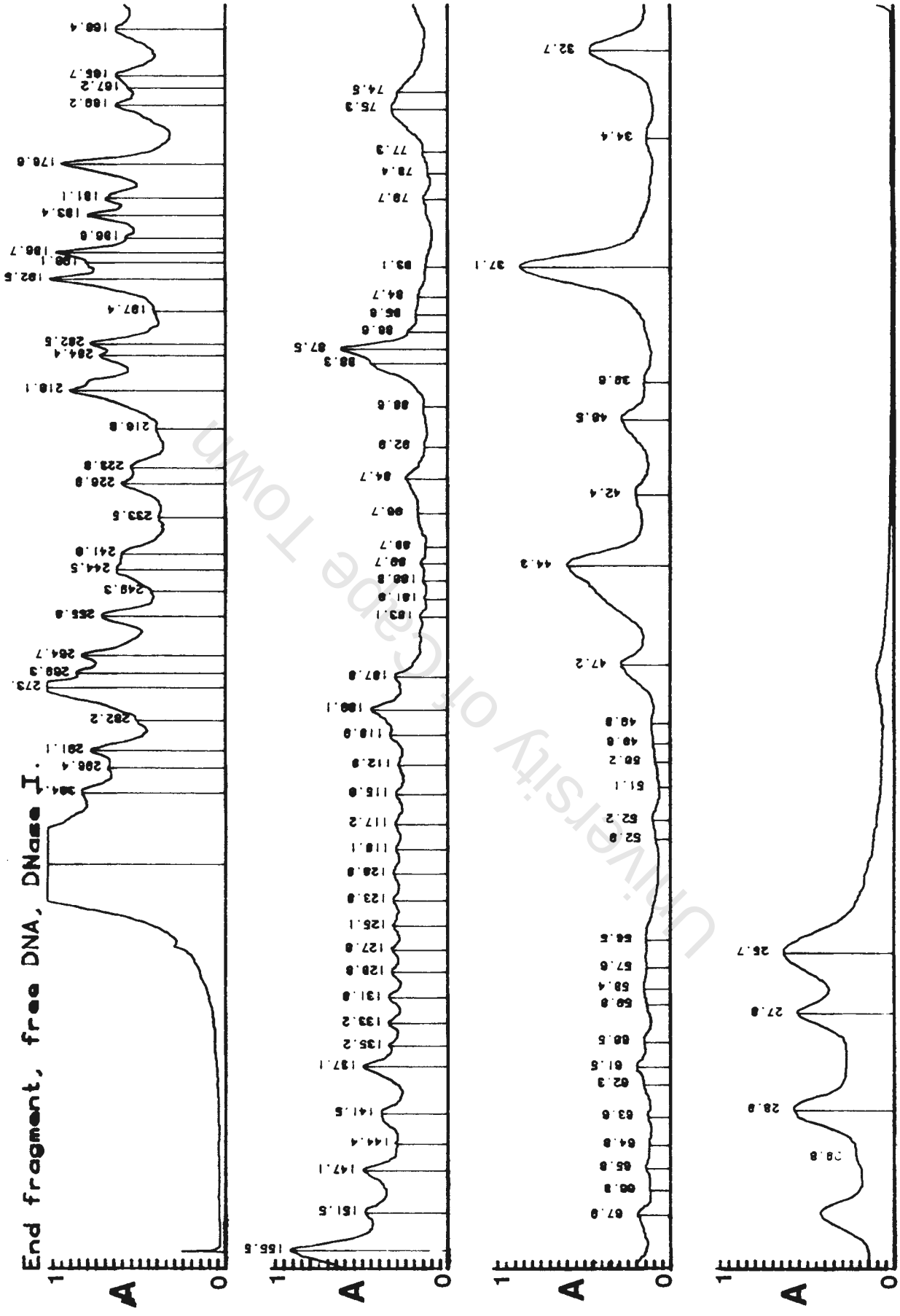
Fig. 1a
Enlargement to show low mobility bands of [^{32}P]dCTP labelled fragments. Lanes 1-3: Stop fragment. Lanes 7-9: End fragment. Lanes 13-15: Upstream fragment. Lanes 5, 11 and 17: size markers. Lanes 4, 6, 10, 12 and 16: sample buffer. DNase I digestions and electrophoresis as in Fig.61



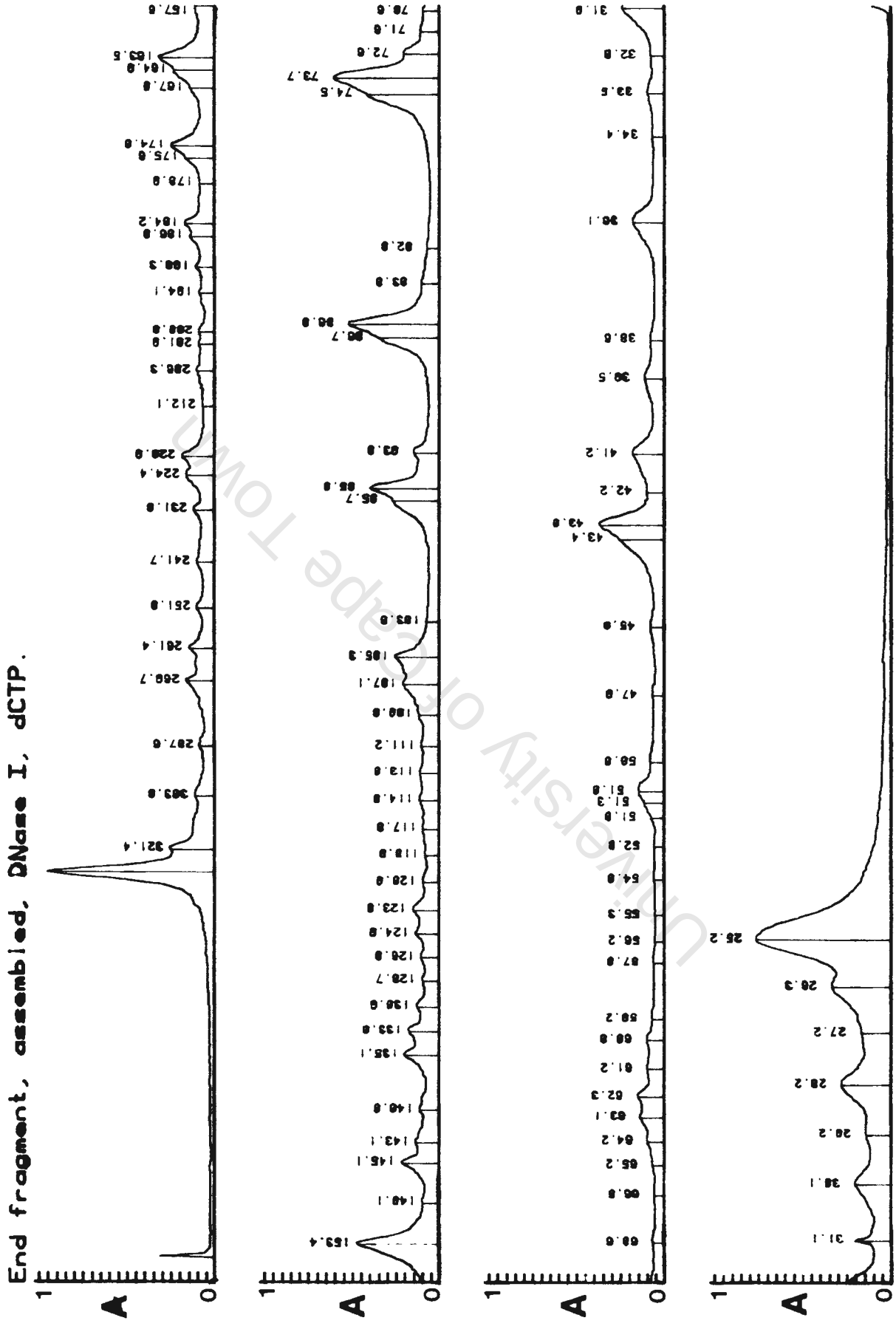
Appendix A Fig. 1b Densitometer trace of Fig. 61 Lane 2.



Appendix A Fig 1c. Densitometer trace of Fig. 61 Lane 3.

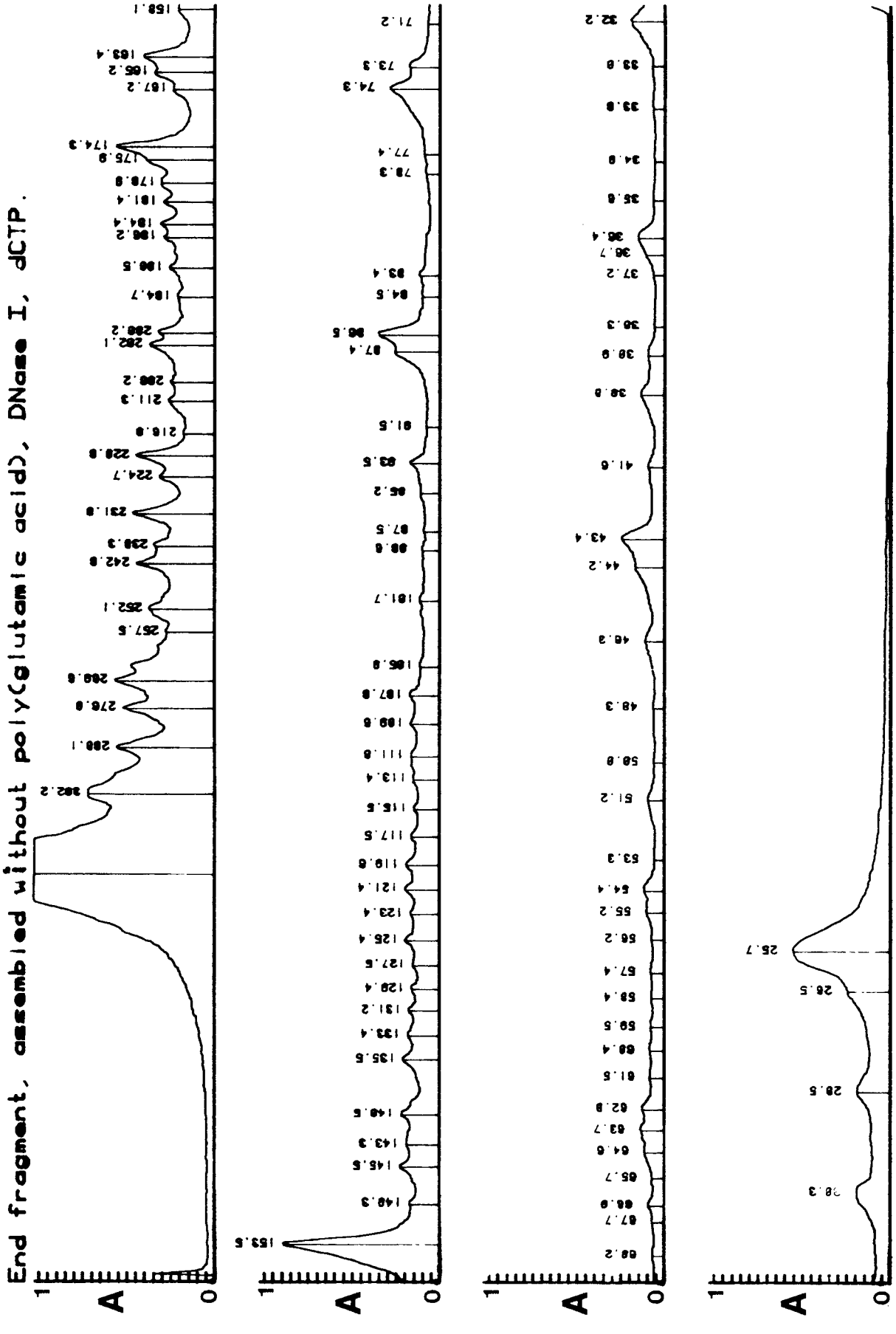


Appendix A; Fig. 2a, lane 4. Densitometer trace of appendix A; Fig. 2b.



Densitometer trace of appendix A; Fig. 2a, lane 11.

Appendix A; Fig. 2c.



Appendix A; Fig. 2d. Densitometer trace of appendix A; Fig. 2a, lane 18.

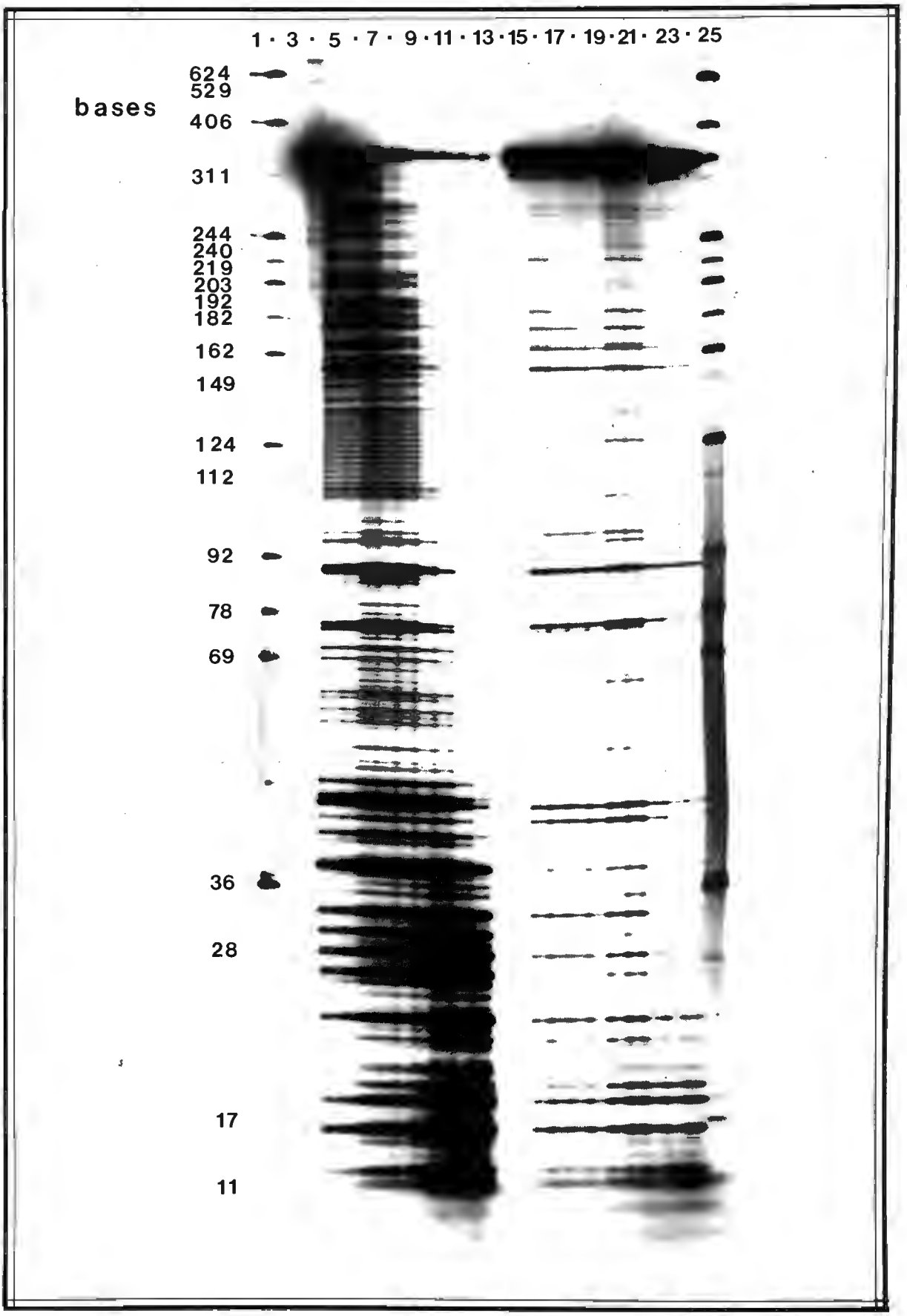


Fig. 3a

End fragment labelled with [^{32}P]dCTP and digested with DNase I. Lanes 4-13: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 15-24: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1, 2 and 25: size markers. Lane 14: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.

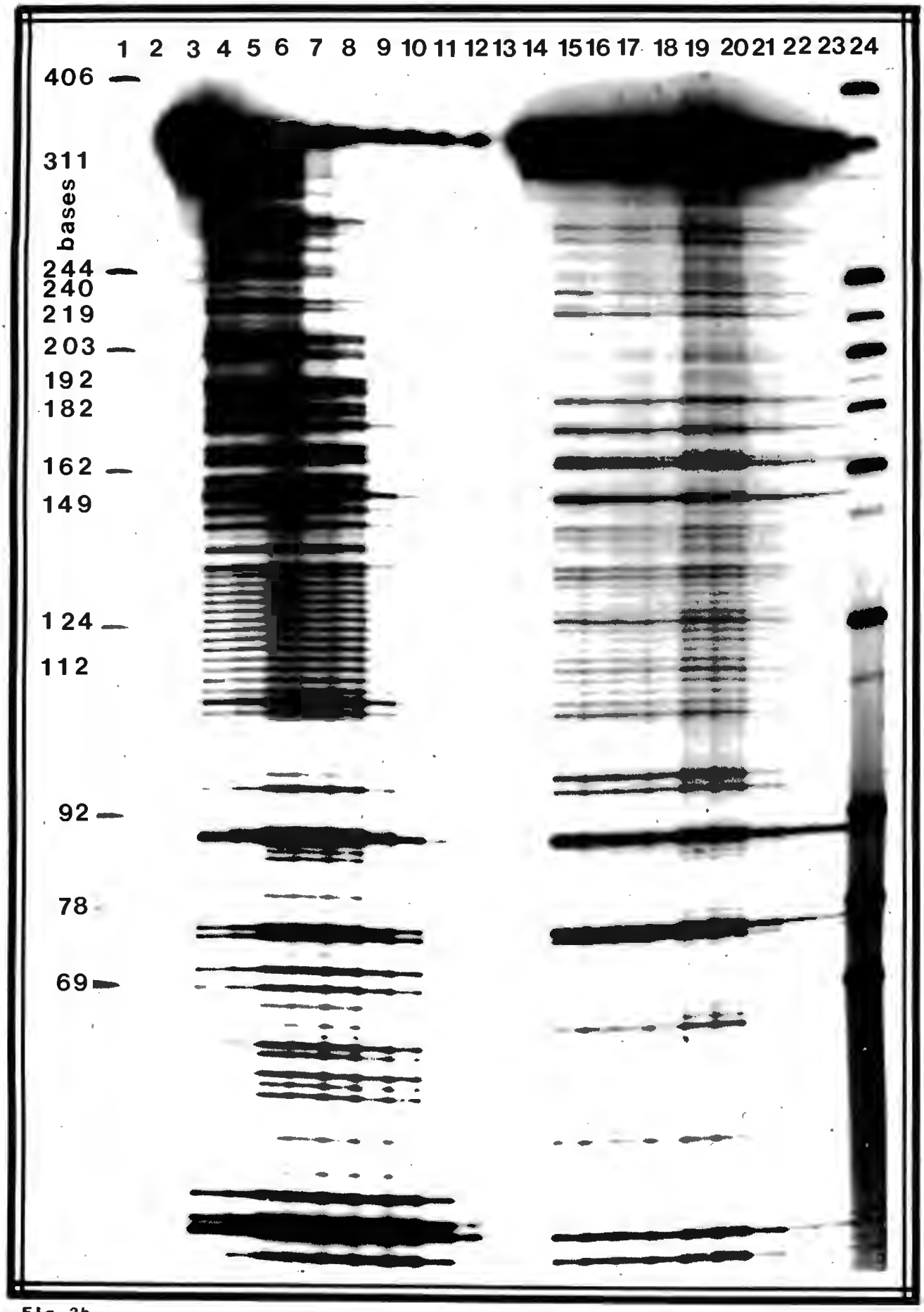
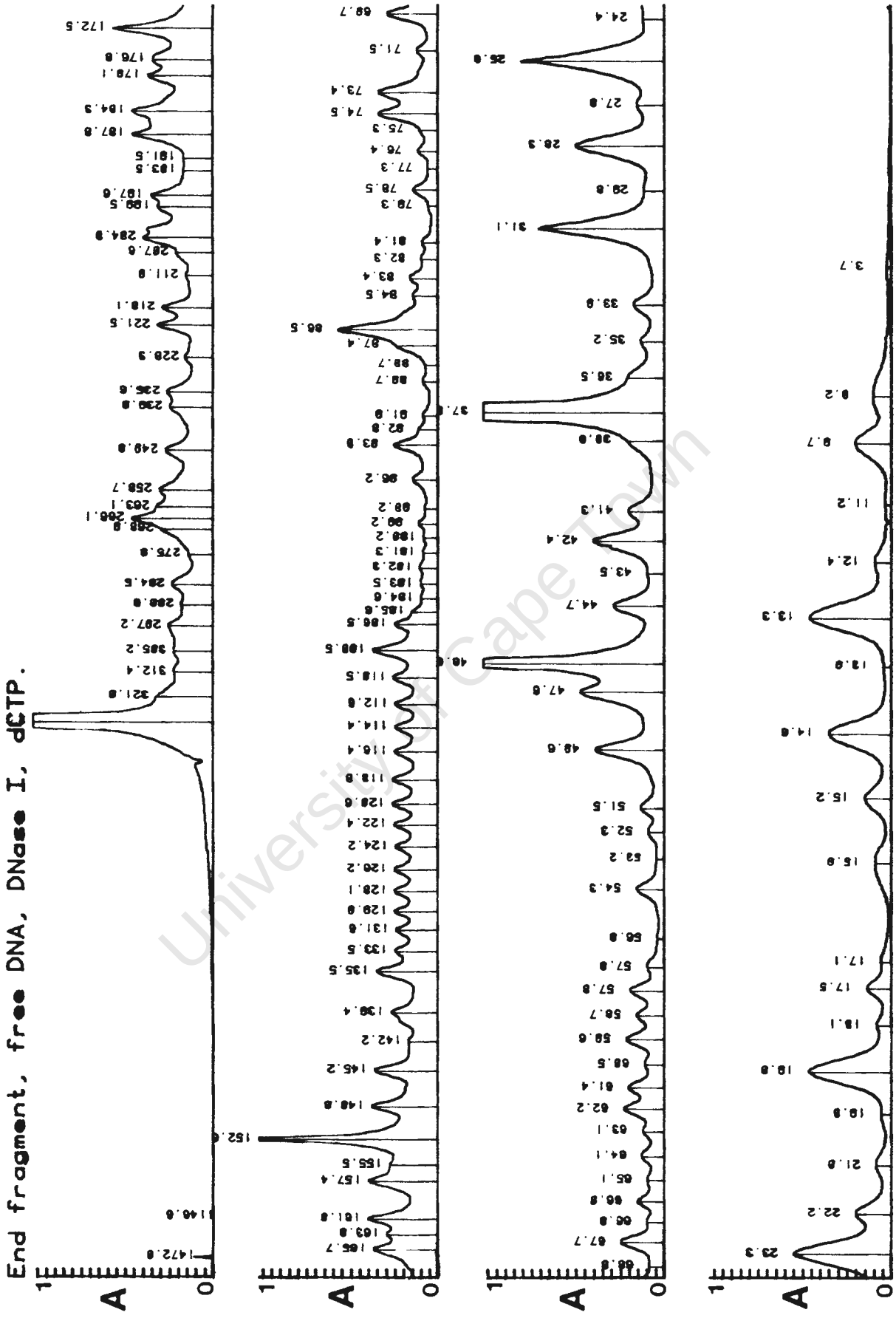
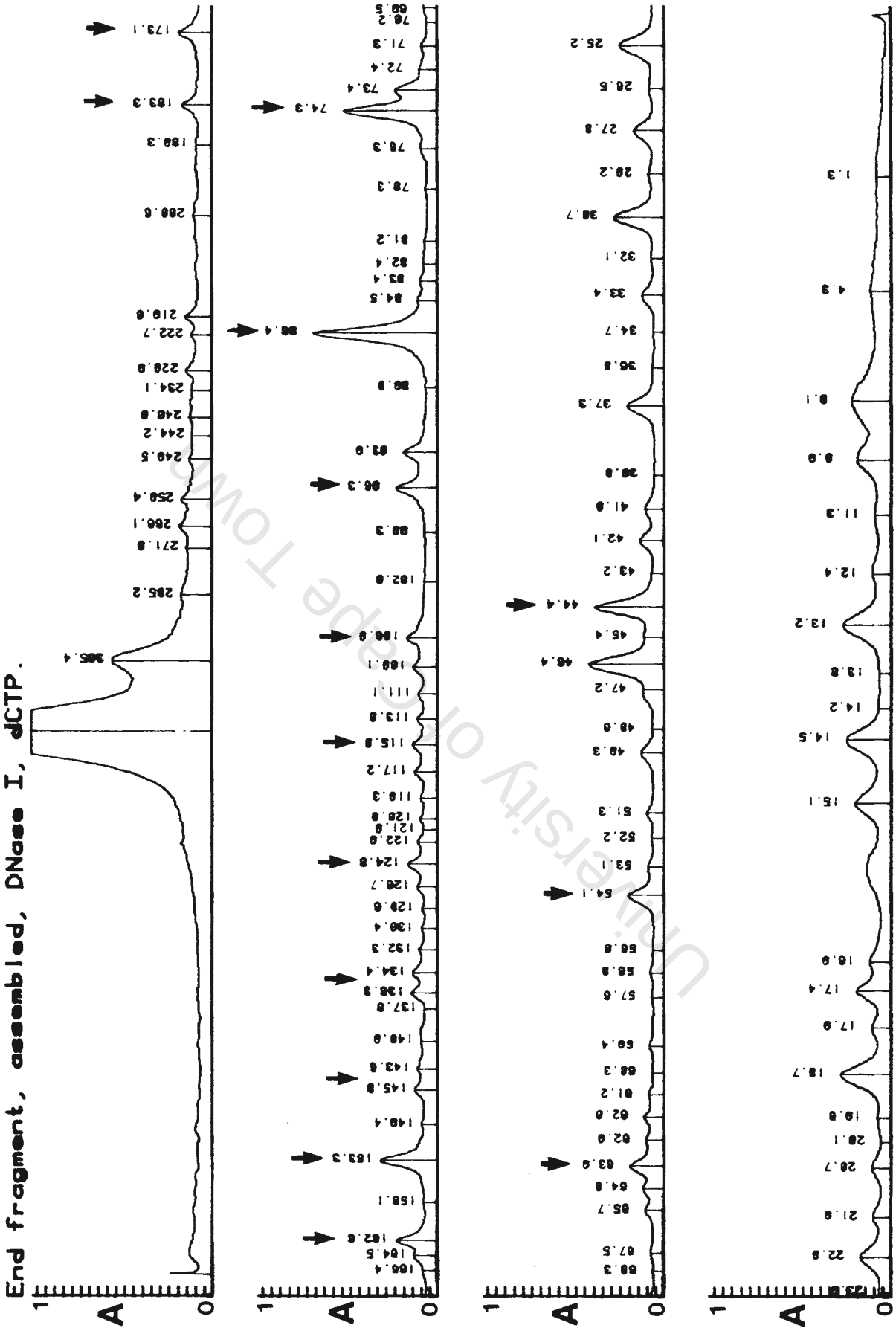


Fig. 3b
End fragment [^{32}P]dCTP labelled and DNase I digested, enlarged to show the low mobility bands.
Lanes 3-12: free DNA. Lanes 14-23: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w). Lanes 1 and 24: size markers. Lanes 2 and 13: sample buffer. Digestions and denaturing DNA gel electrophoresis as in Fig. 3a



Appendix A; Fig. 3c. Densitometer trace of appendix A; Fig. 3a, lane 6.



Appendix A, Fig. 3d. Densitometer trace of appendix A; Fig.3a lane 17.
The arrows indicate an approximately 10bp periodicity.

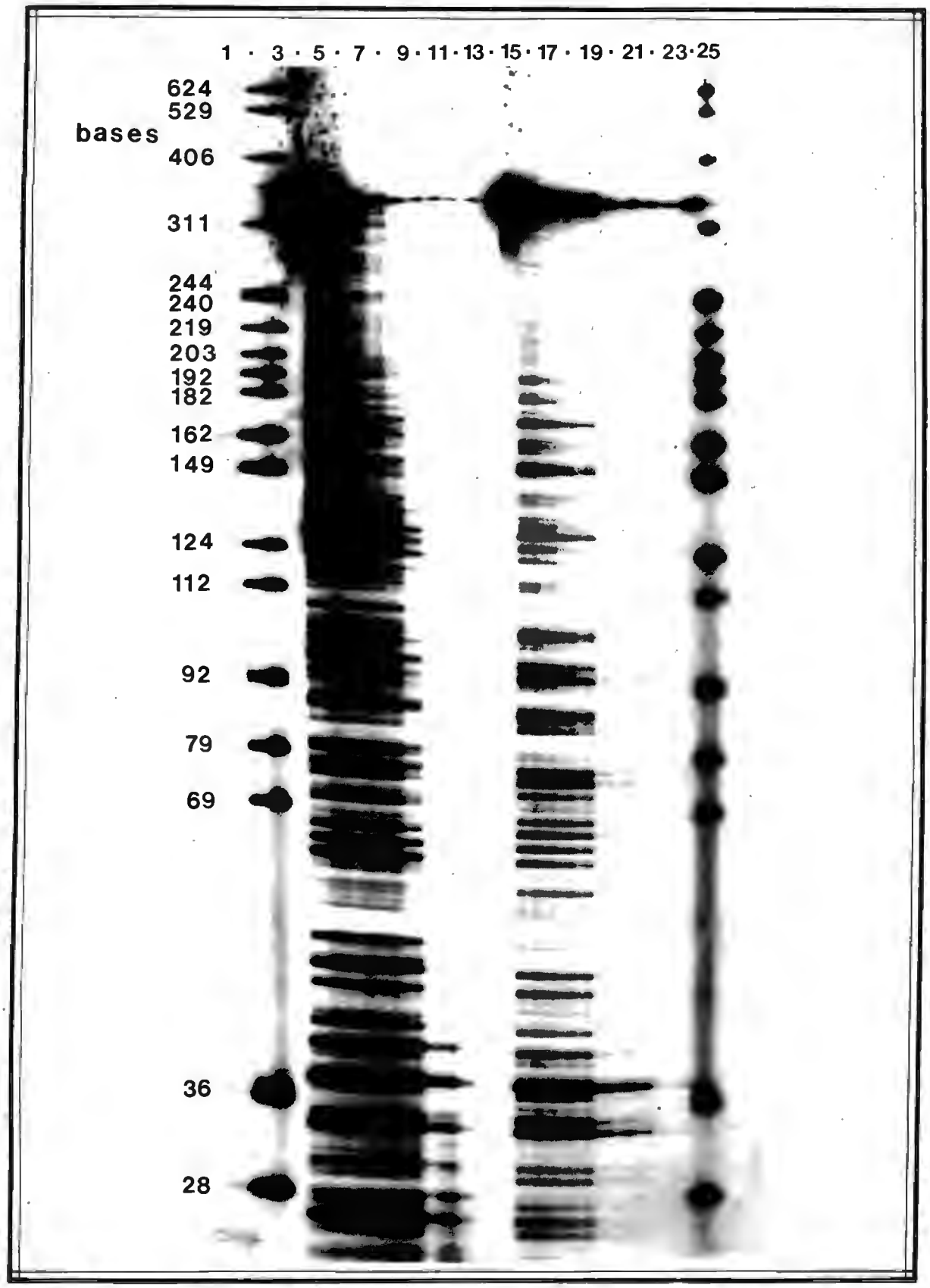


Fig. 4a
End fragment, [^{32}P]dTTP labelled and DNase I digested.
Lanes 4-13: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 15-24: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1,2,3 and 25: size markers. Lanes 12 sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.

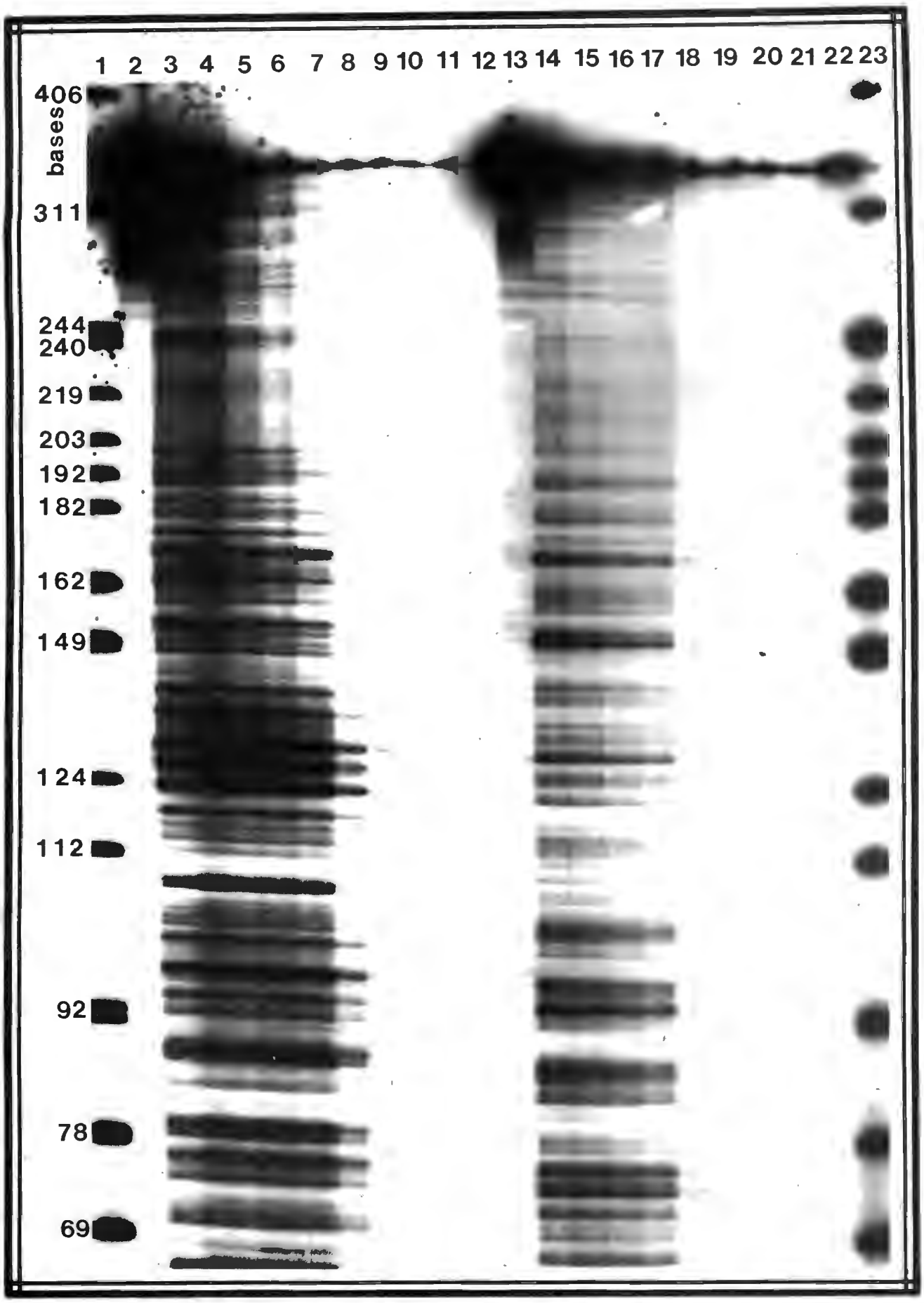
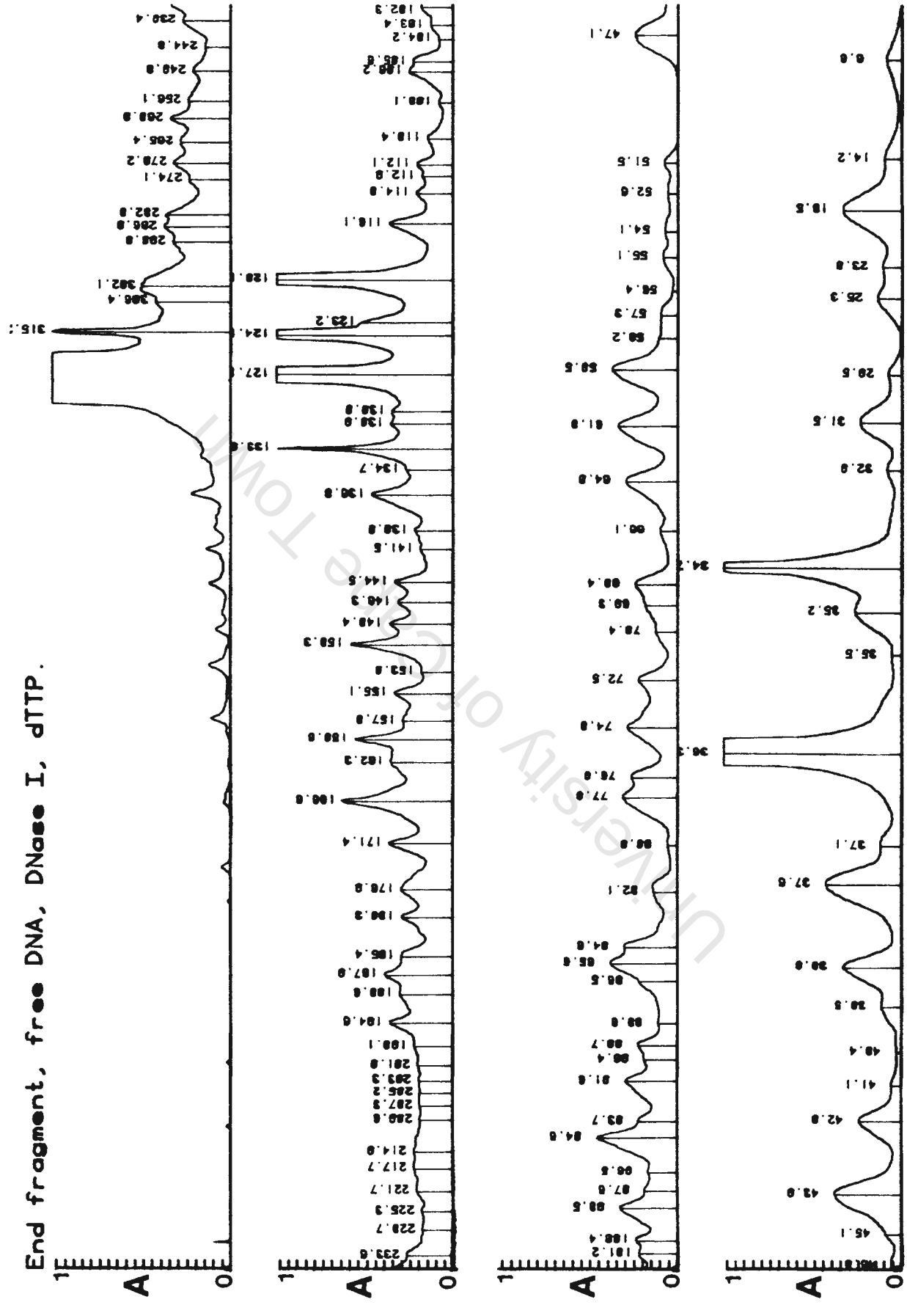
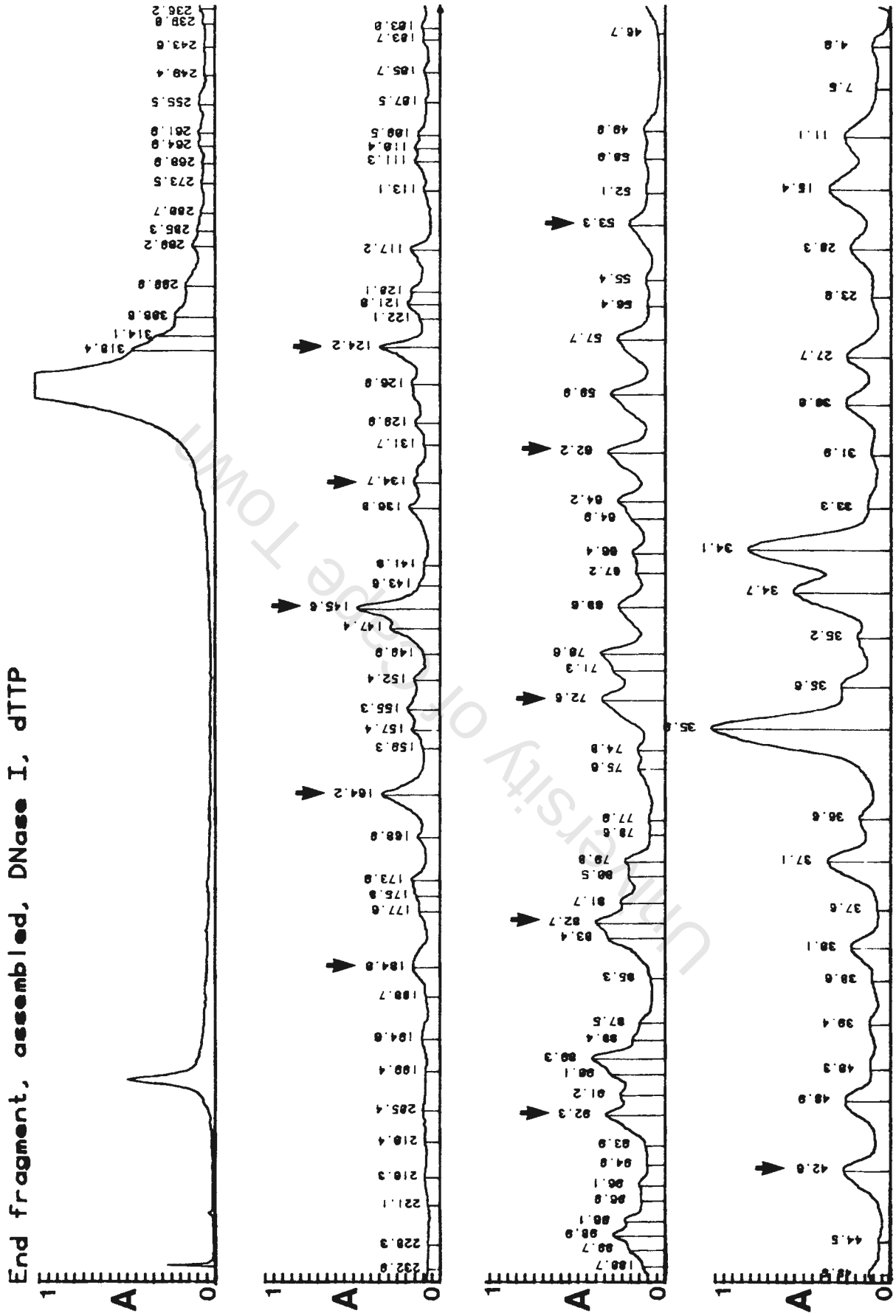


Fig. 4b
End fragment, [^{32}P]dTTP labelled and DNase I digested, enlarged to show the low mobility bands.
Lanes 2-11: free DNA. Lanes 12-21: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w). Lanes 1 and 23: size markers. Lane 22: sample buffer. Digestions and denaturing DNA gel electrophoresis as in Fig. **4a**



Appendix A; Fig. 4c. Densitometer trace of appendix A; Fig. 4a, lane 6.



Appendix A; Fig. 4d. Densitometer trace of appendix A; Fig. 4a, lane 17. The arrows indicate a weak, approximately 10 bp periodicity.

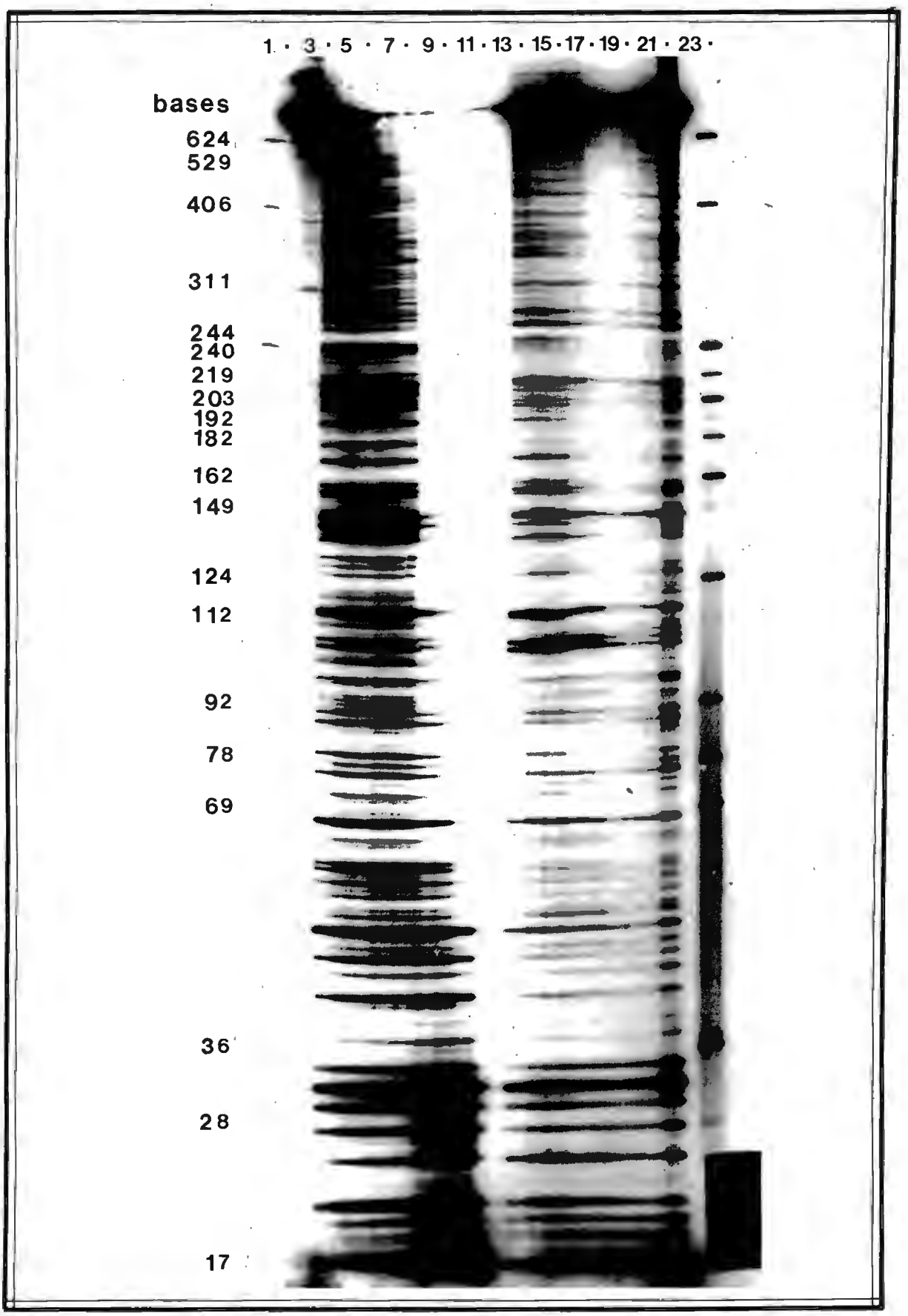


Fig. 5a
Upstream fragment labelled with [^{32}P]dCTP and digested with DNase I. Lanes 3-12: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 13-22: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1 and 24: size markers. Lanes 2 and 23: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.

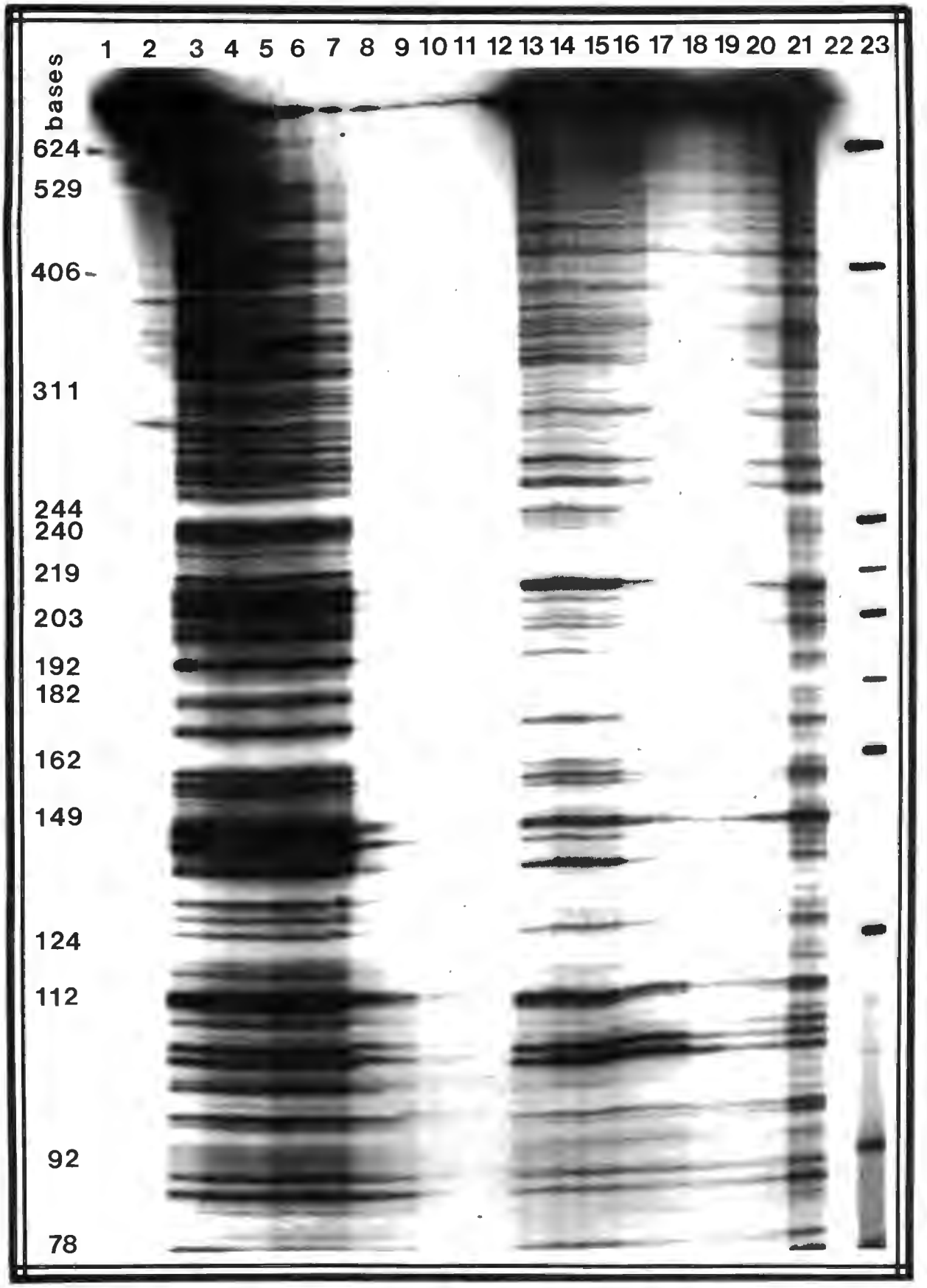
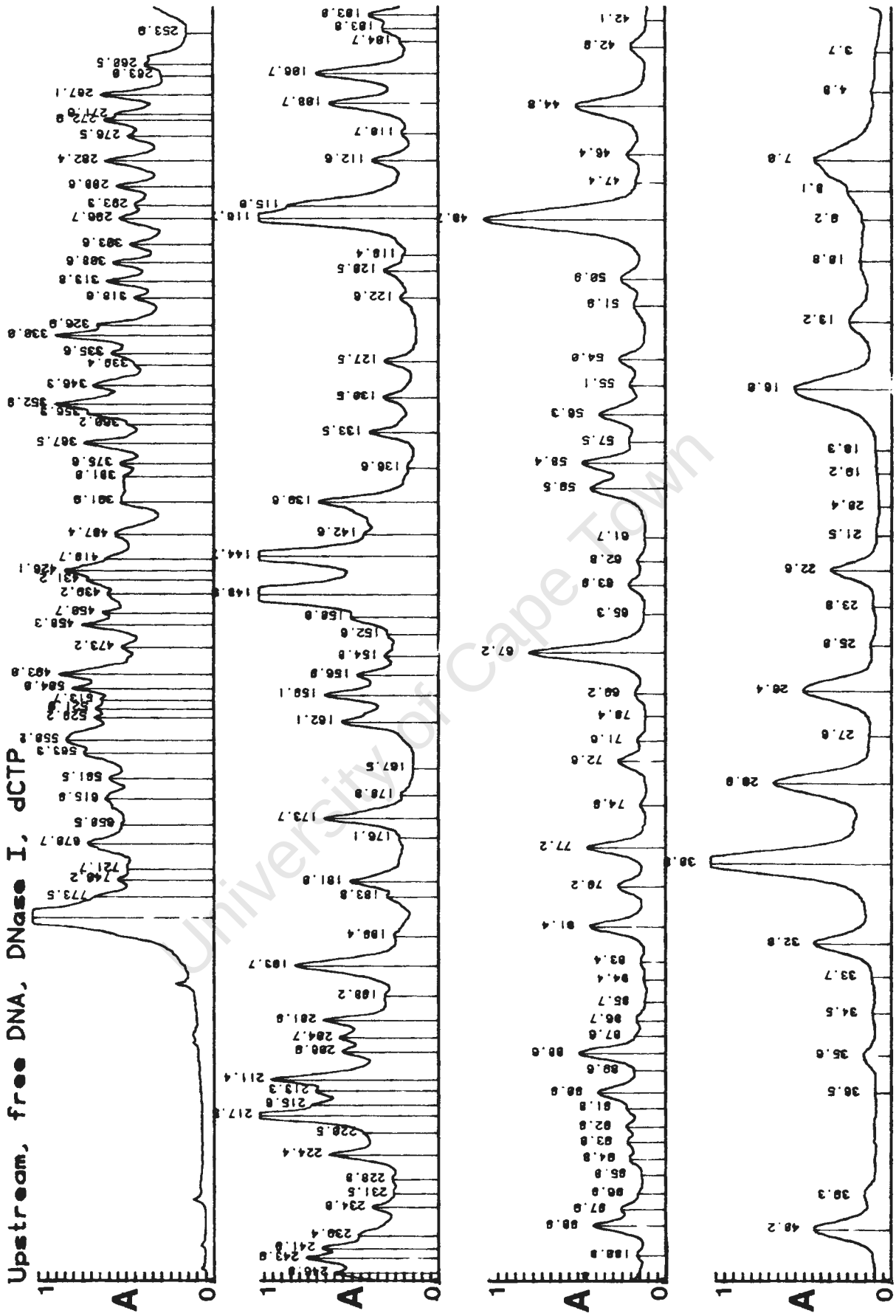
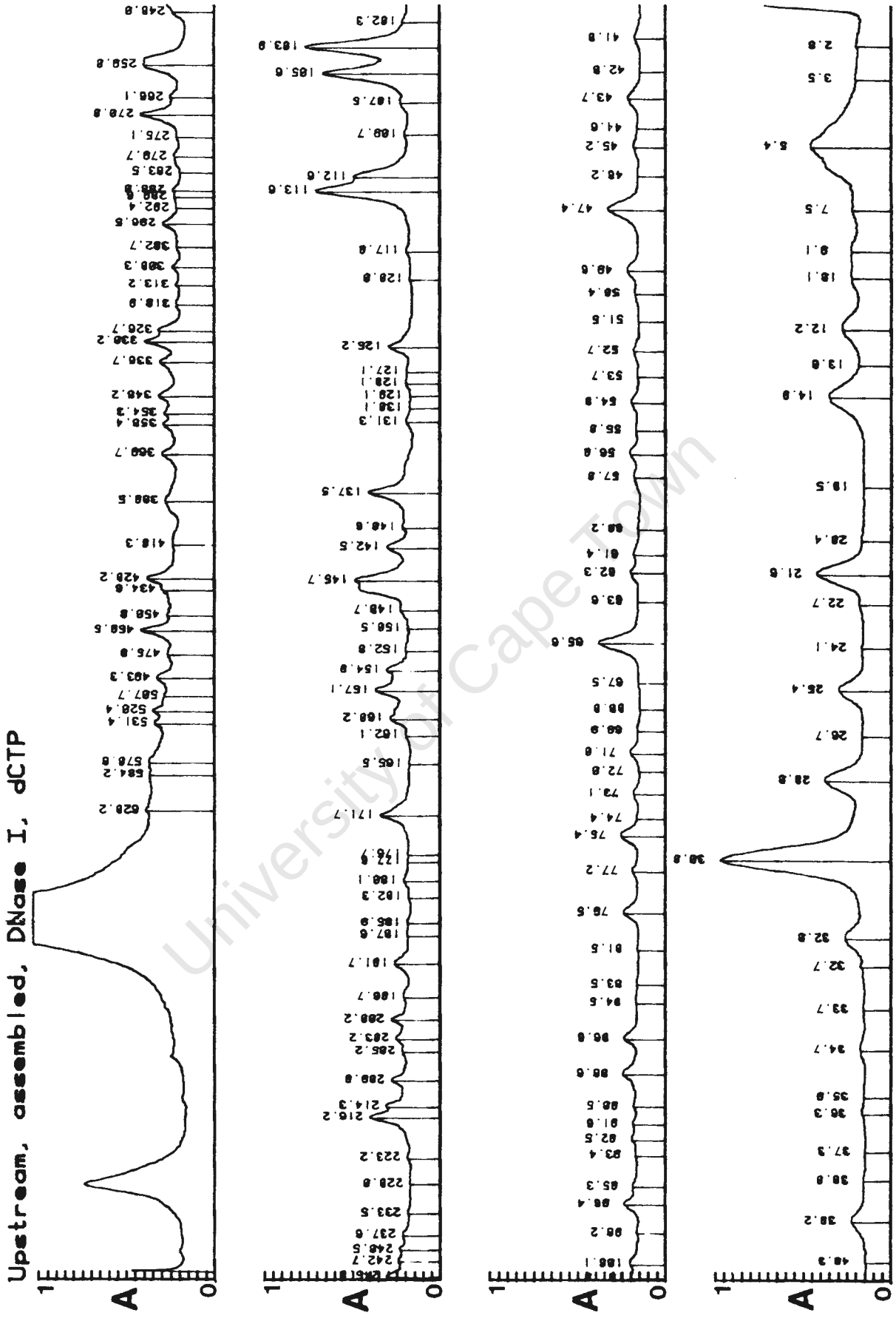


Fig. 5b
Upstream fragment labelled with [^{32}P]dCTP and digested with DNase I, enlarged to show the low mobility bands.
Lanes 2-11: free DNA. Lanes 12-21: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w). Lanes 1 and 23: size markers. Lane 22: sample buffer. Digestions and denaturing DNA gel electrophoresis as in Fig. **5a**



Appendix A; Fig. 5c. Densitometer trace of appendix A; Fig. 5a, lane 5.



Appendix A; Fig. 5d. Densitometer trace of appendix A; Fig. 5a, lane 15.

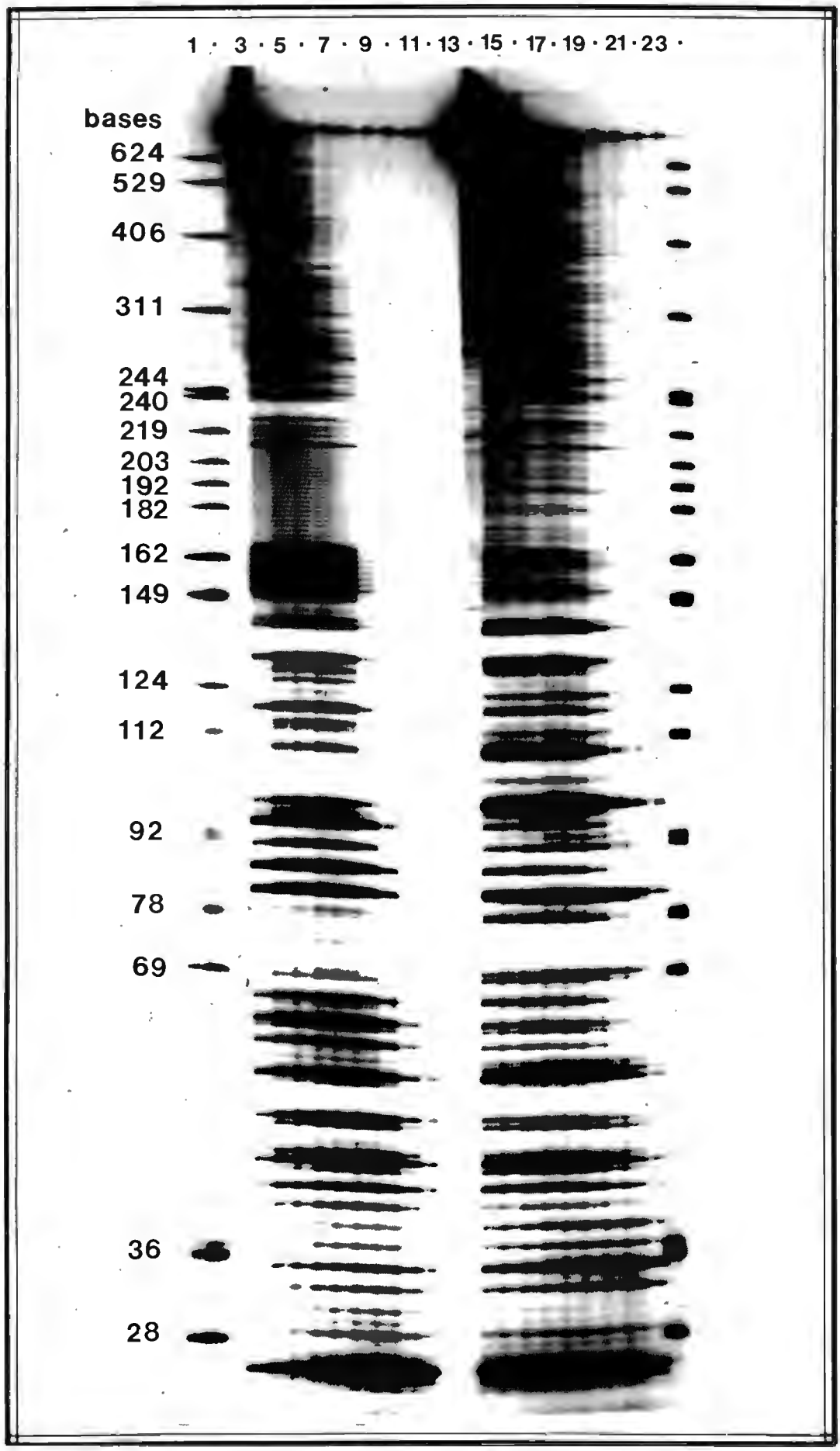


Fig. 6a
Upstream fragment labelled with [^{32}P]dTTP and digested with DNase I. Lanes 3-12: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 14-23: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1,2 and 24: size markers. Lane 13: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.

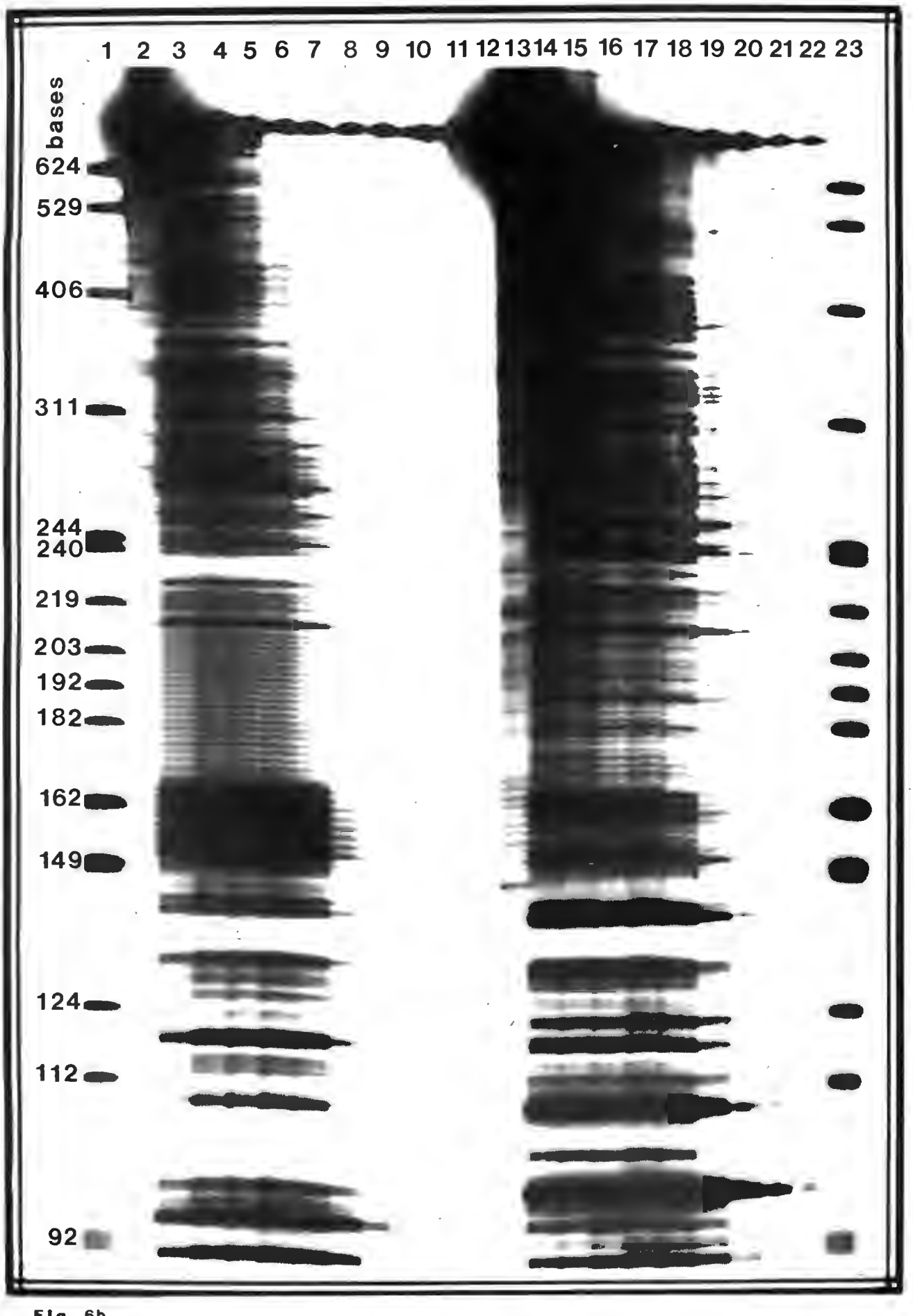
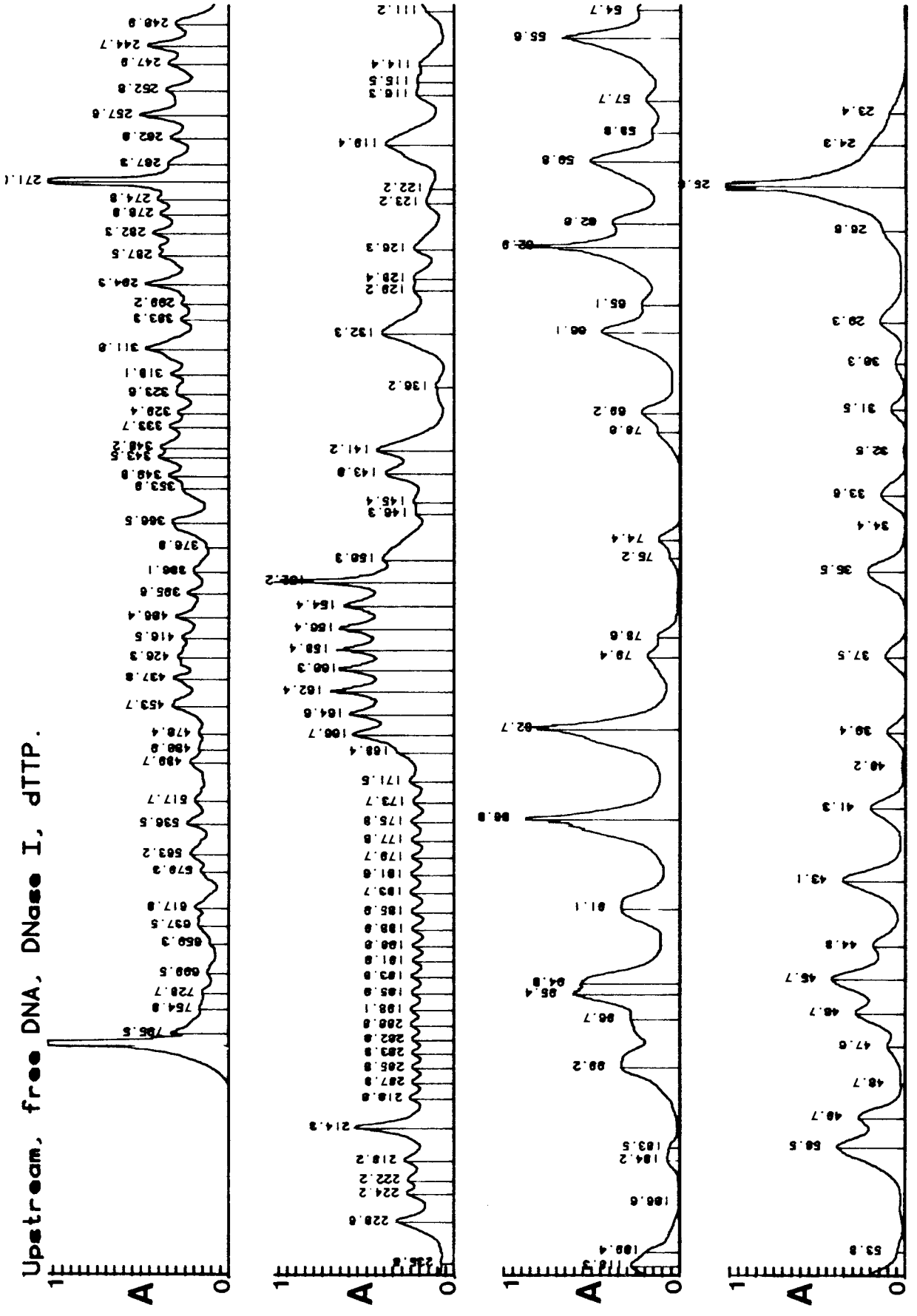
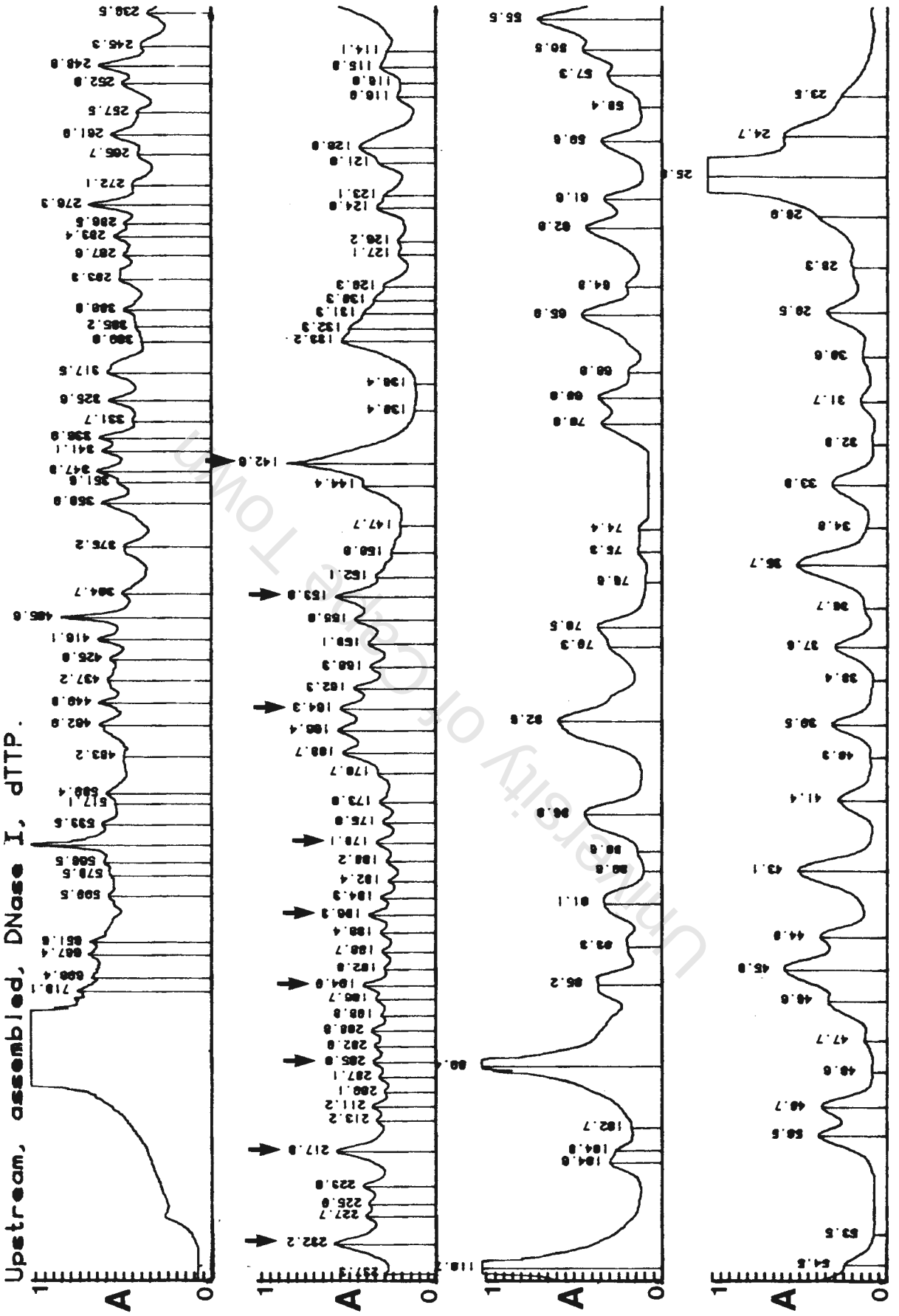


Fig. 6b
Upstream fragment labelled with I^{32}PIdTTP and digested with DNase I, enlarged to show the low mobility bands.
Lanes 2-11: free DNA. Lanes 13-22: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w). Lanes 1 and 23: size markers. Lane 12: sample buffer. Digestions and denaturing DNA gel electrophoresis as in Fig. 6a



Densitometer trace of appendix A; Fig. 6a, lane 5.
Appendix A; Fig. 6c.



Appendix A; Fig. 6d. Densitometer trace of appendix A; Fig. 6a, lane 15. The arrows indicate an approximately 10 base pair periodicity.

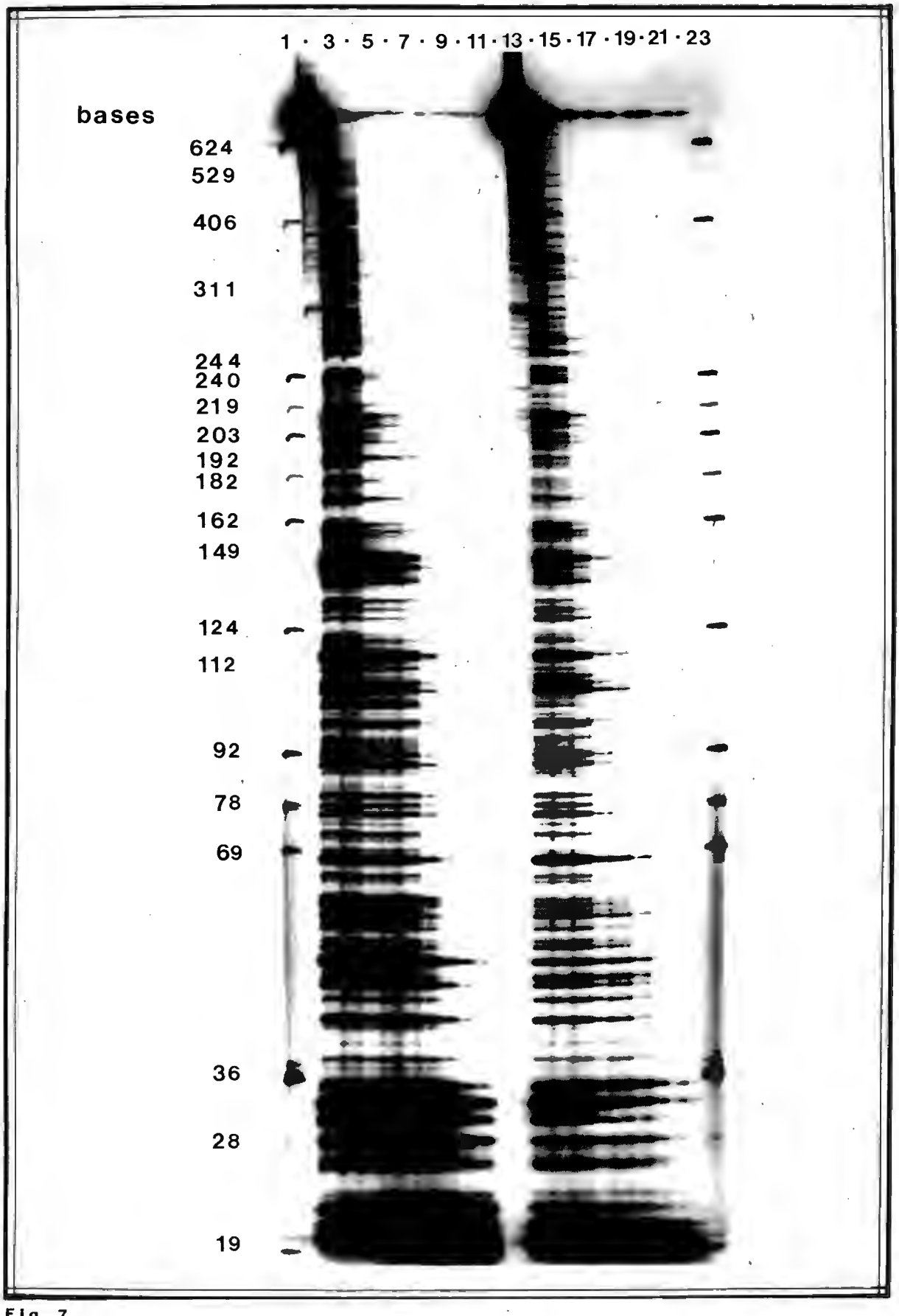


Fig. 7

Upstream fragment labelled with [^{32}P]dCTP and digested with DNase I. Lanes 2-11: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 13-22: DNA assembled at a histone to DNA ratio of 0.32:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1 and 23: size markers. Lane 12: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.

APPENDIX B

Micrococcal nuclease digestions of END, UPSTREAM AND STOP fragments:

Fig. 1a END fragment, dCTP labelled.

1b END fragment, dCTP labelled, enlarged.

1c Densitometer trace, END fragment free DNA.

1d Densitometer trace, END fragment assembled.

Fig. 2a END fragment, dCTP labelled, low protein ratio.

2b Densitometer trace, END fragment free DNA.

2c Densitometer trace, END fragment assembled.

Fig. 3a UPSTREAM fragment, dCTP labelled.

3b Densitometer trace, UPSTREAM fragment free DNA.

3c Densitometer trace, UPSTREAM fragment assembled.

Fig. 4a STOP fragment, dCTP labelled.

4b Densitometer trace, STOP fragment free DNA.

4c Densitometer trace, STOP fragment assembled.

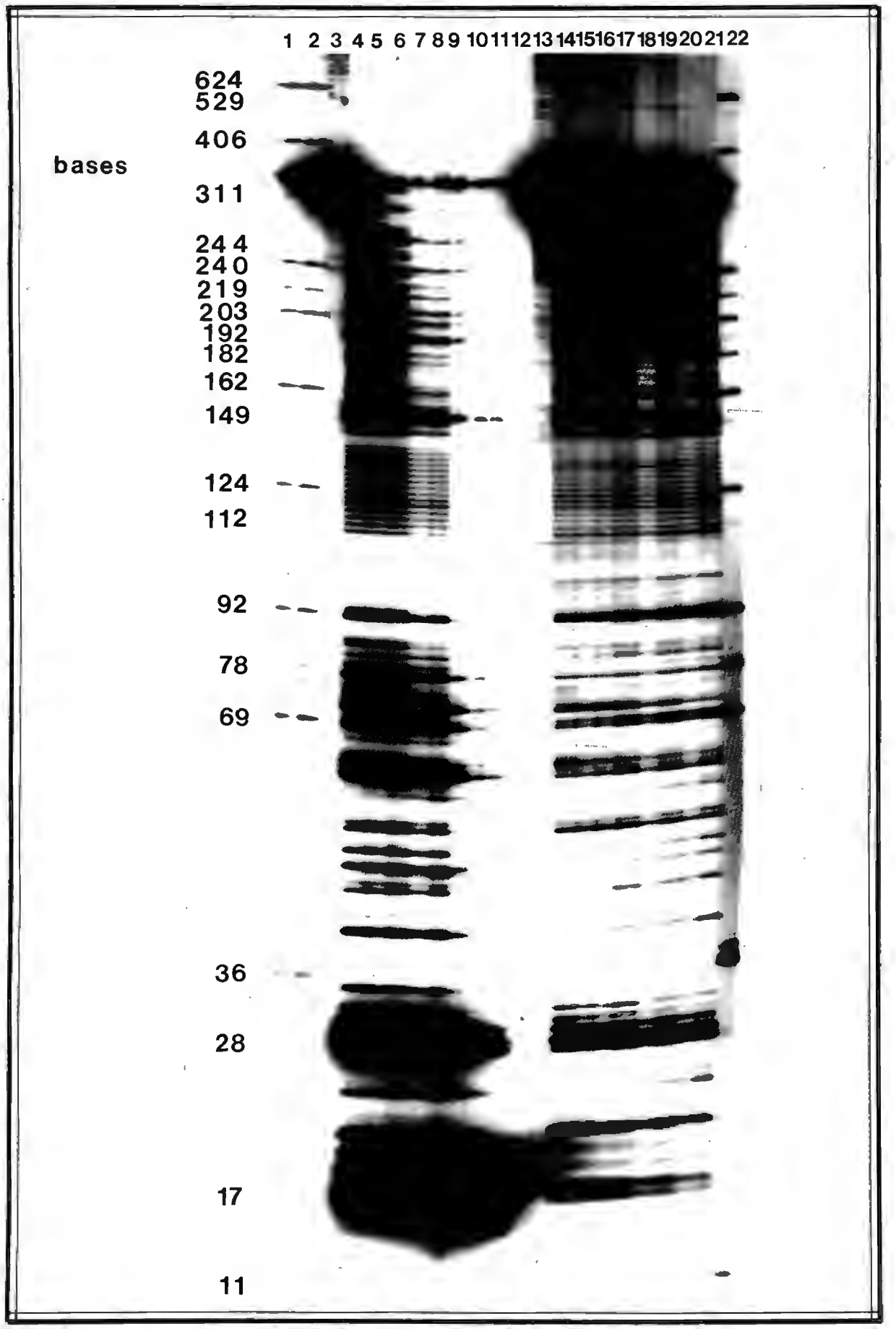


Fig. 1a
End fragment, [^{32}P]dCTP labelled and Micrococcal nuclease digested.
Lanes 3-11: free DNA digested with 1 unit Micrococcal nuclease/µg DNA. Lanes 13-21: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 10 units Micrococcal nuclease/µg DNA. Lanes 1, 2 and 22: size markers. Lane 12: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480 and 720 seconds and the DNA analysed on a denaturing DNA gel.

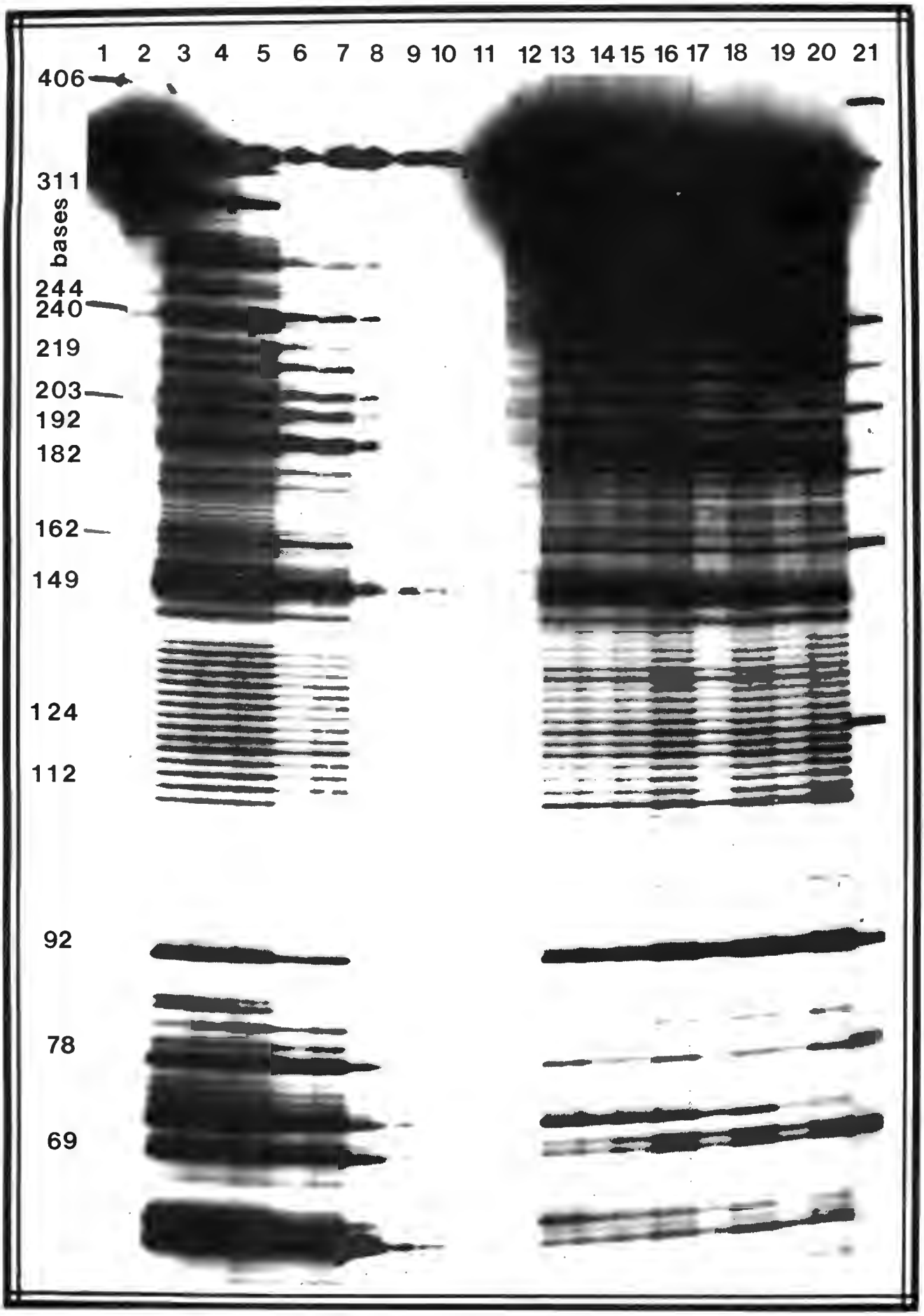
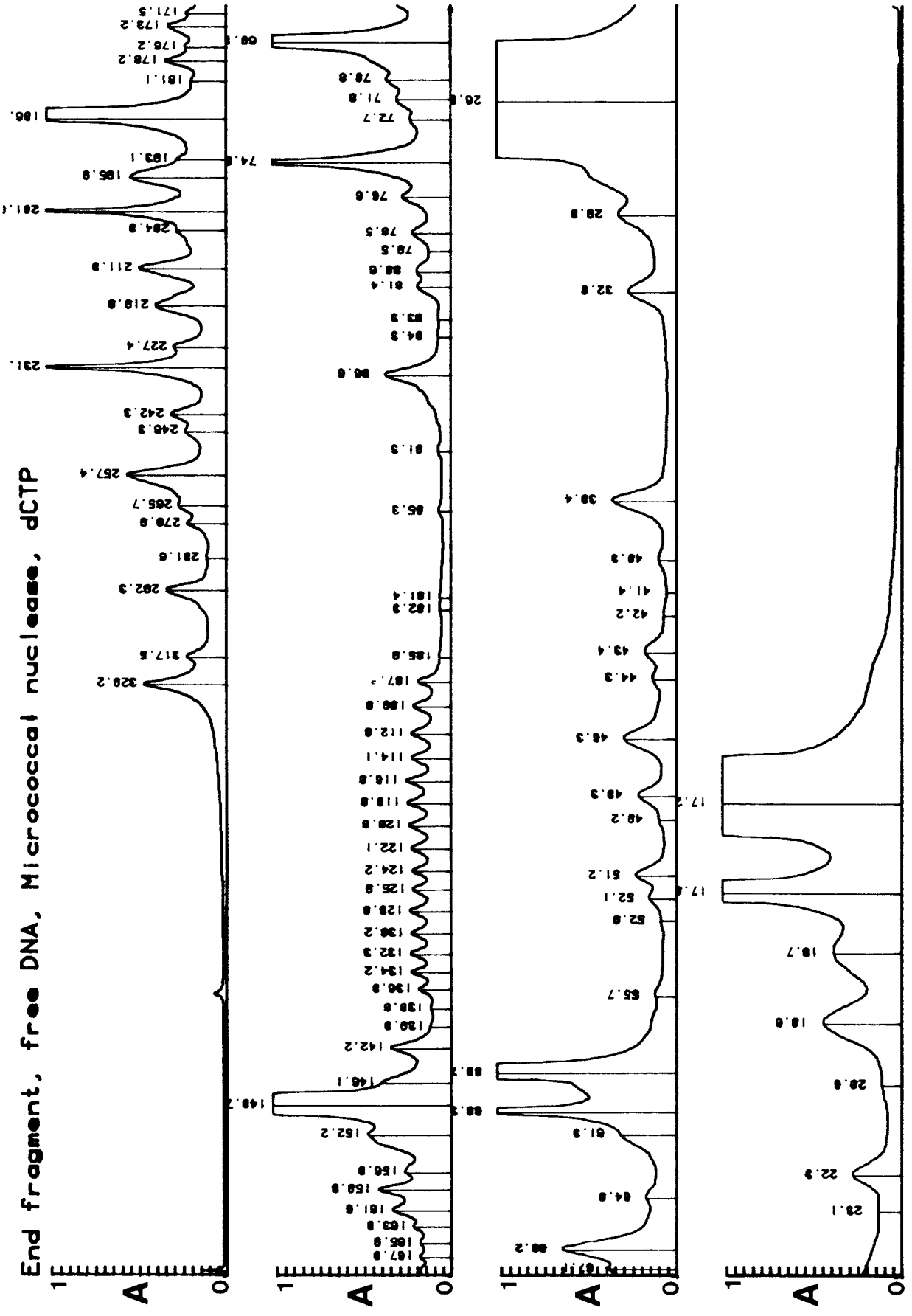
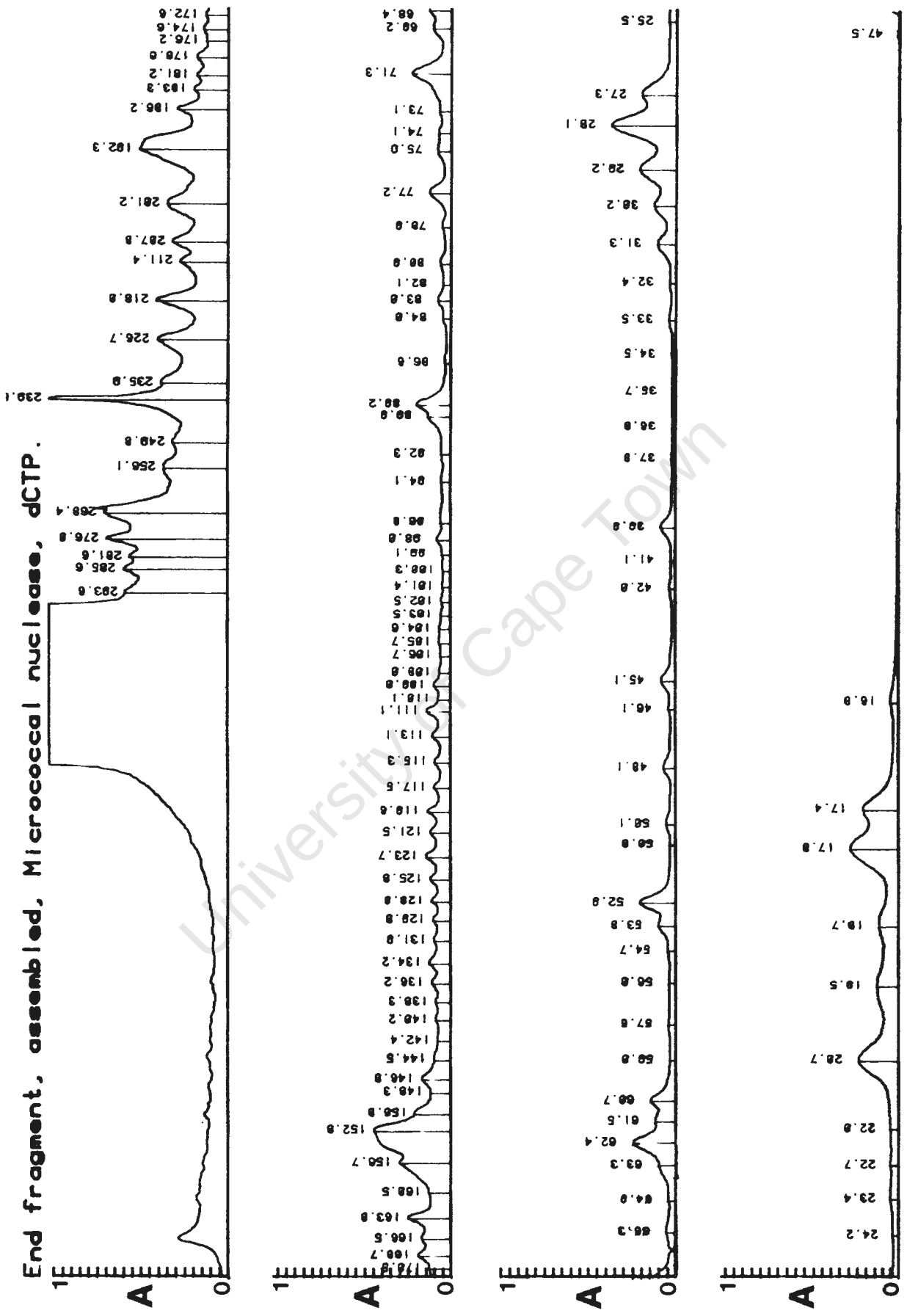


Fig. 1b
End fragment labelled with [^{32}P]dCTP and digested with Micrococcal nuclease enlarged, to show the low mobility bands.
Lanes 2-10: free DNA. Lanes 12-20: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w). Lanes 1 and 21: size markers. Lane 11: sample buffer. Digestions and denaturing DNA gel electrophoresis as in Fig.1a



Densitometer trace of appendix B; Fig. 1a, lane 5.

Appendix B; Fig. 1c.



Appendix B; Fig. 1d. Densitometer trace of appendix B; Fig. 1a, lane 15.

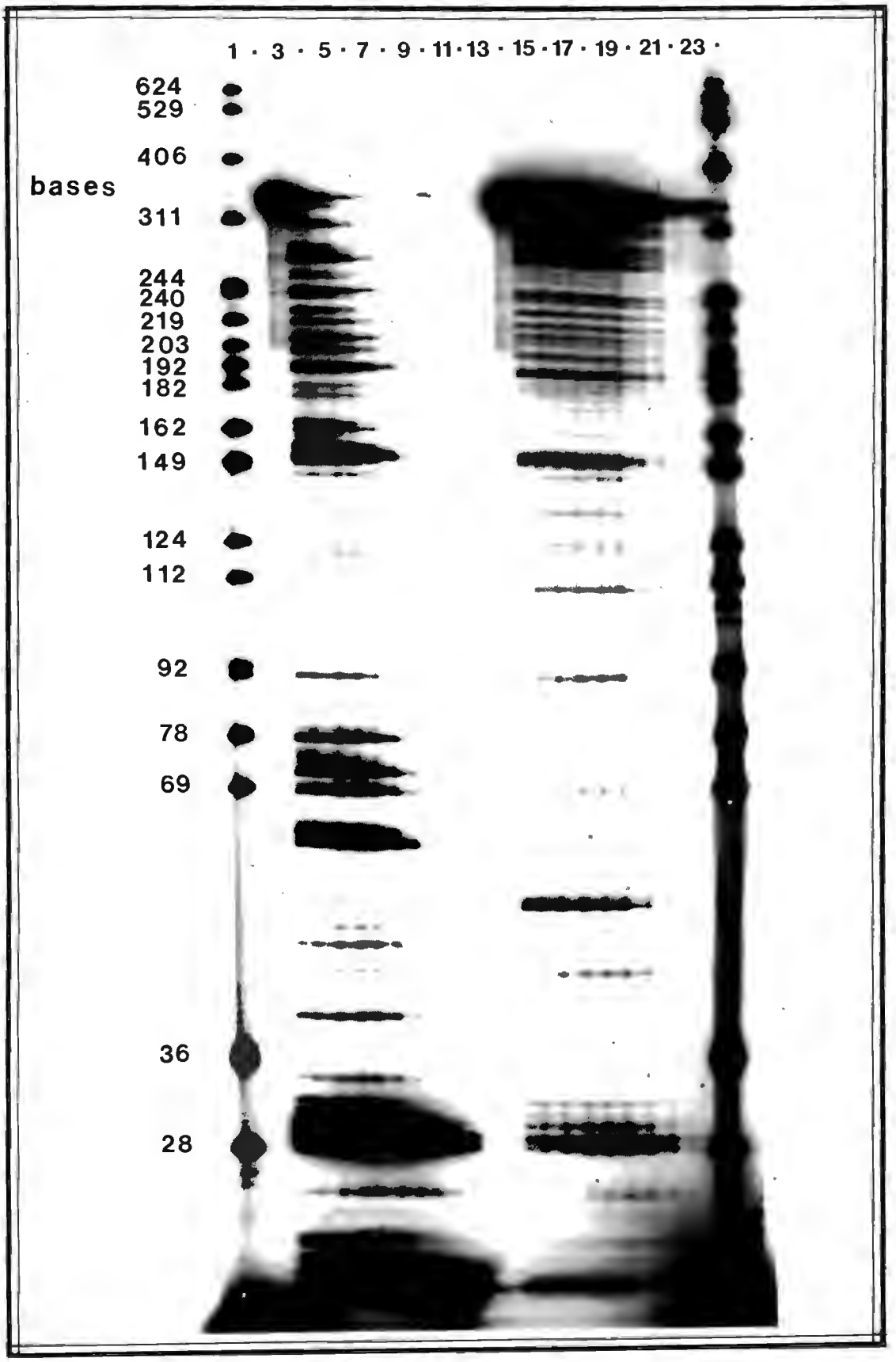
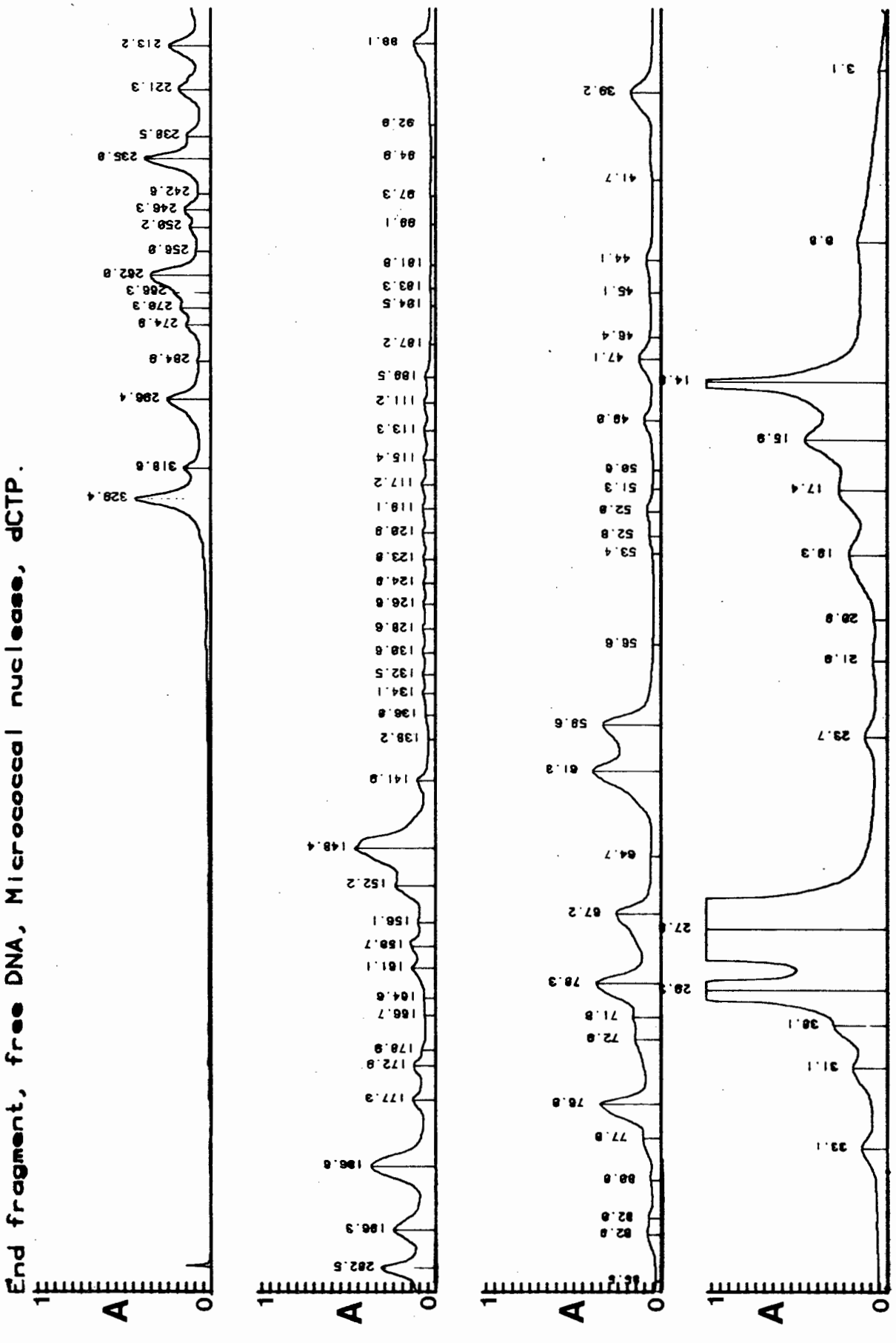
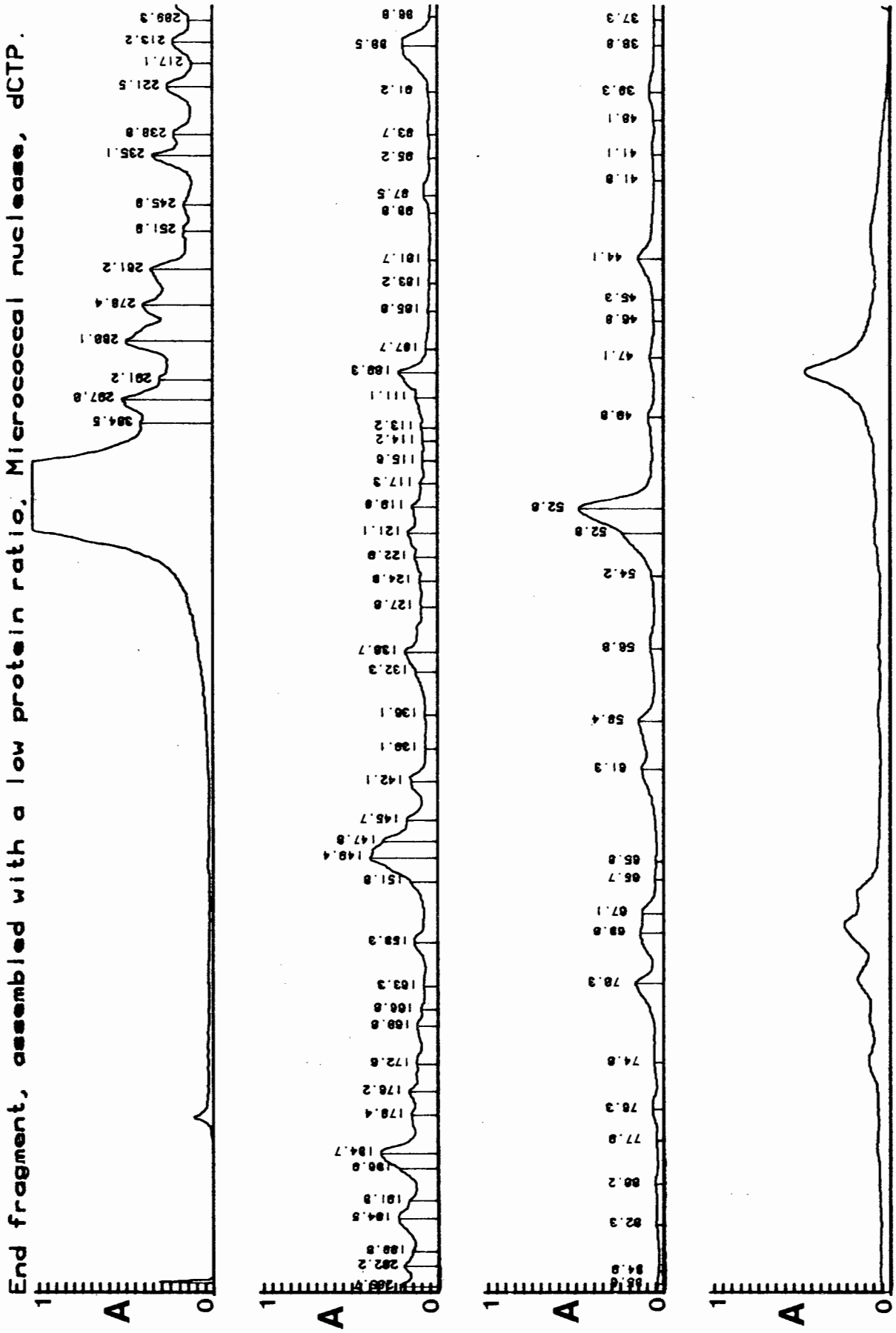


Fig. 2a
End fragment, l^{32}PldCTP labelled and Micrococcal nuclease digested. Lanes 3-12: free DNA digested with 1 unit Micrococcal nuclease/µg DNA. Lanes 14-23: DNA assembled at a histone to DNA ratio of 0.32:1 (w/w), digested with 10 units Micrococcal nuclease/µg DNA. Lanes 1 and 24: size markers. Lanes 2 and 13: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.



Appendix B; Fig. 2b. Densitometer trace of appendix B; Fig. 2a, lane 5.



Densitometer trace of appendix B; Fig. 2a, lane 16.

Appendix B; Fig. 2c.

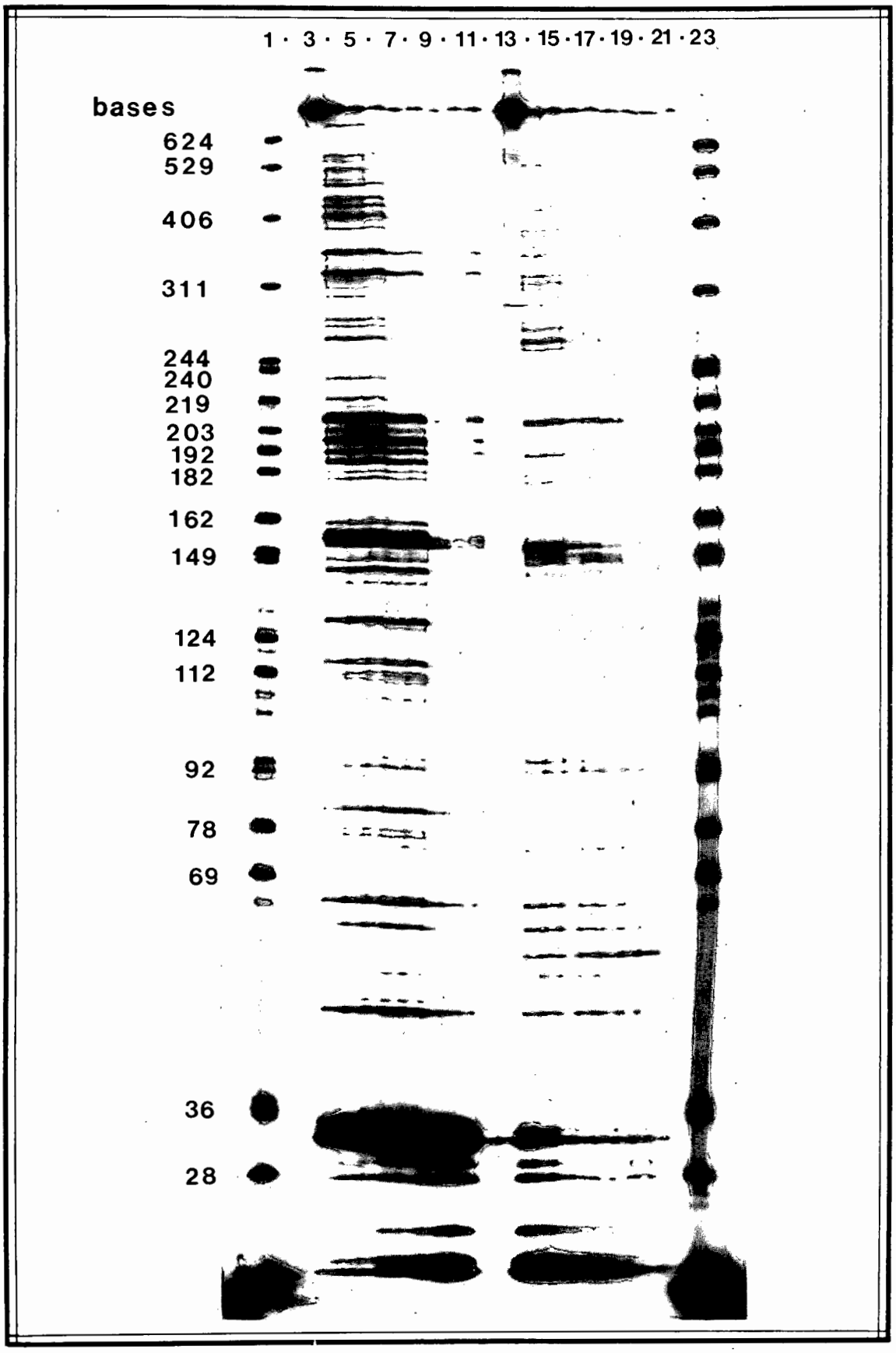
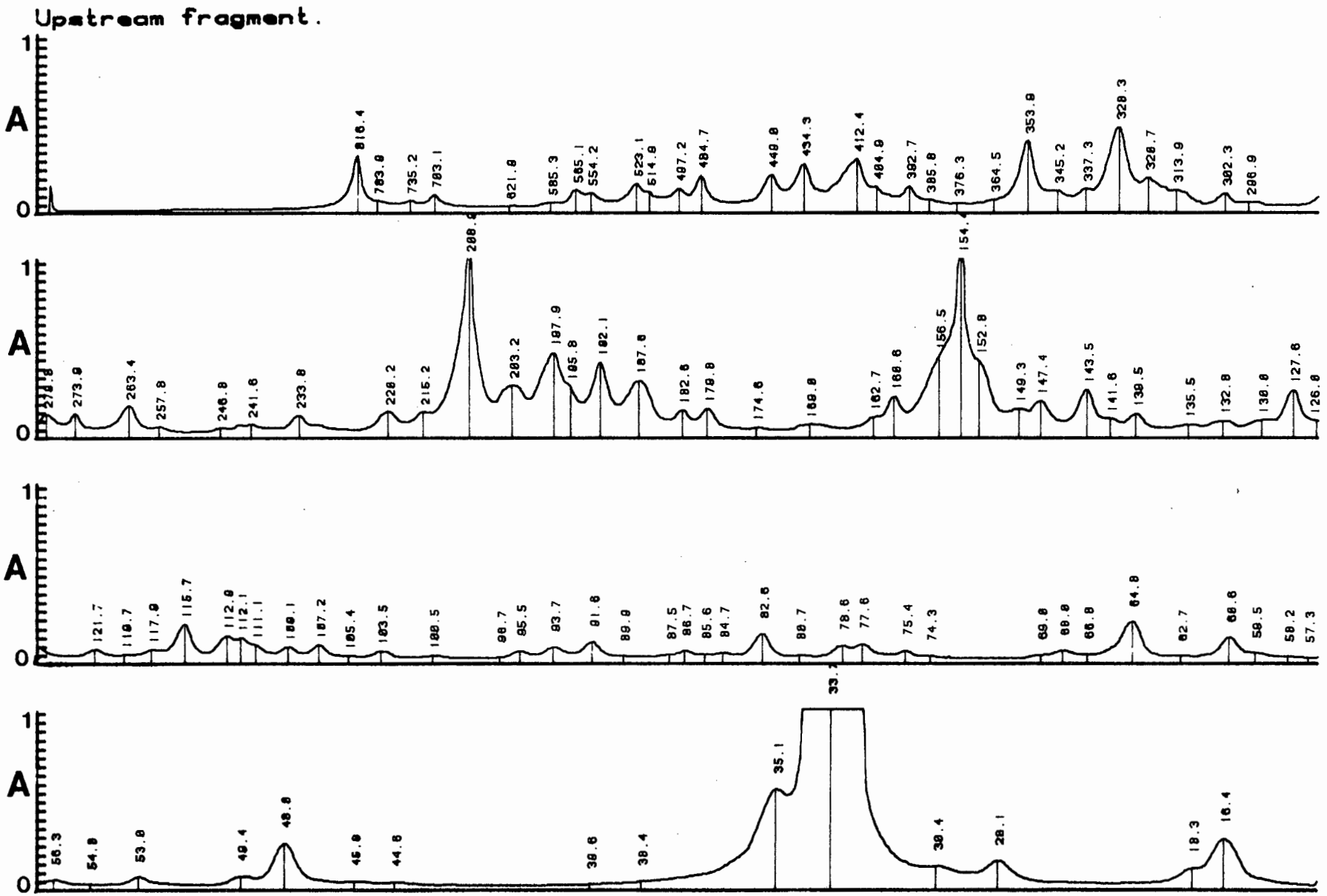
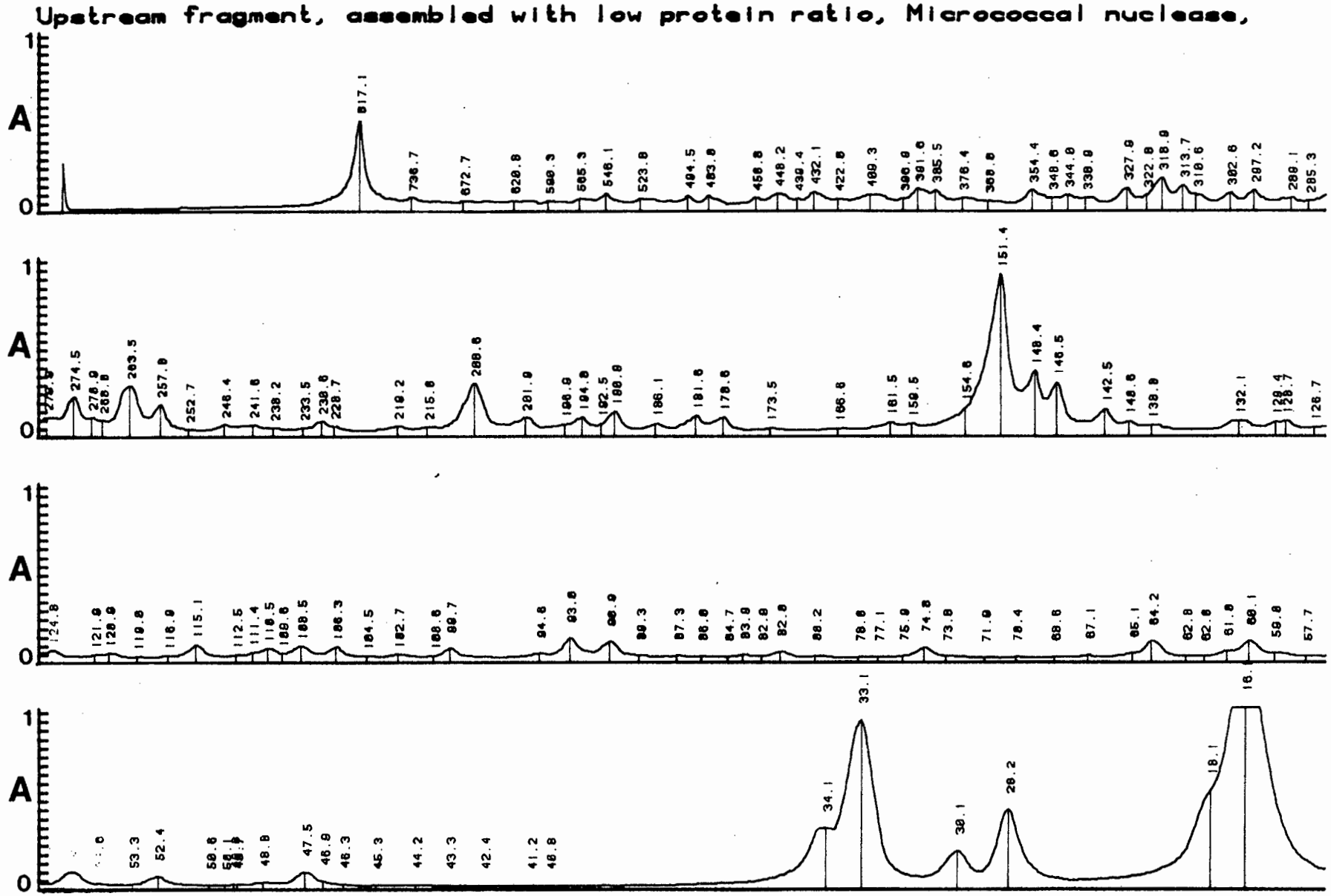


Fig. 3a
Upstream fragment, [^{32}P]dCTP labelled and Micrococcal nuclease digested. Lanes 3-11: free DNA digested with 1 unit Micrococcal nuclease/µg DNA. Lanes 13-21: DNA assembled at a histone to DNA ratio of 0.32:1 (w/w), digested with 10 units Micrococcal nuclease/µg DNA. Lanes 1 and 23: size markers. Lanes 2, 12 and 22: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480 and 720 seconds and the DNA analysed on a denaturing DNA gel.



Appendix B; Fig..3b. Densitometer trace of appendix B; Fig. 3a, lane 5.



Appendix B; Fig. 3c. Densitometer trace of appendix B; Fig. 3a, lane 15.

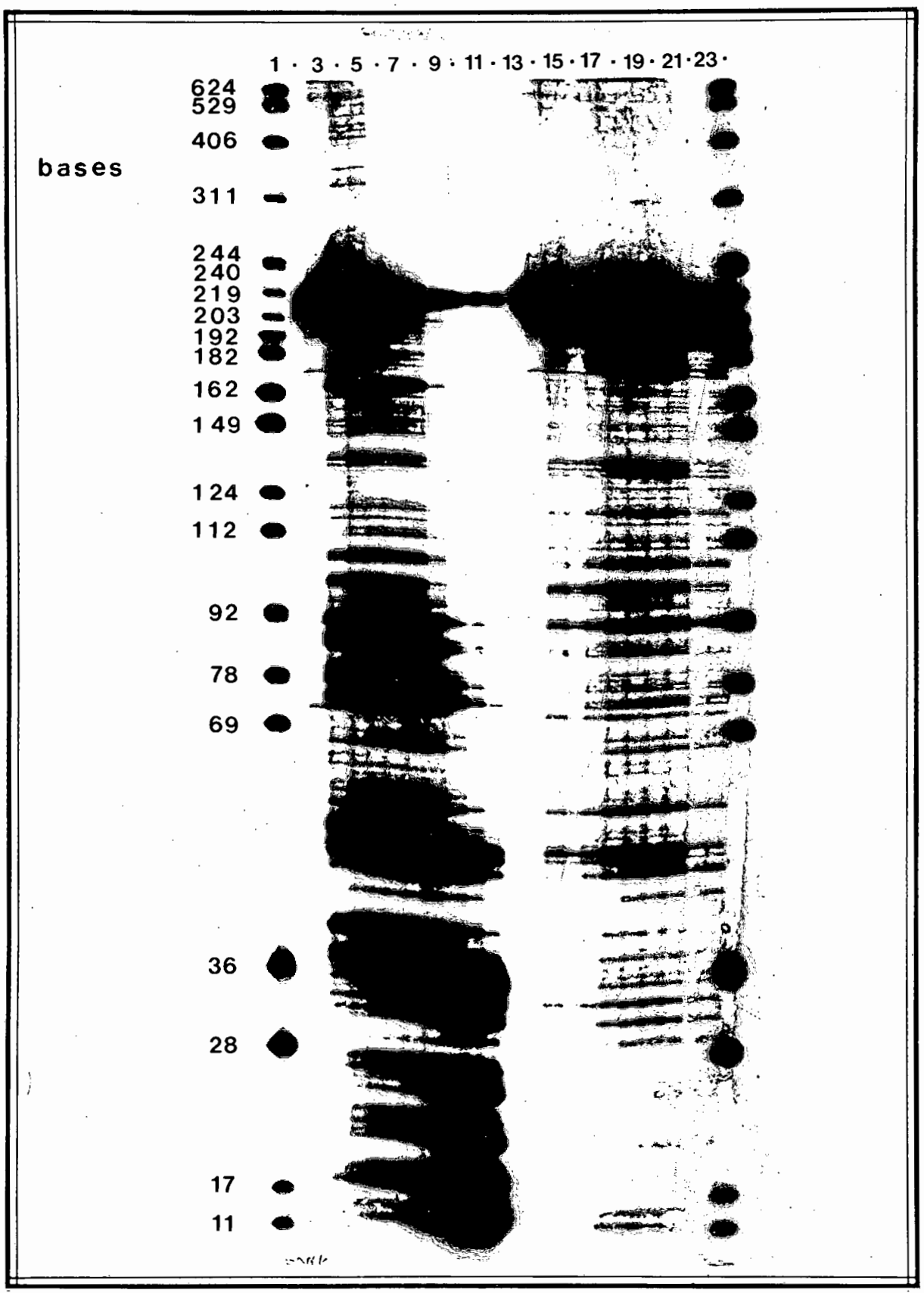
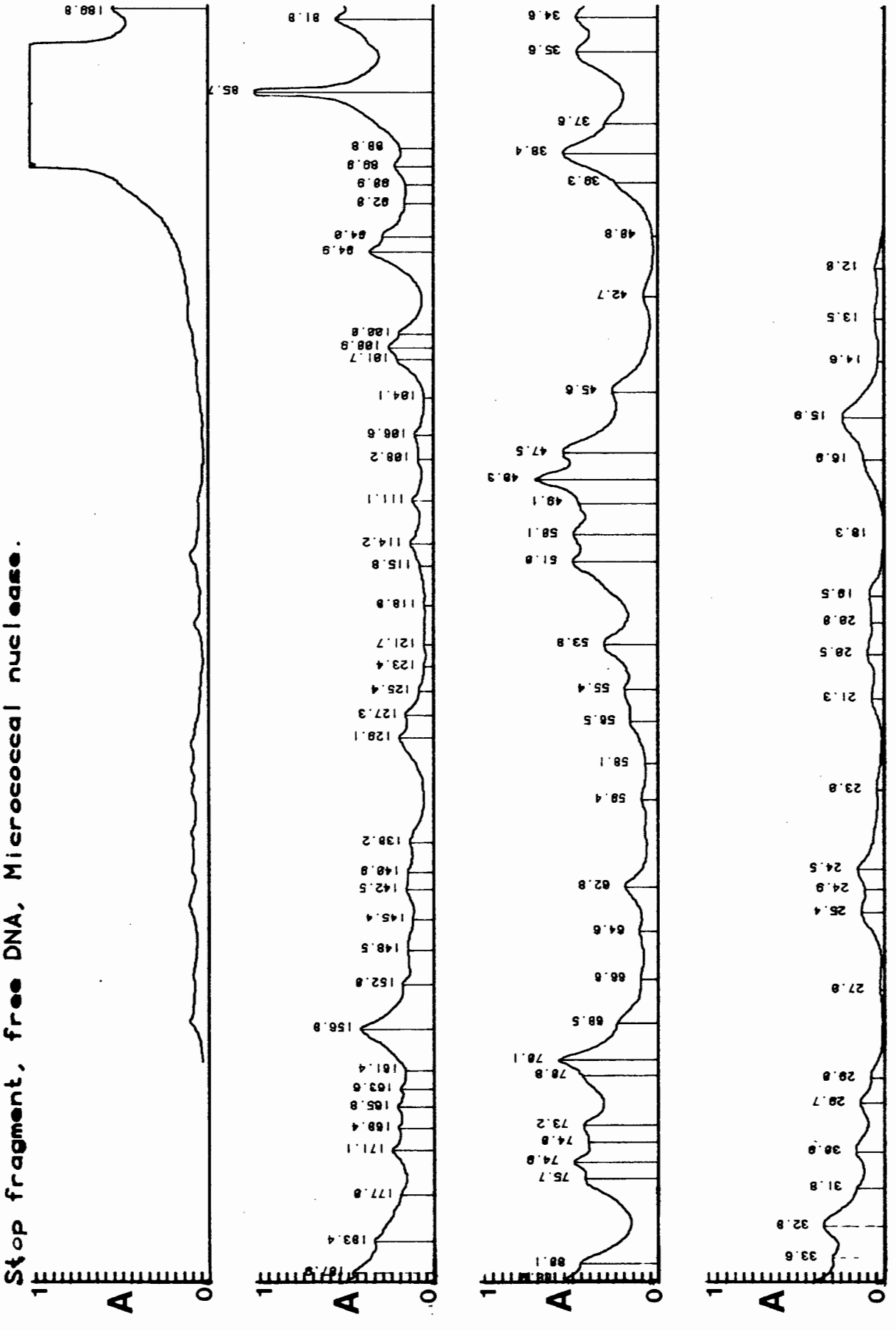
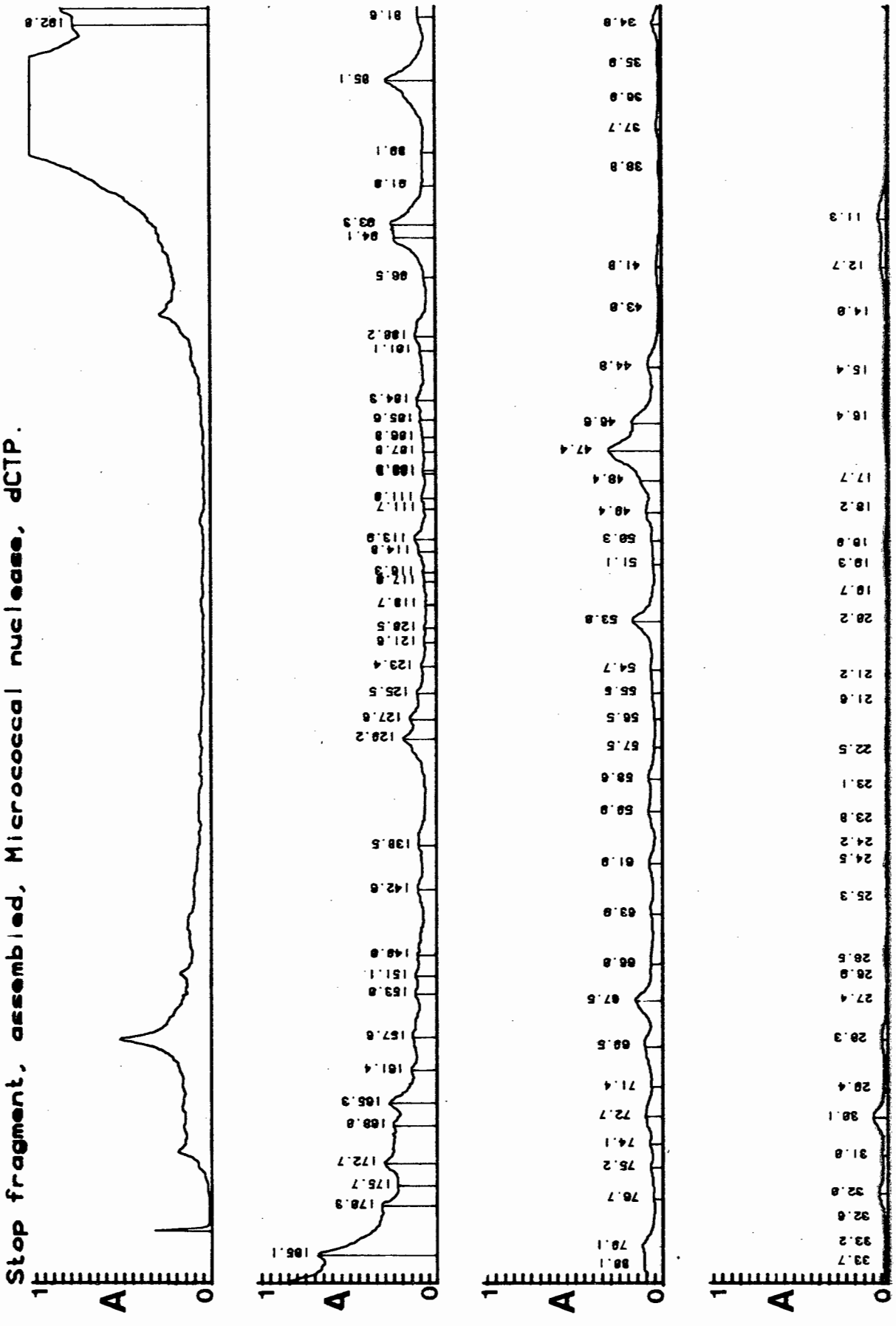


Fig. 4a

Stop fragment, labelled with [^{32}P]dCTP and digested with DNase I. Lanes 3-12: free DNA digested at 1.25 units DNase I/µg DNA. Lanes 14-23: DNA assembled at a histone to DNA ratio of 0.64:1 (w/w), digested with 12.5 units DNase I/µg DNA. Lanes 1 and 24: [illegible] size markers. Lanes 2 and 13: sample buffer. Digestions were carried out for 0, 15, 30, 60, 90, 120, 240, 480, 720 and 1 200 seconds and the DNA analysed on a denaturing DNA gel.



Appendix B; Fig. 4b. Densitometer trace of appendix B; Fig. 4a, lane 5.



Densitometer trace of appendix B; Fig. 4a, lane 16.
Appendix B; Fig. 4c.

APPENDIX C

Calladine's rules (See section 8 Figs. 75 and 76), applied to the sequences of the UPSTREAM, STOP and END fragments (Figs. 51, 52 and 53), as well as the 5S RNA gene sequence (Lu et al. 1980). The sum functions (Fig. 76) have been applied to the coding strand and can therefore be directly compared to the sequences in Figs. 51, 52 and 53.

UPSTREAM FRAGMENT SEQUENCE:

Fig.	1	Twist
Fig.	2	Roll
Fig.	3	Torsion (sigma)
Fig.	4	Propeller twist

STOP FRAGMENT SEQUENCE:

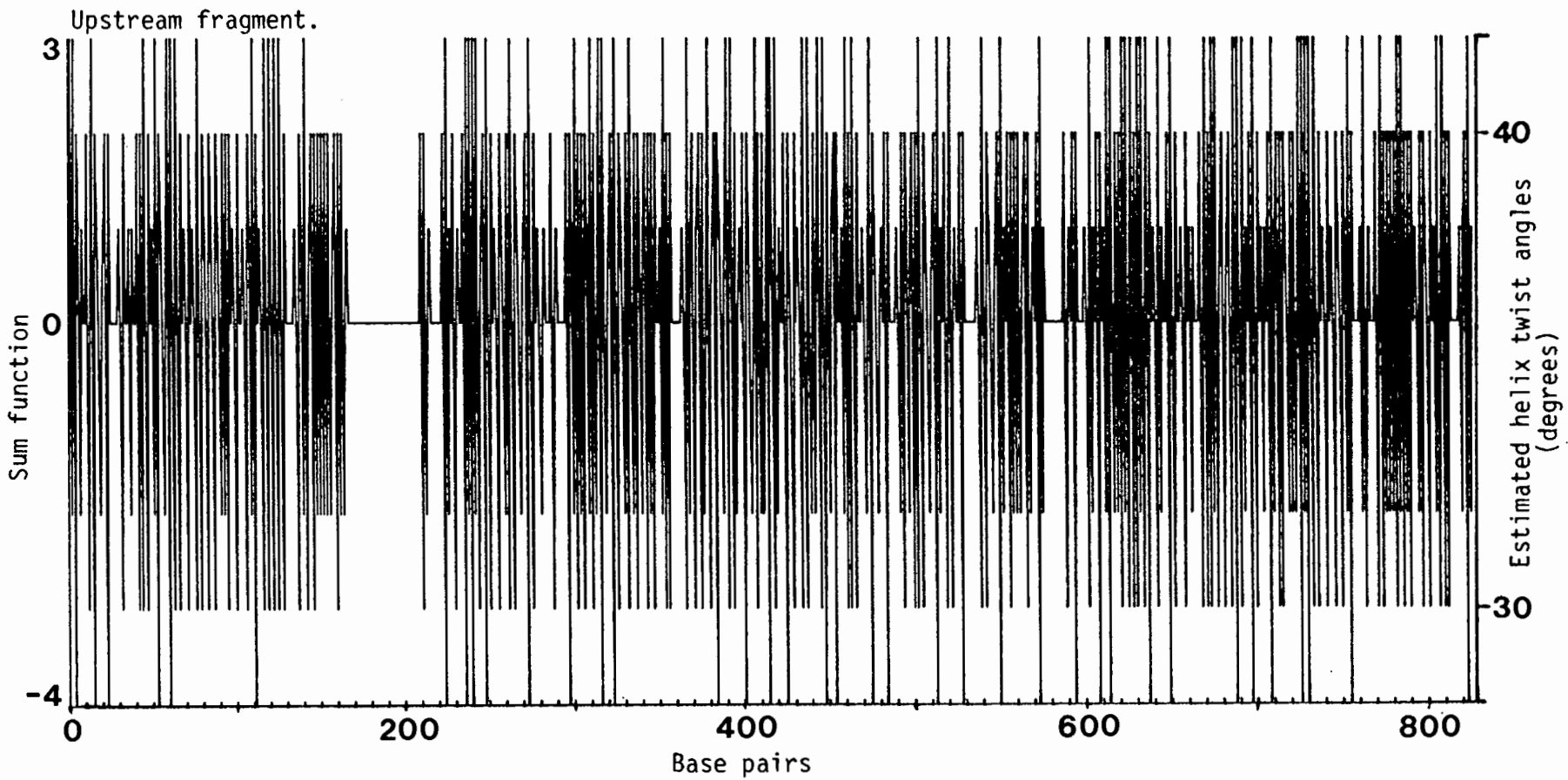
Fig.	5	Twist
Fig.	6	Roll
Fig.	7	Torsion (sigma)
Fig.	8	Propeller twist

END FRAGMENT SEQUENCE:

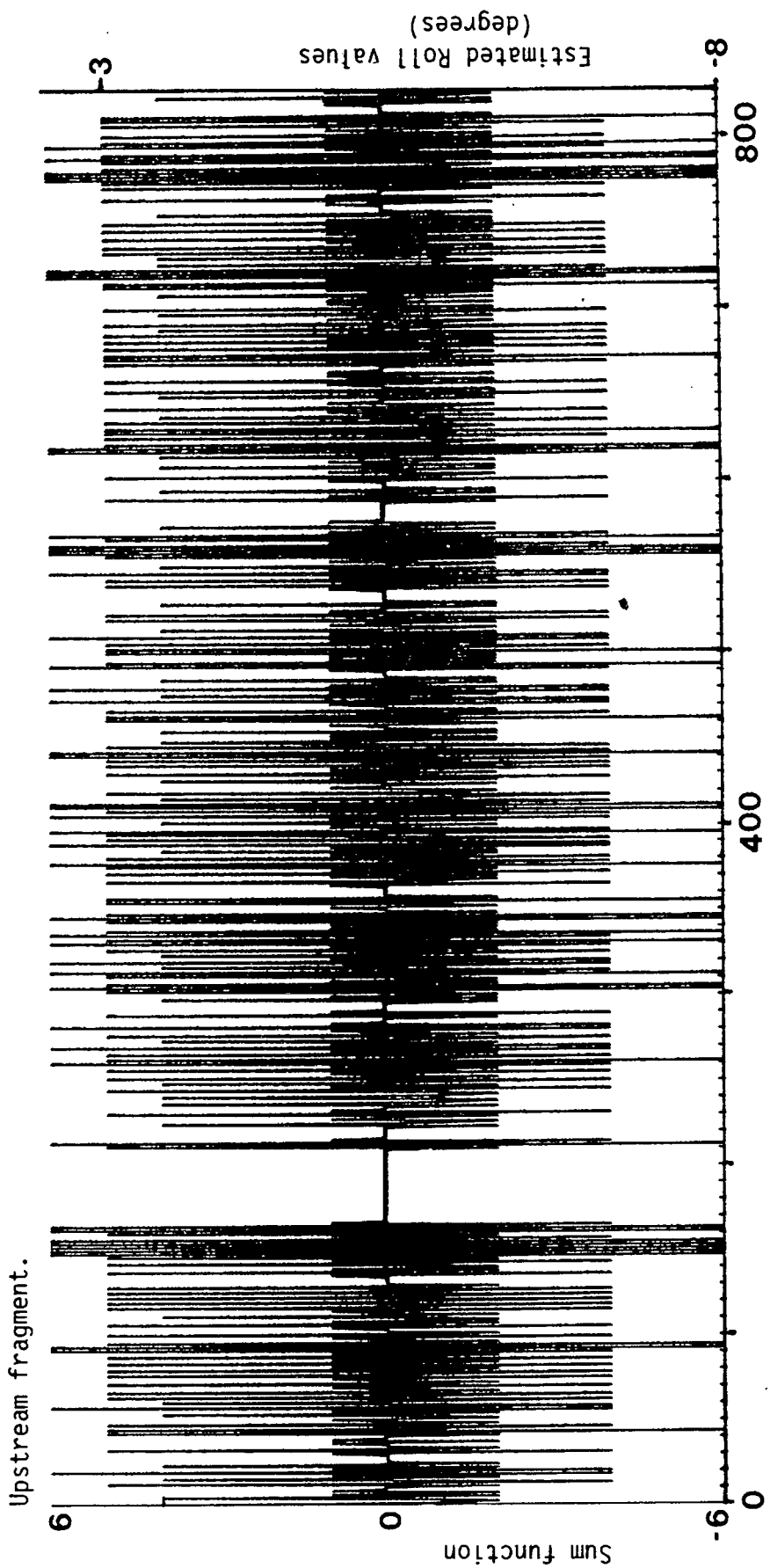
Fig.	9	Twist
Fig.	10	Roll
Fig.	11	Torsion (sigma)
Fig.	12	Propeller twist

5S RNA SEQUENCE:

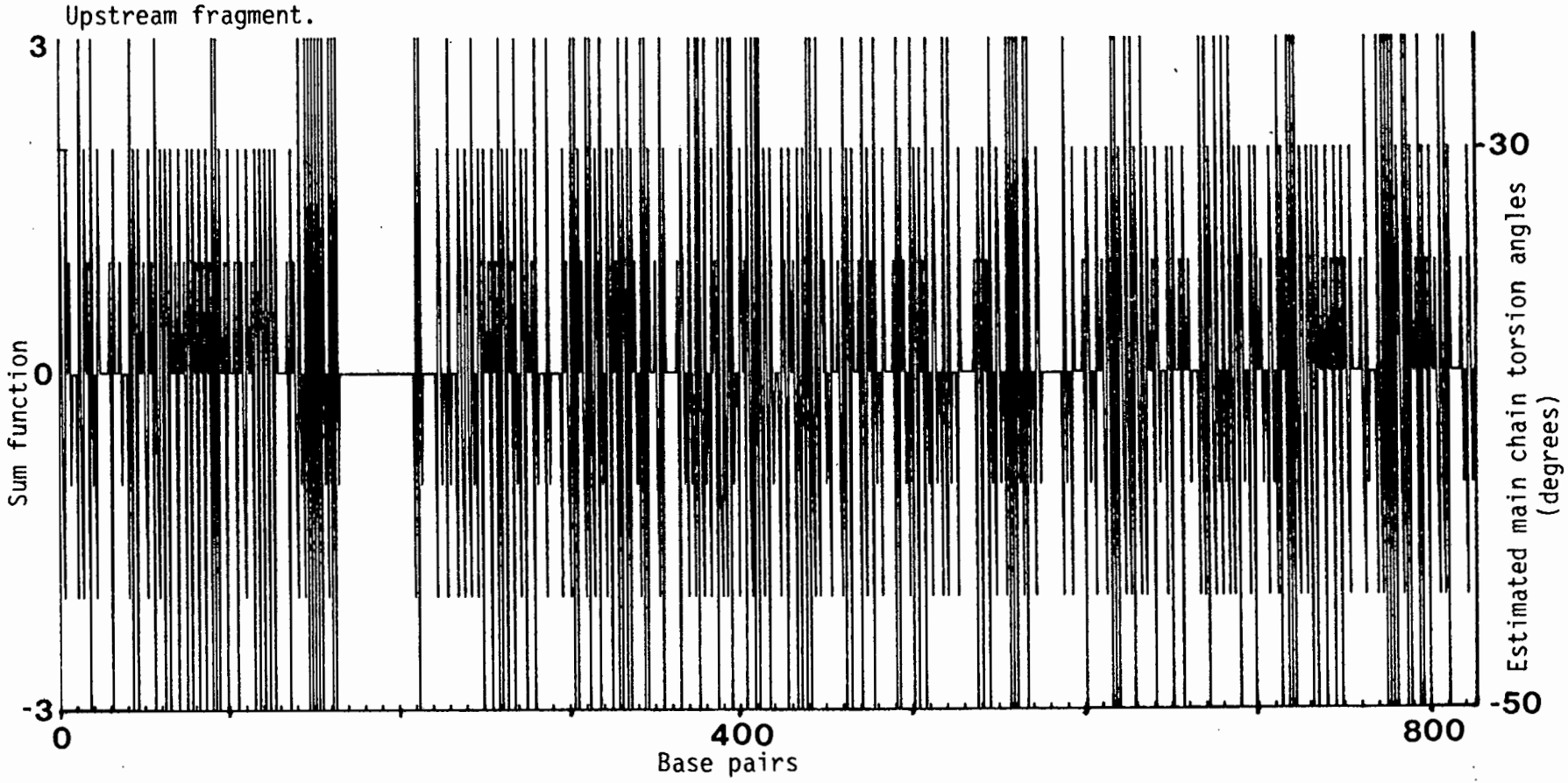
Fig.	13	Twist
Fig.	14	Roll
Fig.	15	Torsion (sigma)
Fig.	16	Propeller twist



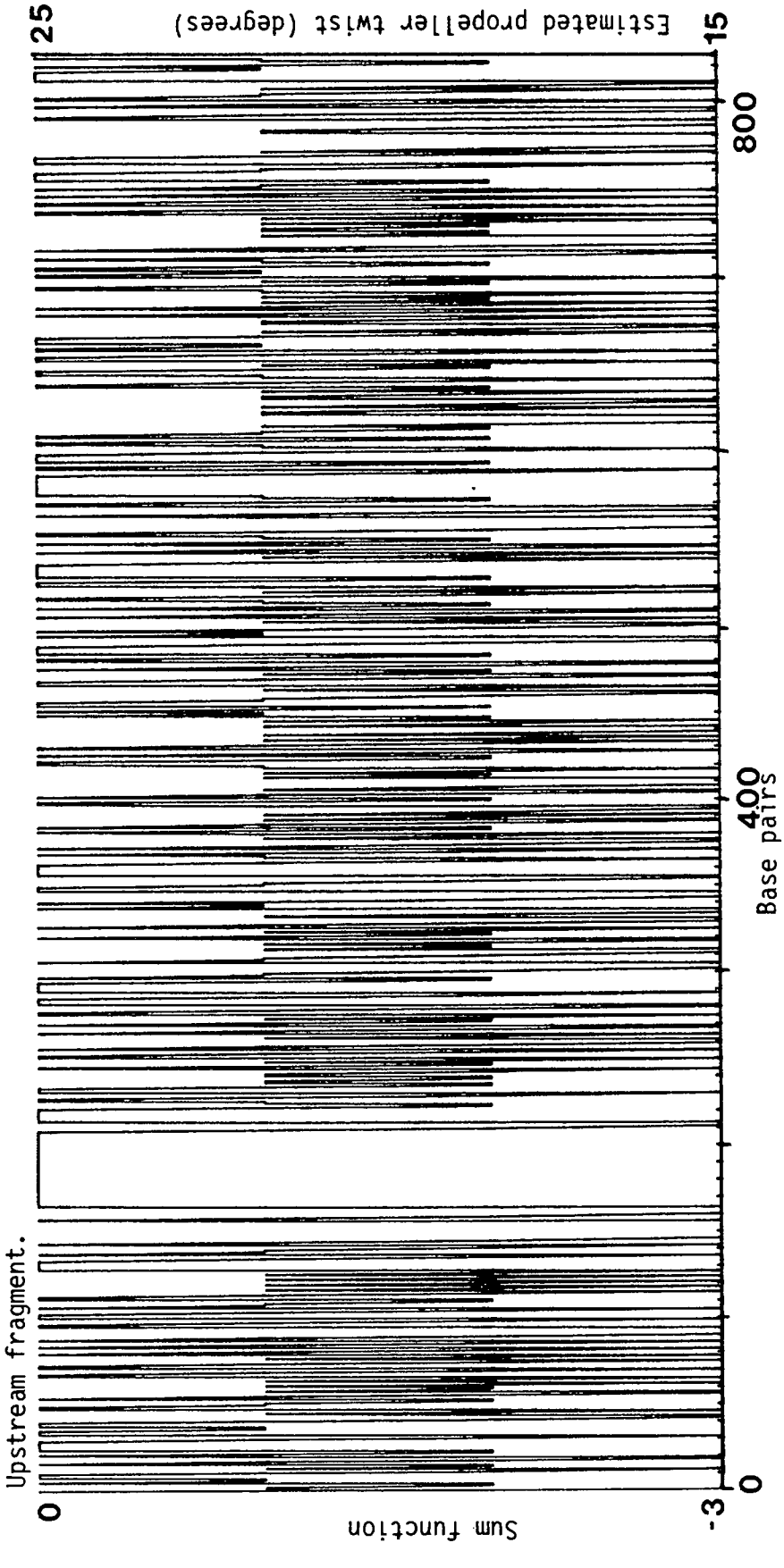
Appendix C Fig 1



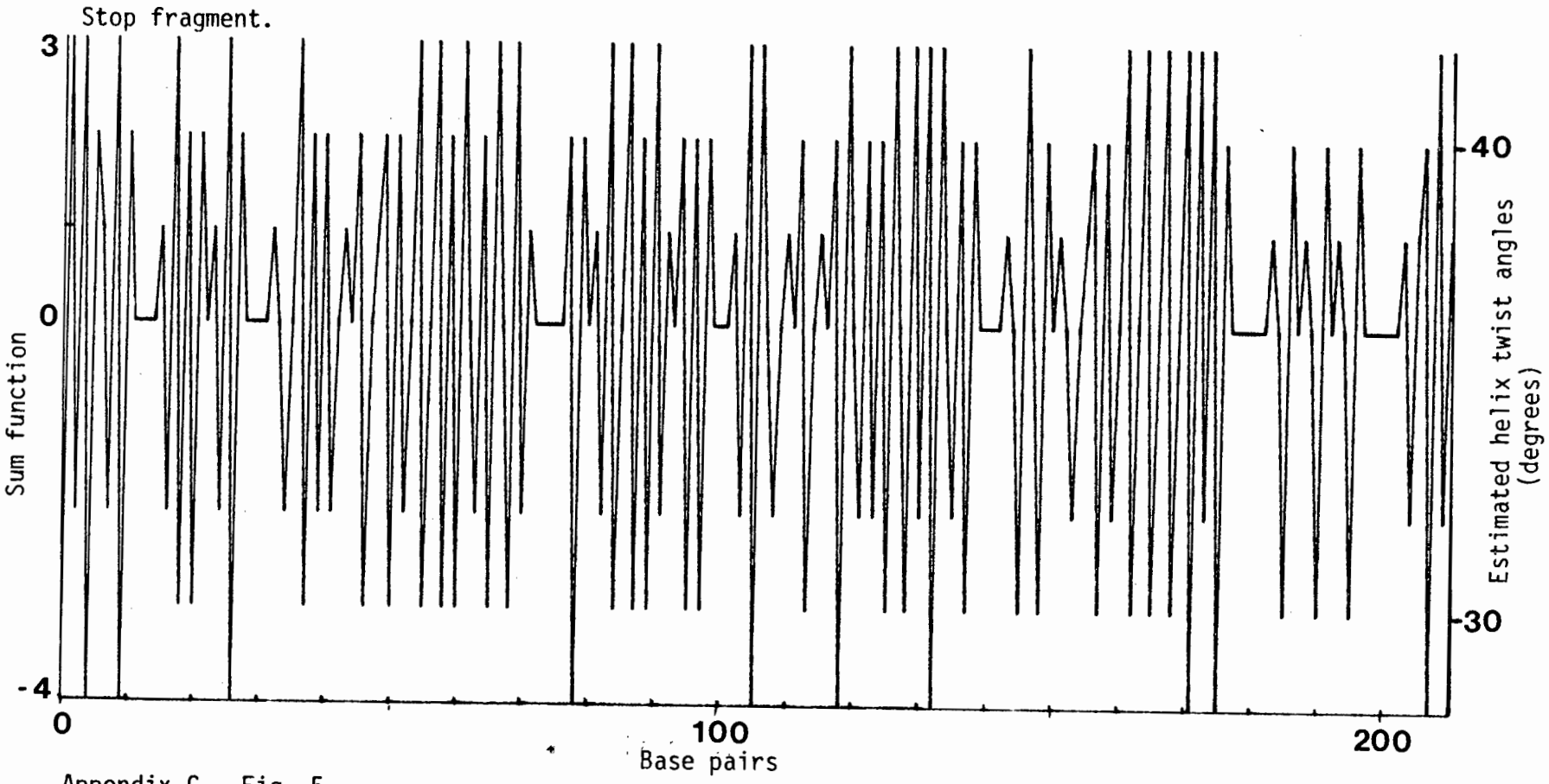
Appendix C Fig. 2



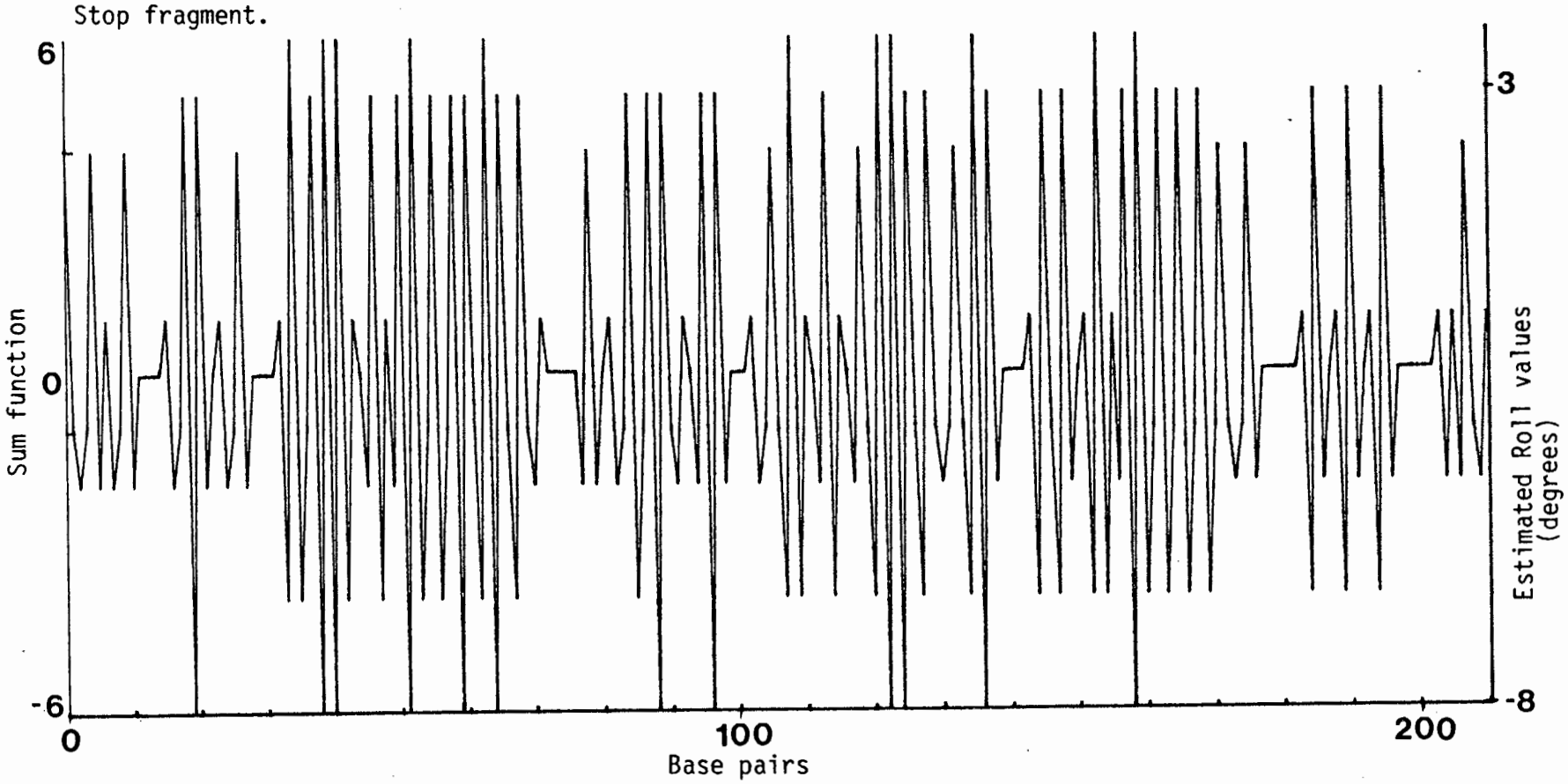
Appendix C Fig. 3



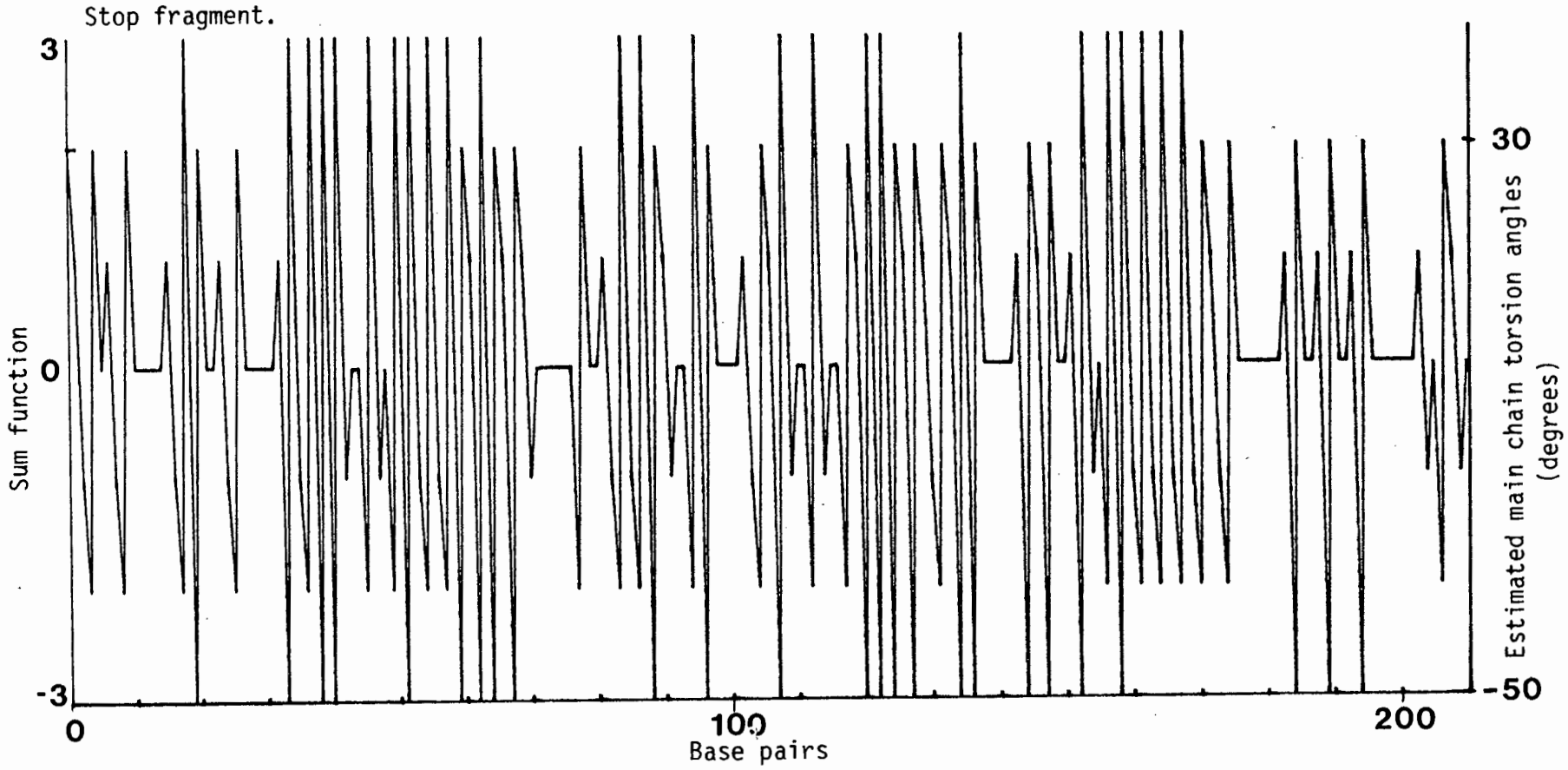
Appendix C Fig. 4



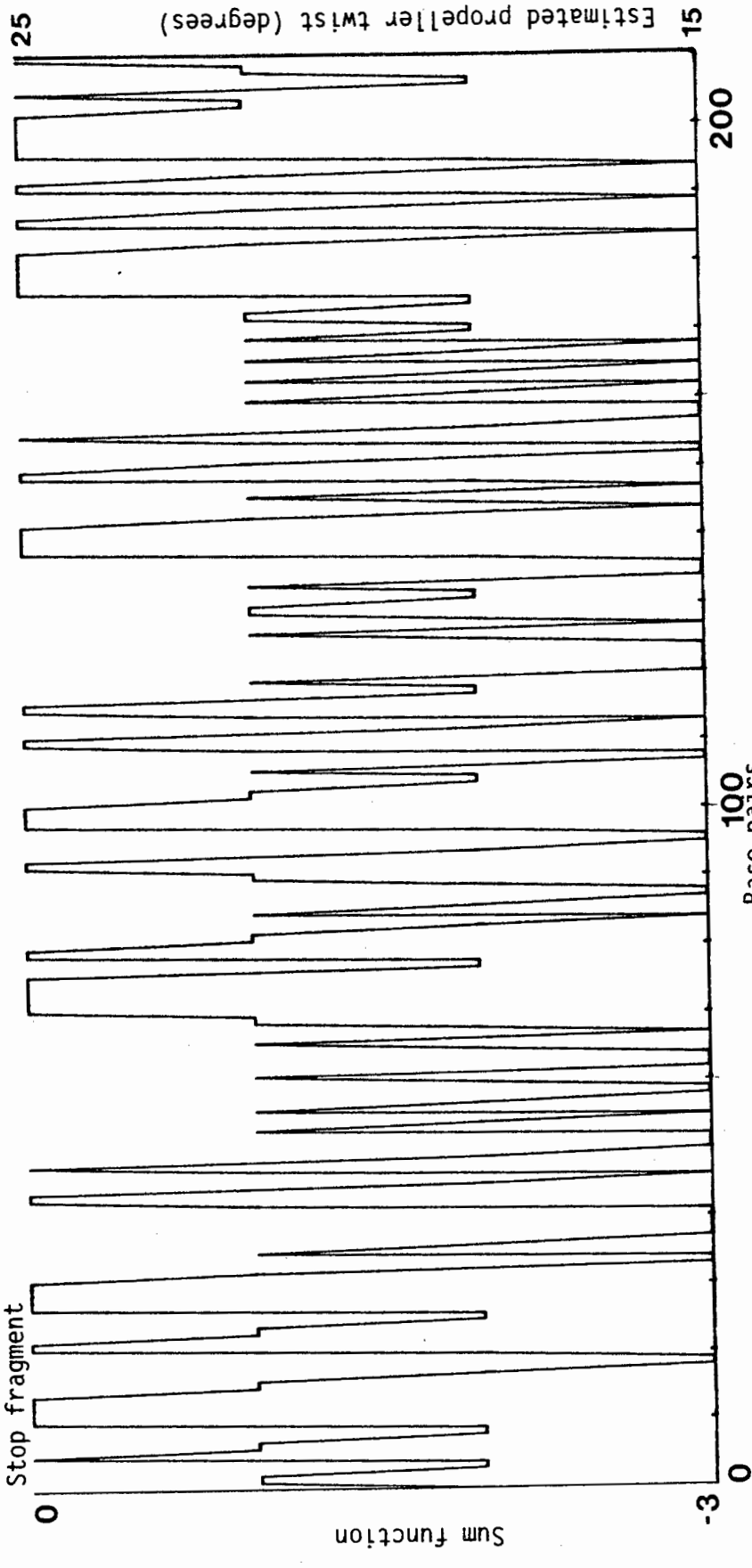
Appendix C Fig. 5



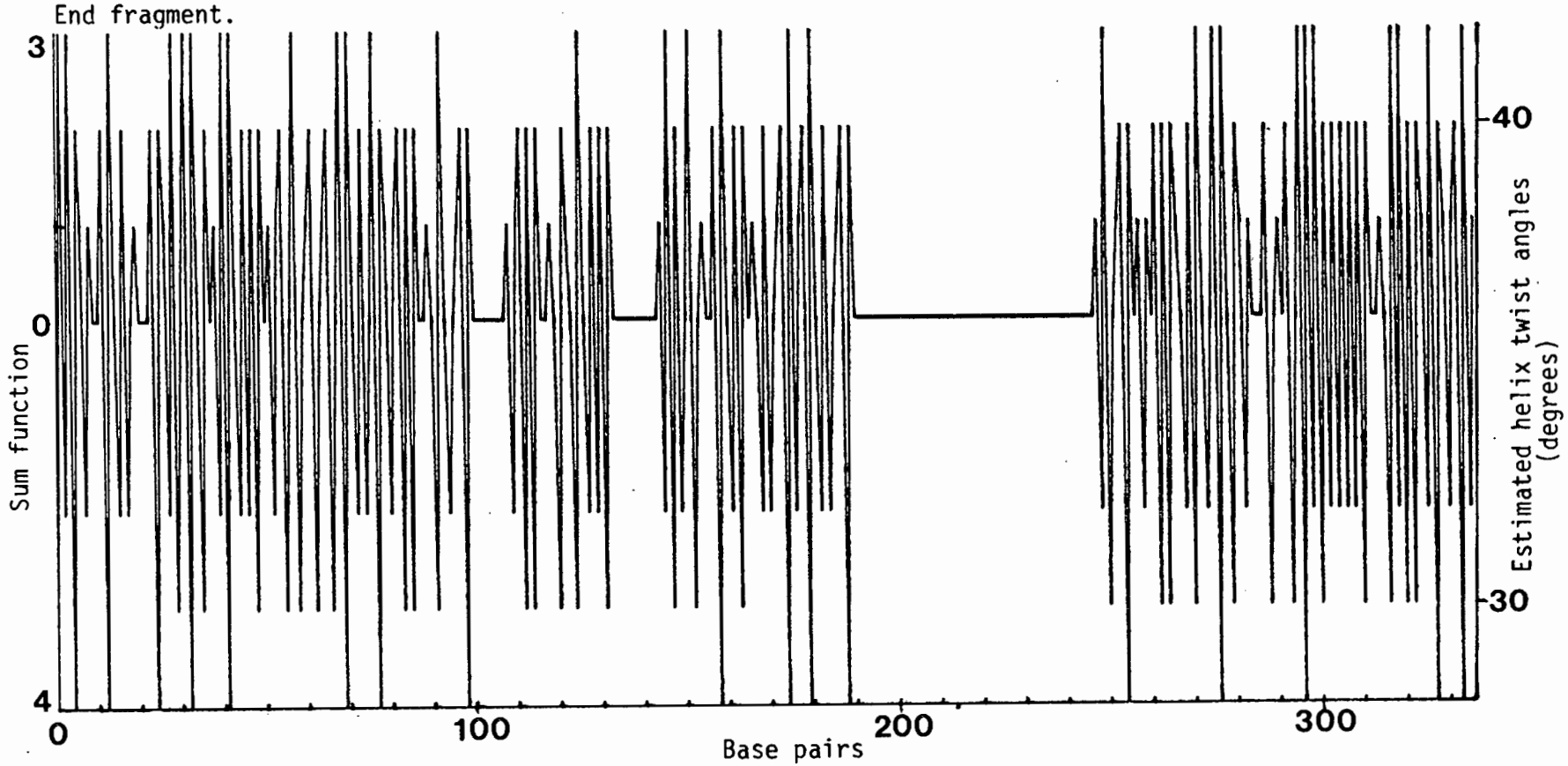
Appendix C Fig. 6



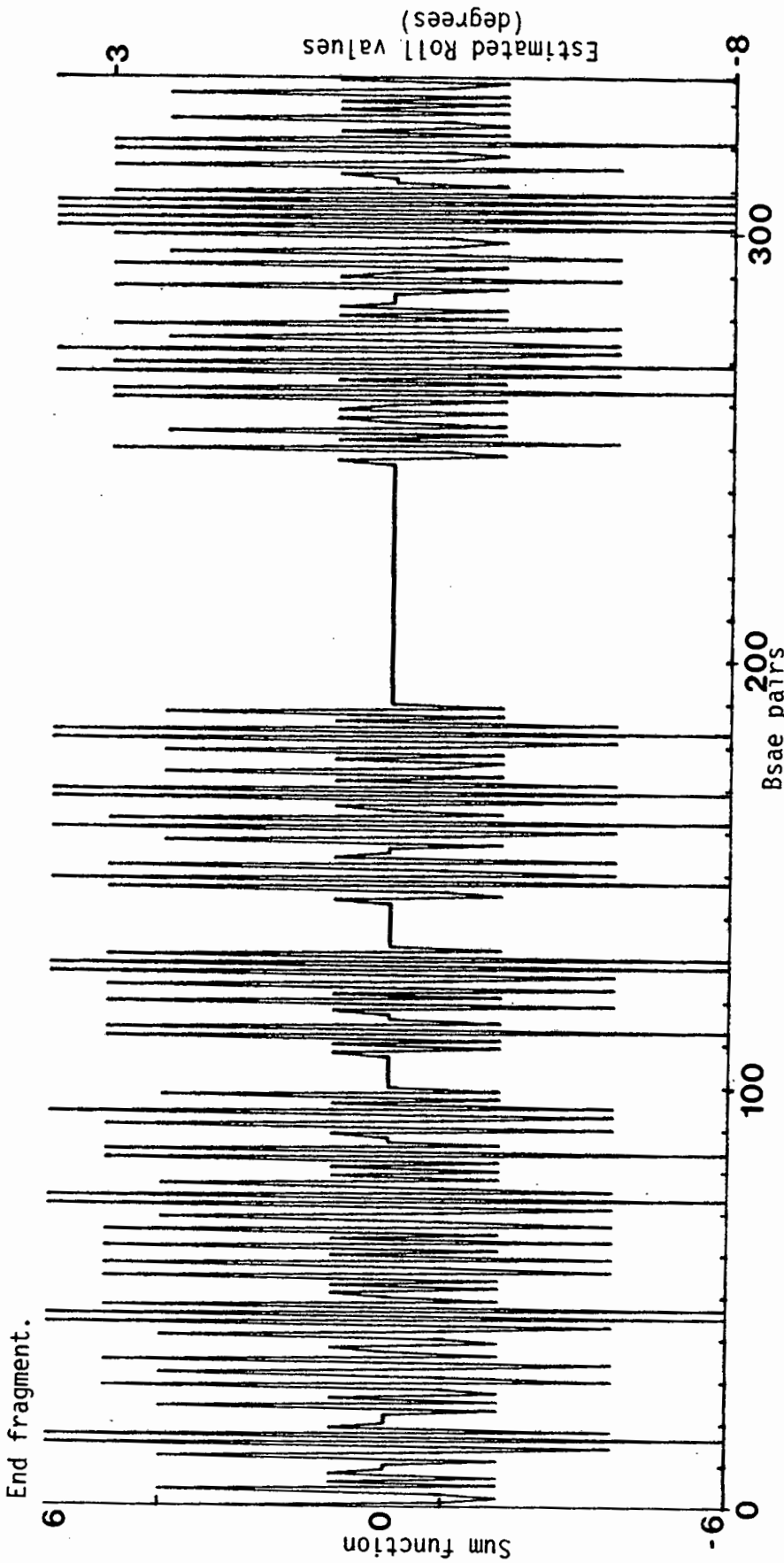
Appendix C Fig. 7



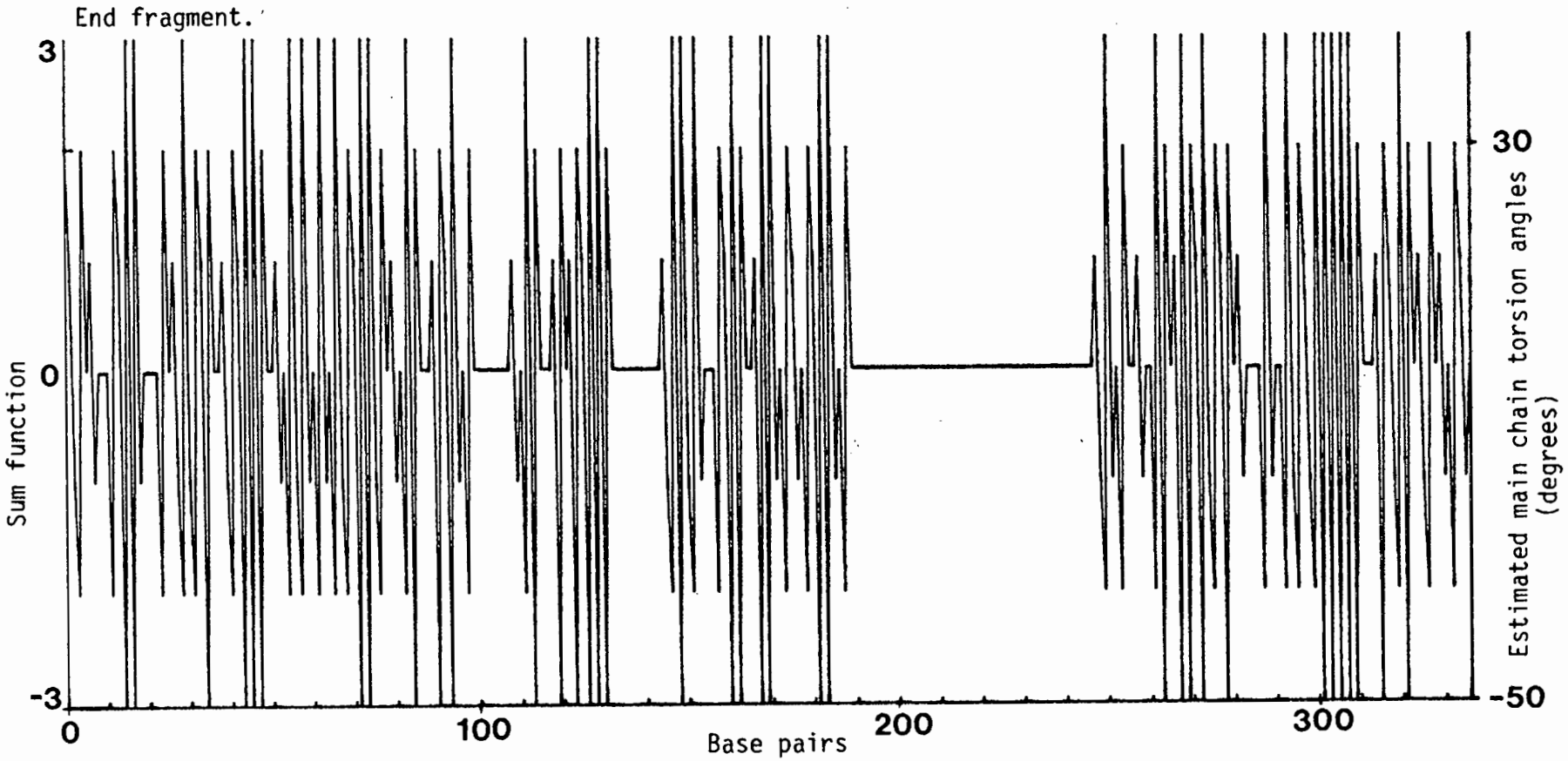
Appendix C Fig. 8



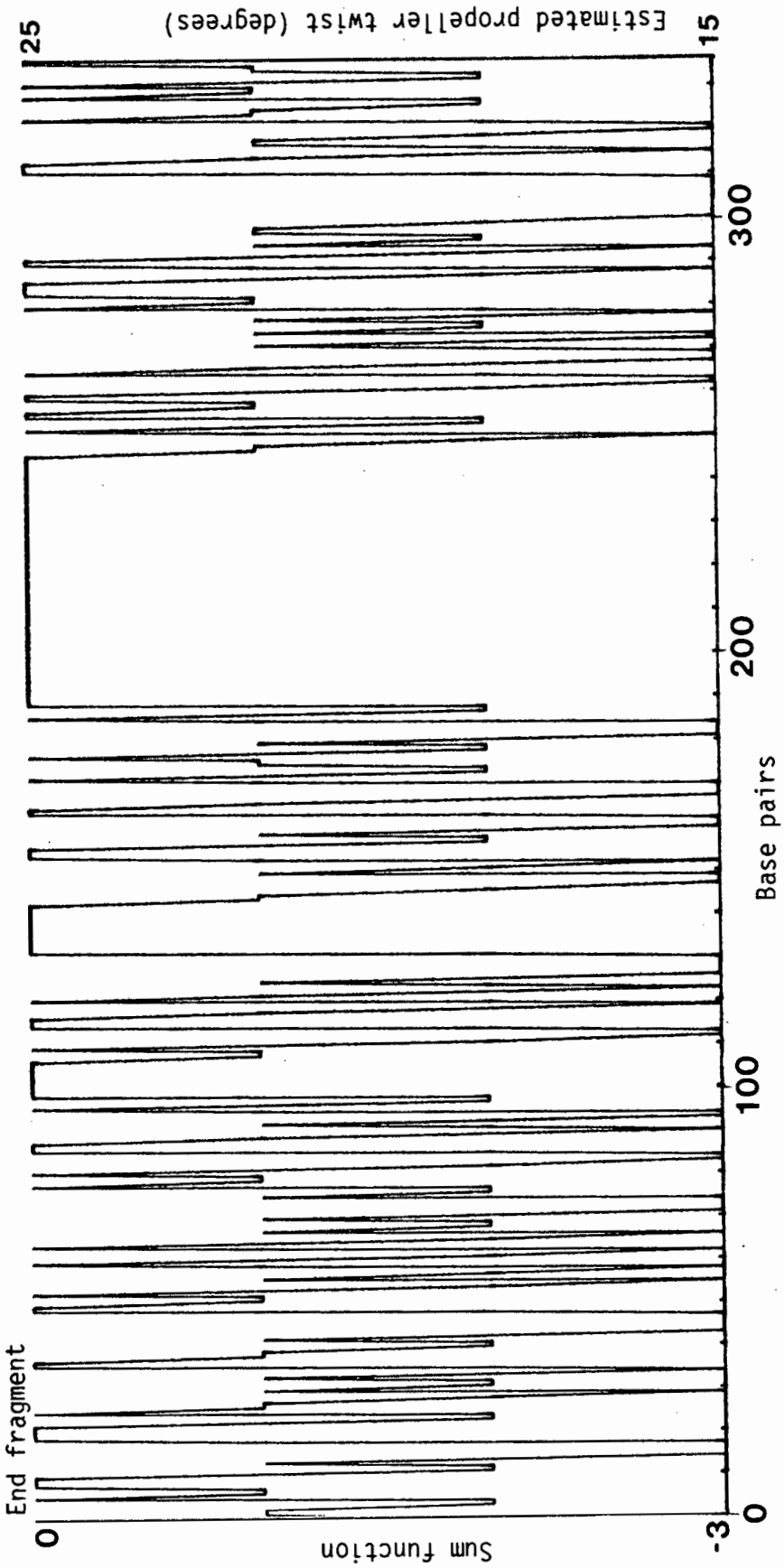
Appendix C Fig. 9



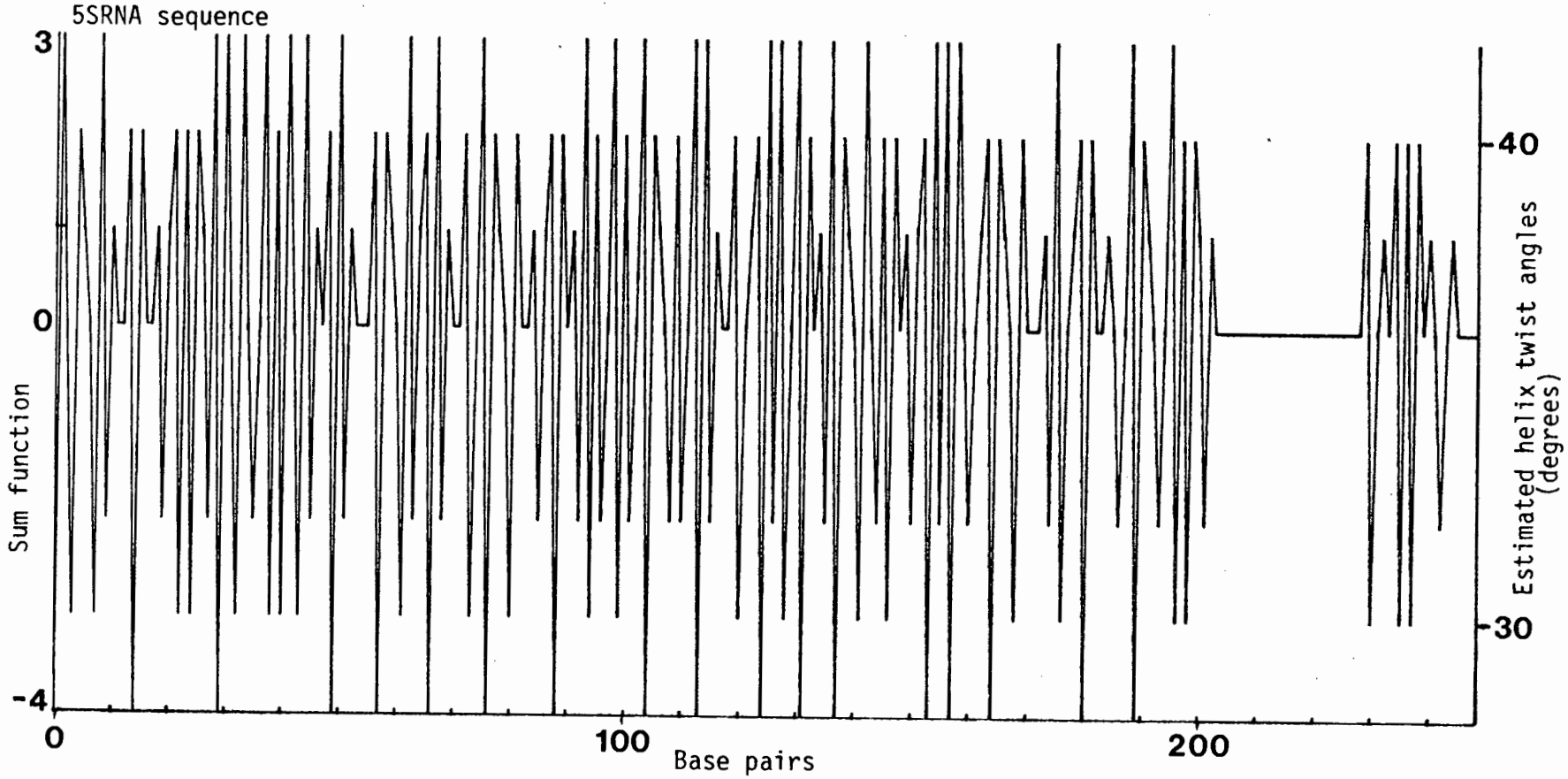
Appendix C Fig. 10



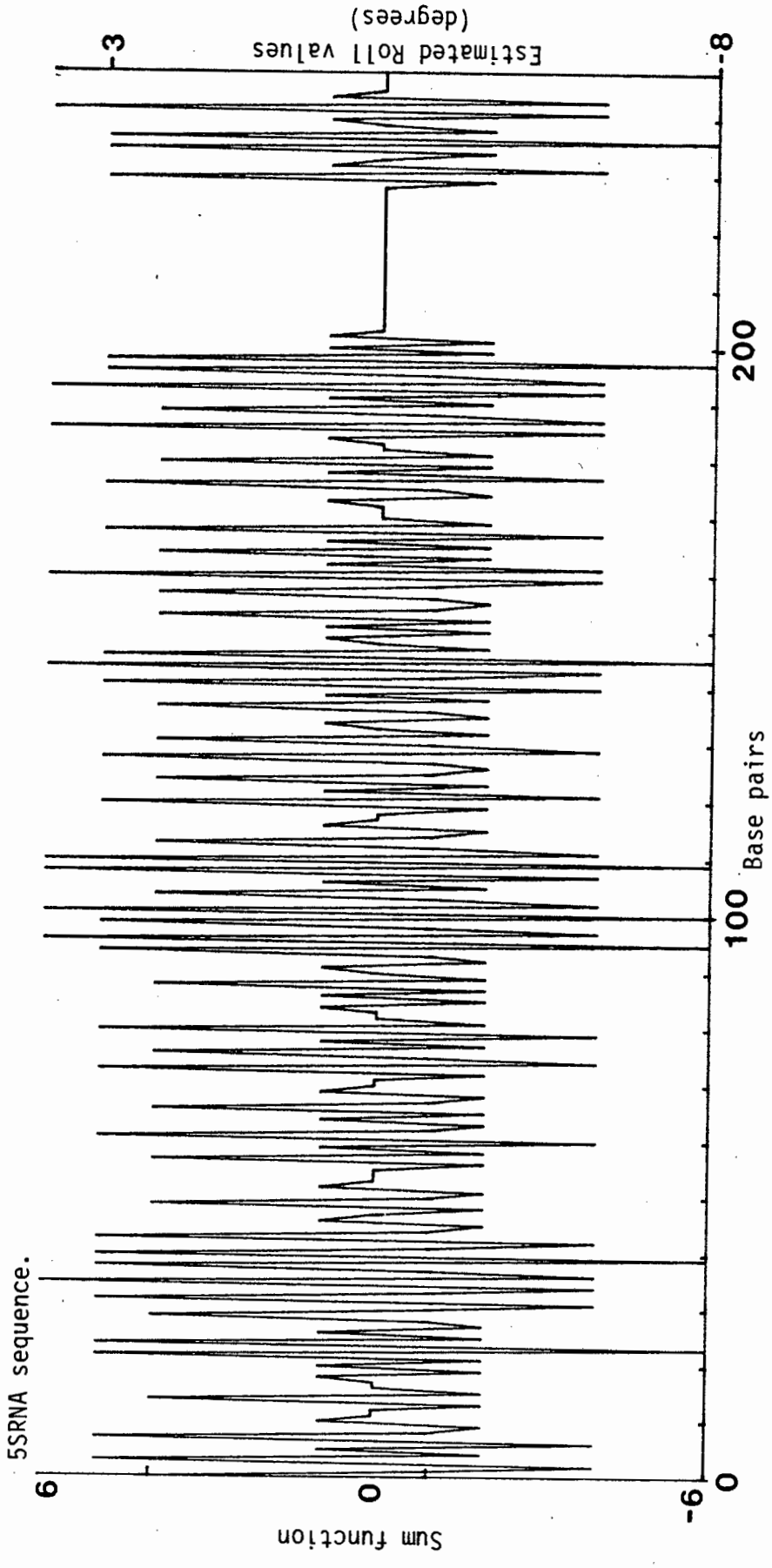
Appendix C Fig. 11



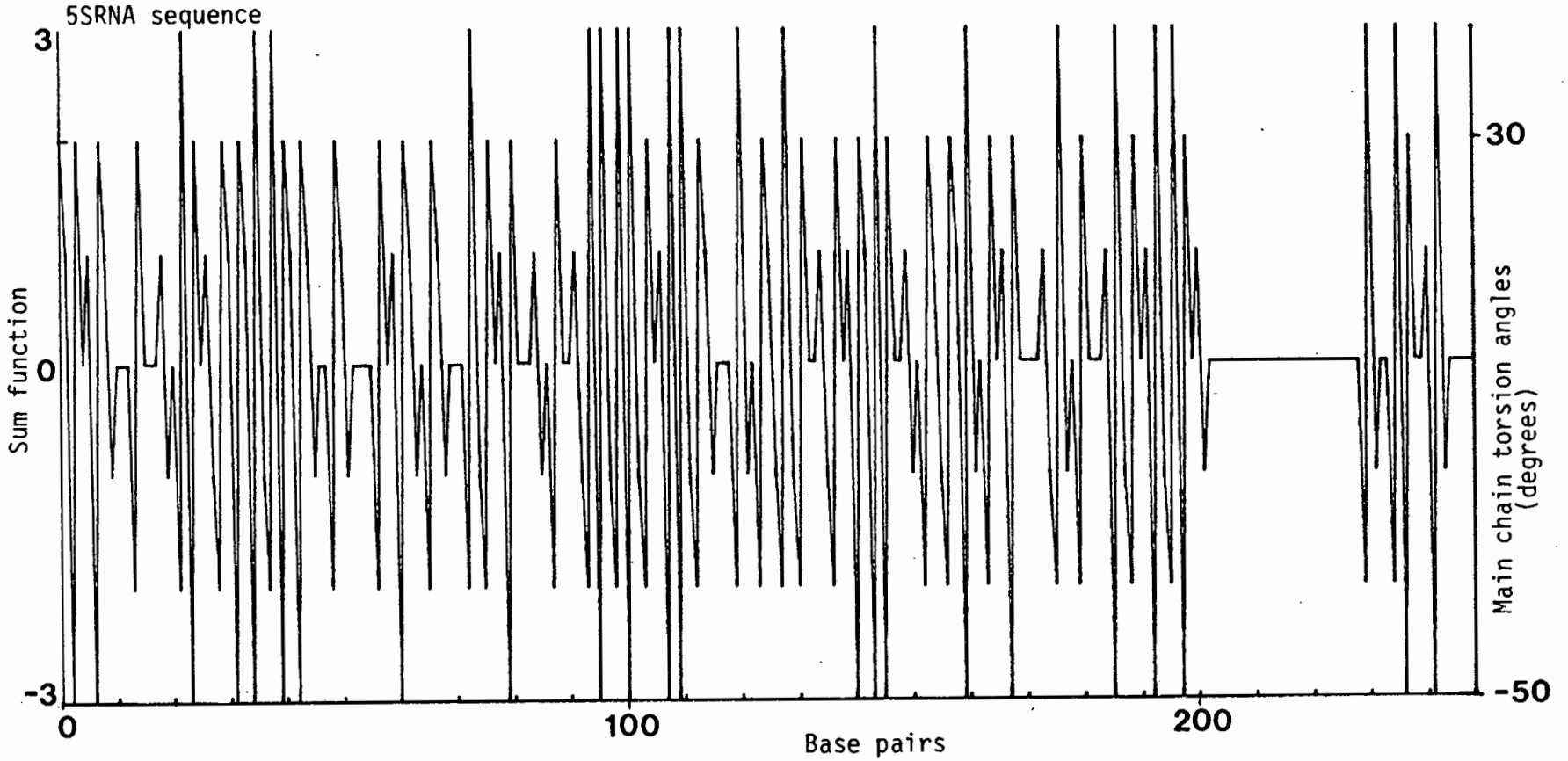
Appendix C Fig. 12



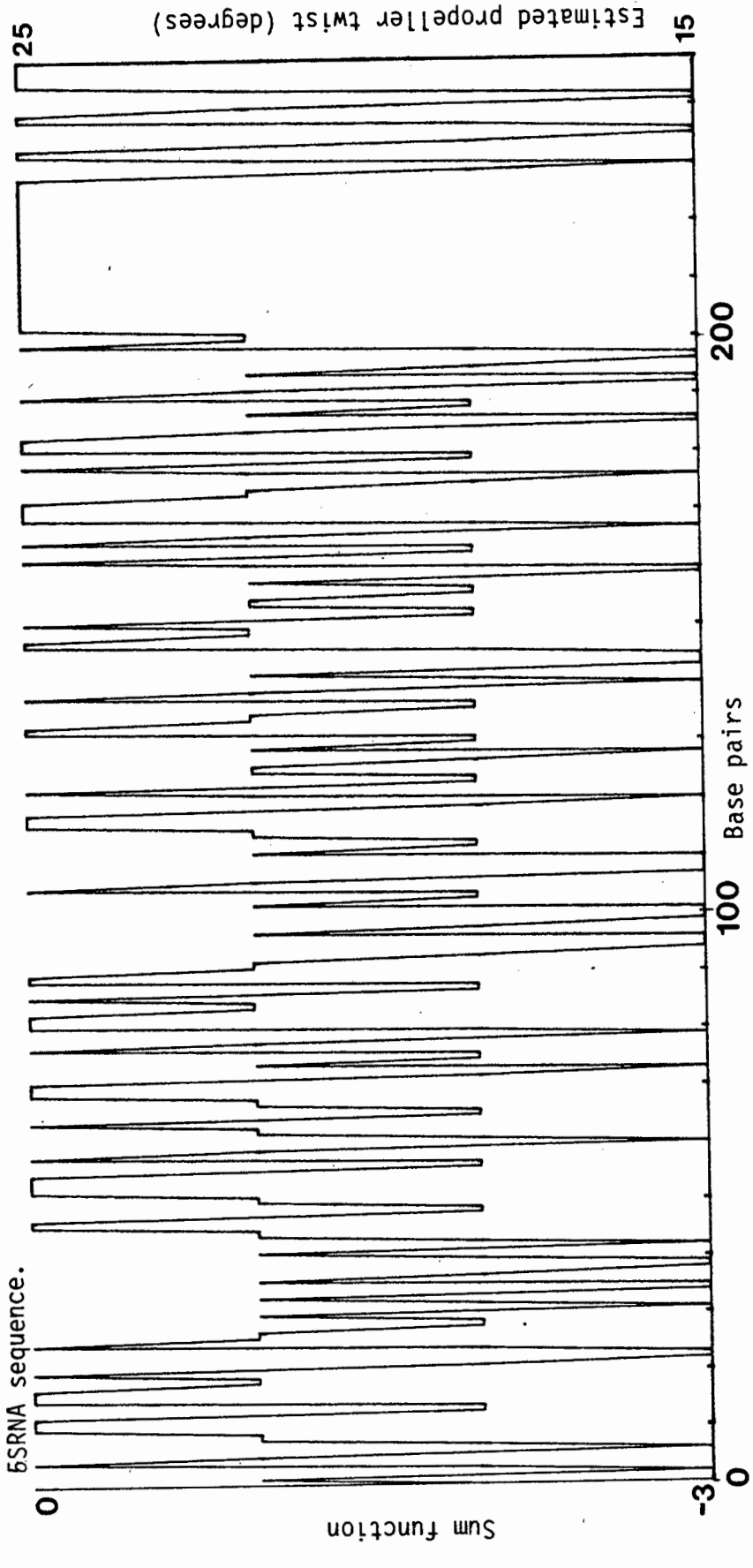
Appendix C Fig. 13



Appendix C Fig. 14



Appendix C Fig. 15



Appendix C Fig. 16

APPENDIX D

Predictions of core positions according to Zhurkin (1983) (Section 8). The functions have been calculated for the coding strands of the UPSTREAM STOP and END fragments (Figs. 51, 52 and 53) as well as the 5S RNA gene sequence (Lu et al. 1980) and can therefore be directly compared to the sequences in Figs. 51, 52 and 53.

UPSTREAM FRAGMENT SEQUENCE:

Fig.	1	f(m)	10 base pair repeat
Fig.	2	f'(m)	10.5 base pair repeat

STOP FRAGMENT SEQUENCE:

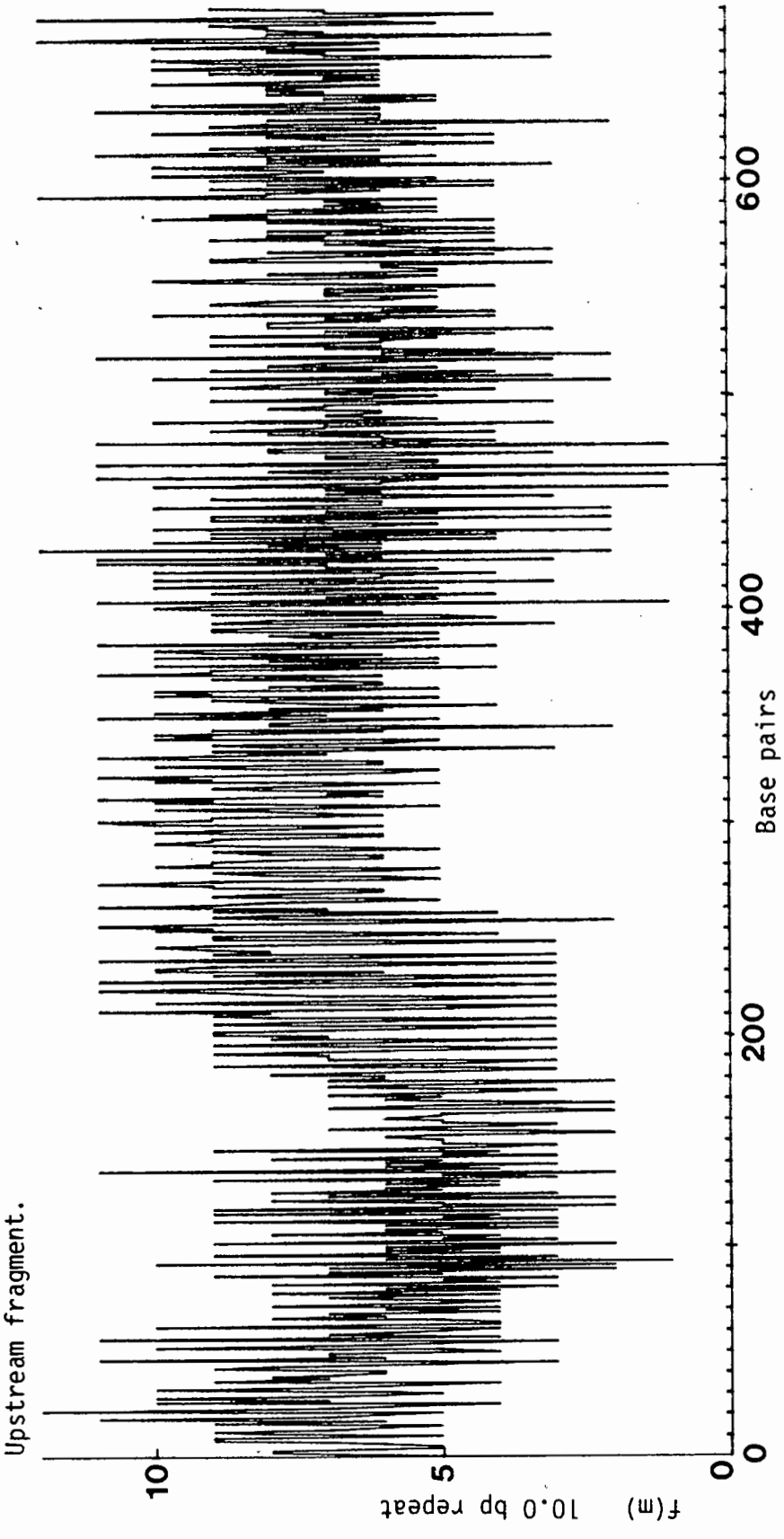
Fig.	3	f(m)	10 base pair repeat
Fig.	4	f'(m)	10.5 base pair repeat

END FRAGMENT SEQUENCE:

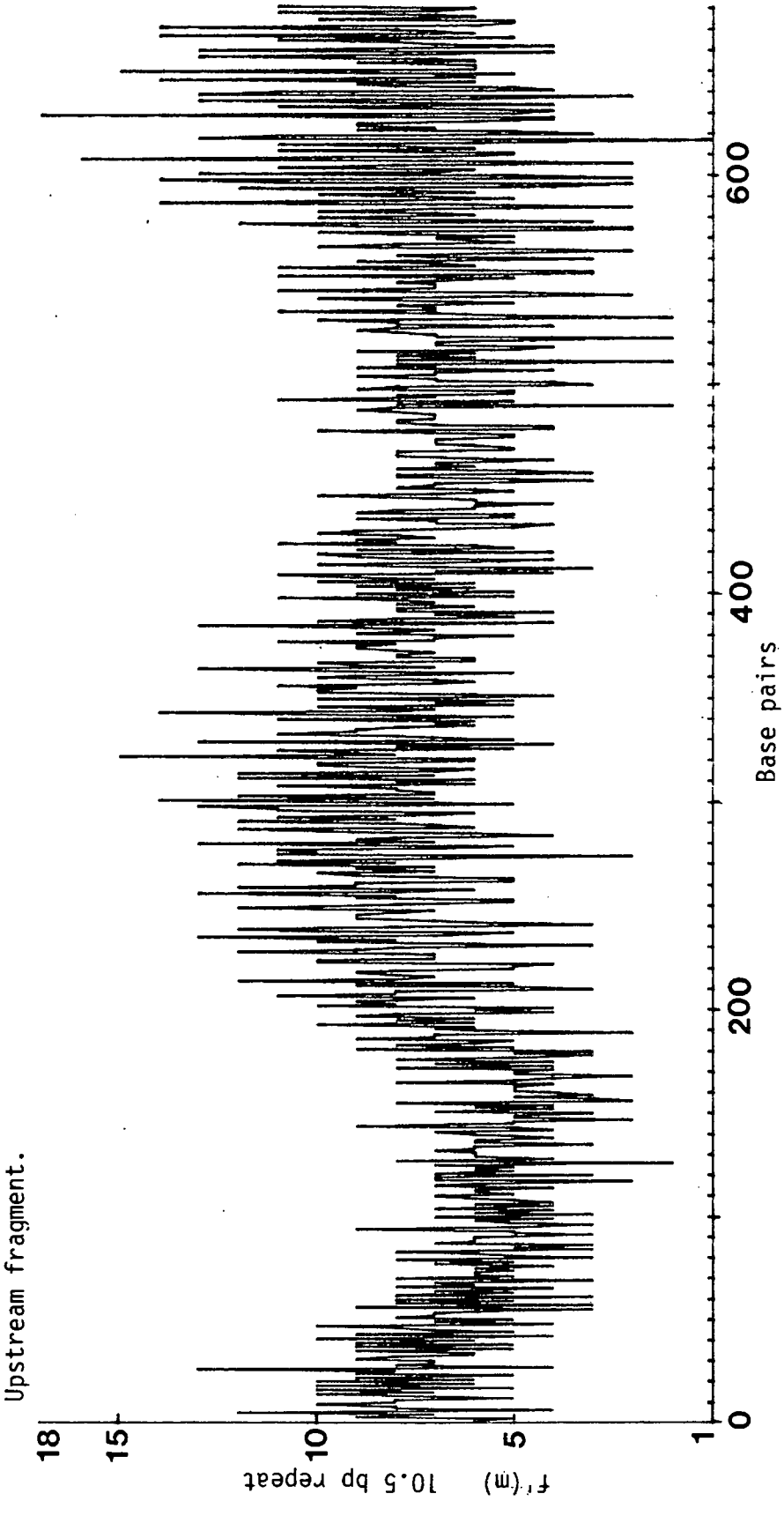
Fig.	5	f(m)	10 base pair repeat
Fig.	6	f'(m)	10.5 base pair repeat

5S RNA SEQUENCE:

Fig.	7	f(m)	10 base pair repeat
Fig.	8	f'(m)	10.5 base pair repeat

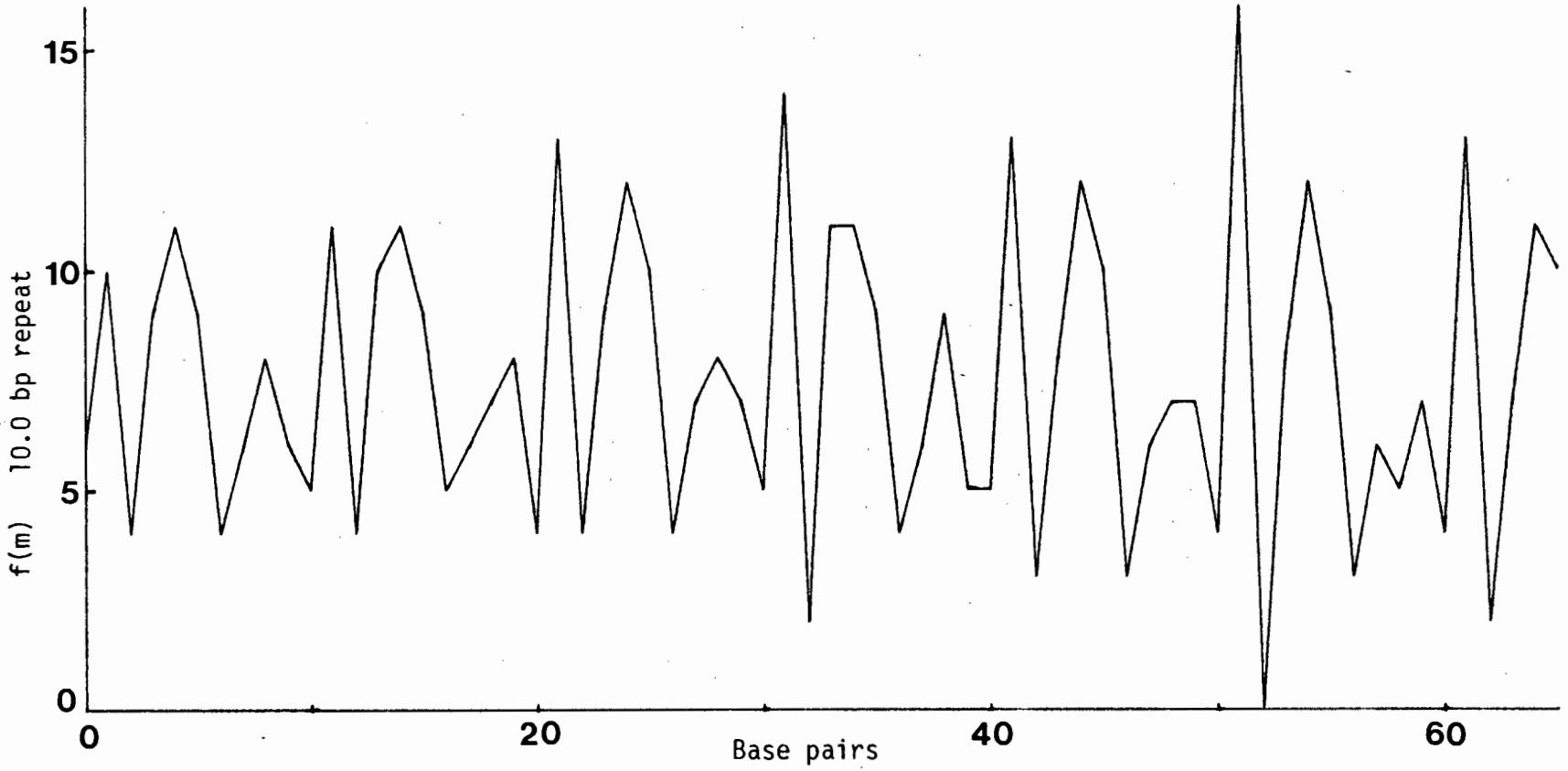


Appendix D Fig. 1

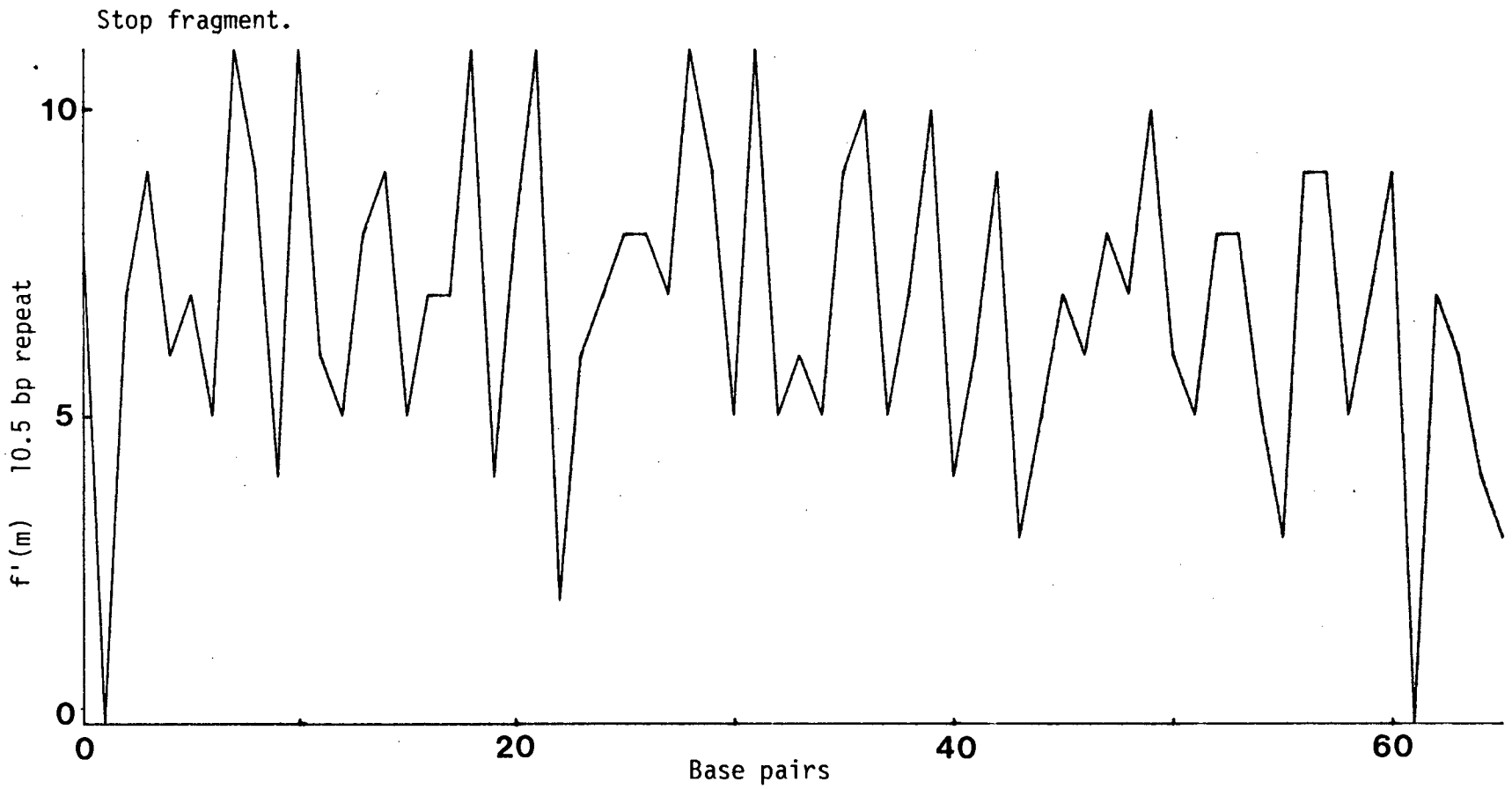


Appendix D Fig. 2

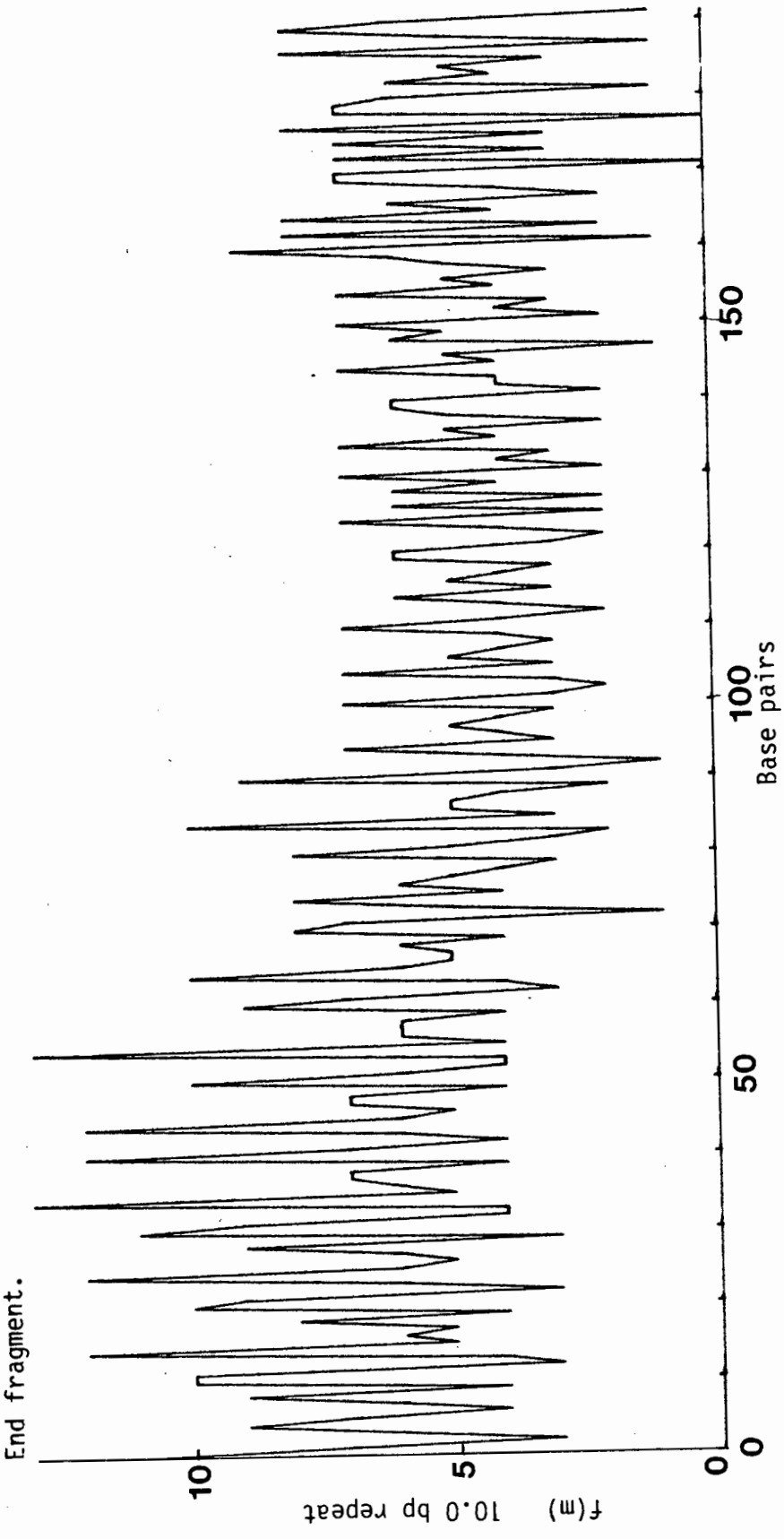
Stop fragment.



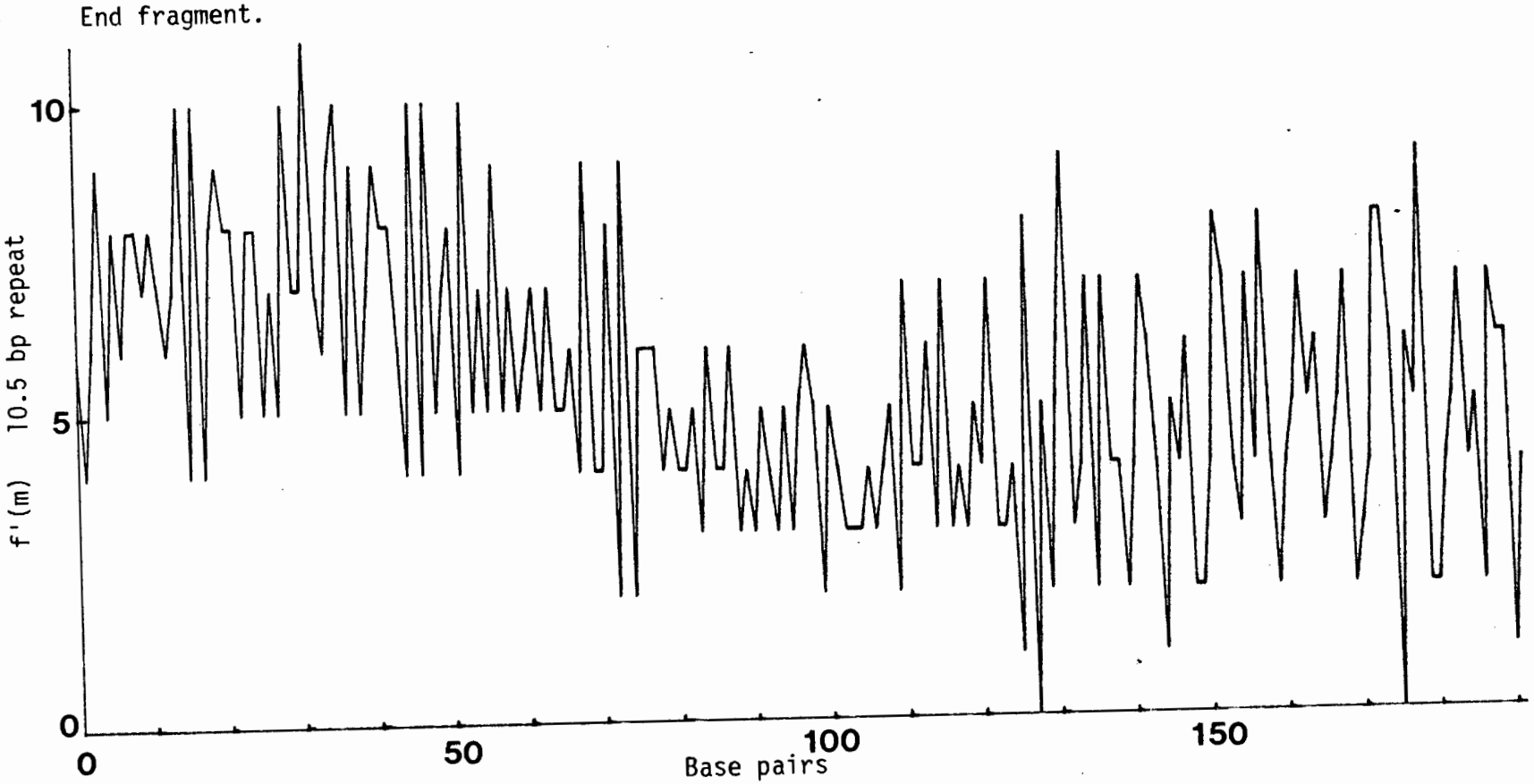
Appendix D. Fig. 3



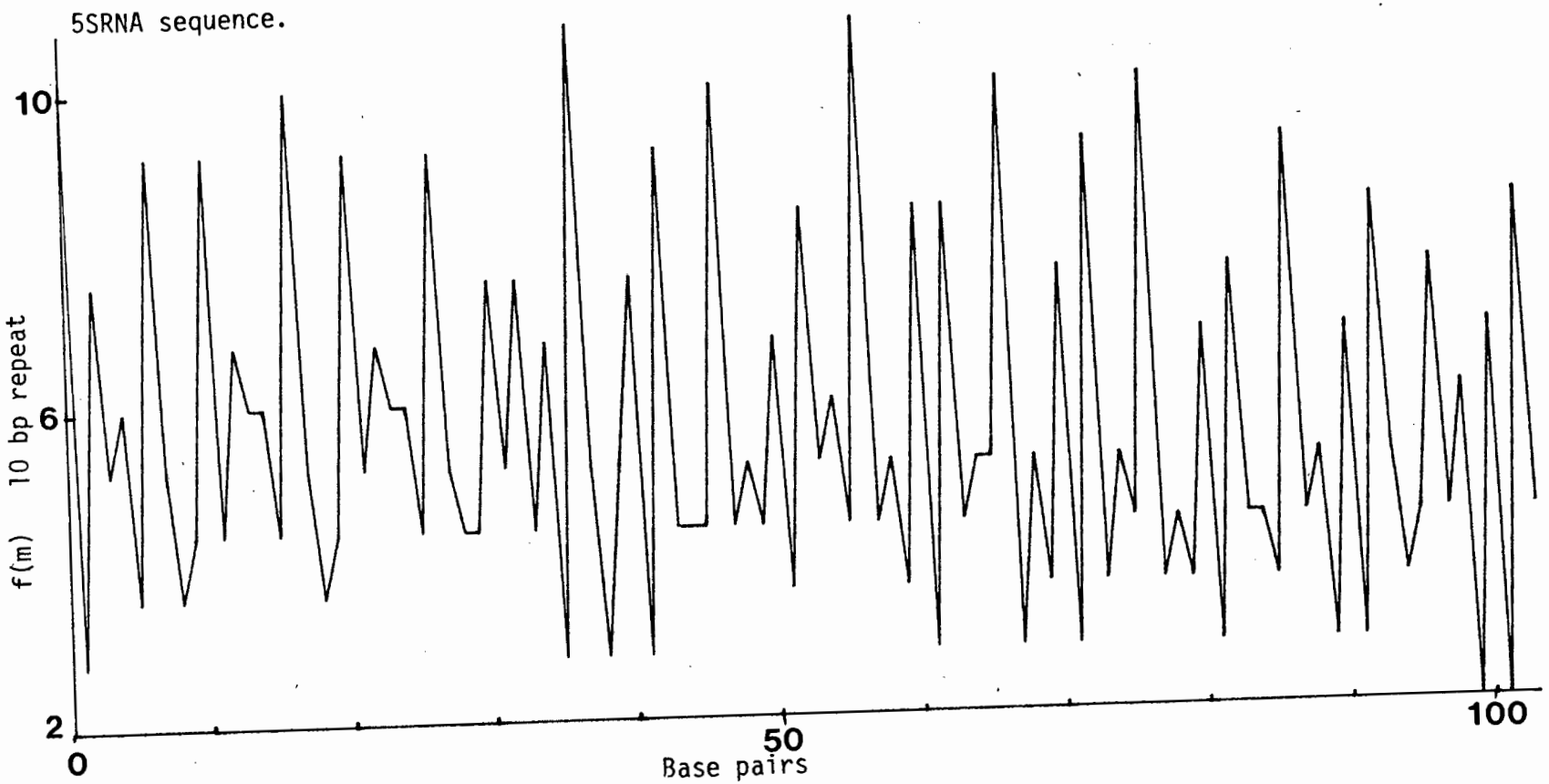
Appendix D Fig. 4



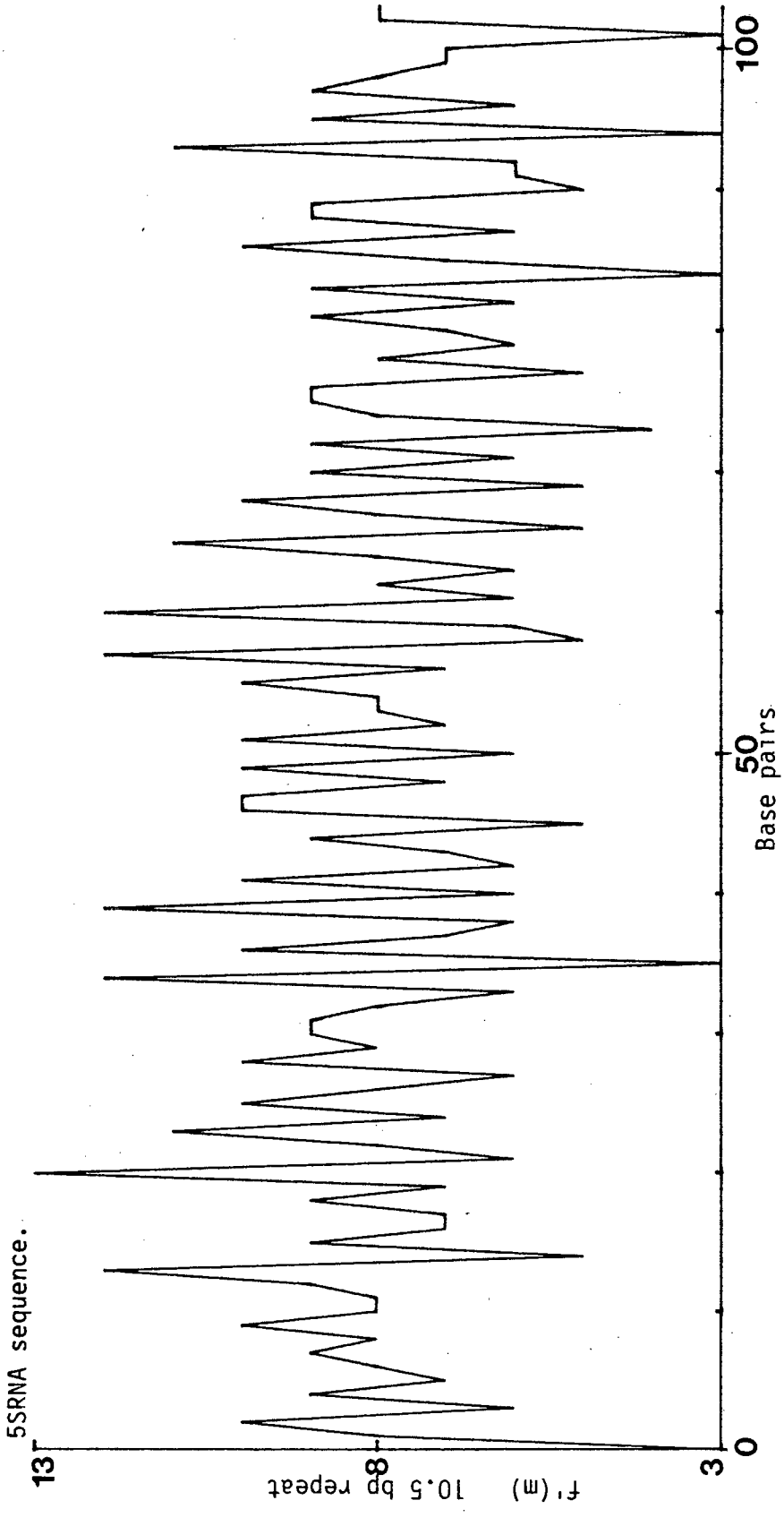
Appendix D Fig. 5



Appendix D Fig. 6



Appendix D Fig. 7



Appendix D Fig. 8