THE DISTRIBUTION OF DNA SEQUENCES WITHIN INTERPHASE CHROMATIN

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The work contained in this thesis is my own original work and has not been submitted for a degree at any other university.

SUMMARY

Investigation into the distribution of DNA squences within interphase chromatin using various nucleases yielded the following results:

By digestion of isolated chromatin with a nuclease specific for single-stranded DNA, the N.crassa endonuclease, it has been shown that a significant and reproducible amount (1±0.5%) of the DNA in interphase chromatin is present in the unpaired form.

These single-stranded regions are not enriched for the rare base 5-methylcytosine to any significant extent, if at all.

When nuclei prepared from mammalian cells are treated with micrococcal nuclease their DNA is cleaved into regularly sized fragments (Noll, 1974a). These fragments are approximate multiples of a monomer which is 180±20 n.p. in length. If most of the DNA is digested to monomer fragments, then about 20% of the total nuclear DNA is rendered acid-soluble. This 20% acid-soluble nucleotide material is not enriched for 5-methylcytosine. In fact if anything the opposite appears to be true , namely that the DNA within the subunits seems to contain a slightly higher proportion of this base.

Most eukaryotes have a repeating nucleohistone substructure. Those organisms tested were human, mouse, kangaroo rat, marsupial mouse, Drosophila, Xenopus, sea urchin and barley. Ambiguities exist with yeast and Tetrahymena.

From the amount of nuclear DNA rendered acid-soluble by micrococcal nuclease it was deduced that the satellite DNA sequences of the kangaroo rat must be packed into chromatin subunits. By nucleic acid hybridisation it was shown that both satellite DNA sequences and ribosomal DNA sequences are present

in the subunits of mouse L cells.

It is suggested, but not unequivocally proven, that the histone genes of the sea urchin are packed into chromatin subunits in sperm; during early embryogenesis these same sequences become more accessible to nuclease digestion indicating that genes undergoing rapid rates of transcription are not present in the same type of nucleohistone structure as transcriptionally inactive DNA sequences.

During the course of this work a simple, rapid and accurate method for the detection of mycoplasma contamination was devised.

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ABBREVIATIONS

Throughout this thesis an attempt has been made to use only recognised abbreviations. Exceptions are:

5-MeCyt	5-methylcytosine
6-MAP	6-methylaminopurine
n.p.	nucleotide pairs
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
DNP	deoxyribonucleoprotein
vol	volumes
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
DMSO	dimethylsulphoxide
PPO	2,5-diphenyloxazole
POPOP	p-Bis(2-(5-phenyloxazolyl))-benzene
FBS	foetal bovine serum
FLA	foetal amnion cell line

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

The major part of this Introduction will try to summarise the extent of our present knowledge on the structure of eukaryotic chromatin by discussing some of the major advances made in this field using a variety of physical and biochemical techniques. It will not be possible, however, to cover this vast and expanding field exhaustively. This will be followed by a brief and selective summary of the work done in attempts to analyse possible control regions within chromatin.

Section 1. Definition of Terms.

'Chromatin' was first applied by Fleming in 1879 to the deeply staining material of nuclei. Chromatin can now be defined as the extended form of chromosomes found in the nucleus between mitotic cell divisions (Bonner et al.,1968a; Mirsky,1971). Purified interphase nuclei can be lysed and their contents collected by centrifugation. The most rapidly sedimenting component has also been termed chromatin, although nucleohistone or deoxyribonucleoprotein (DNP) complex may be more precise terms. In this work I shall refer to the material prepared from lysed nuclei as 'isolated chromatin,' nucleohistone, or DNP complex, reserving the term chromatin to refer only to the material in the interphase nucleus.

Section 2. Constituents of Isolated Chromatin.

There are numerous published methods for the isolation of DNP complexes, some from whole tissues, others from

purified nuclei (see Bonner et al.,1968b). Those methods that use nuclei as the starting material eliminate to a large extent the non-specific adsorption of cytoplasmic constituents onto the nuclear material during isolation (Johns and Forrester,1969; Hill et al.,1971), and presumably also reduce the risk of lysosomal enzymatic attack on the DNA and chromosomal proteins. Isolated calf thymus chromatin has the following overall composition relative to DNA content:

DNA	100
Histone	114
Non-histone proteins	33
RNA	7

Bonner et al.(1968a). The histone nomenclature used throughout this thesis will be the one agreed upon at the 1974 CIBA Symposium on Chromatin Structure, and which is shown in Table 1 together with the older nomenclatures and some properties of histones. In addition to these major components some minor components such as poly(ADP)ribose (Chambon et al.,1966; Reeder et al.,1967; Hasegawa et al., 1967; Nishizuka et al.,1969; Shall,1972; Sugimura,1973) have been found. Several excellent reviews summarise the constituents of isolated chromatin and their roles in the structure and function of chromatin (Stellwagen and Cole, 1969; Hearst and Botchan,1970; Dupraw,1970; Elgin et al., 1971; DeLange and Smith,1971; Huberman,1973; Lewin,1974; Elgin and Weintraub,1975).

Table 1

Nomenclature of Histones

Descriptive	Rasmussen et al.(1962)	Jones and Butler(1962)	Proposed 1972(a)	Proposed 1974(b)	Molecular Weights	Total Residues
lys-rich	I	f1	KAP	H1	21,500	215
	IIb2	f2b	KAS	H2B	13,774	125
slightly lys-rich	IIb1	f2a2	LAK	H2A	14,004	129
	III	f3	ARE	H3	15,324	135
arg-rich	IV	f2a1	GRK	H4	11,282	102
erythrocyte specific	V	f2c		H5	18,400	150

Section 3. X-ray Diffraction Studies.

Over the past 16 years or so X-ray diffraction has been applied to the study of the structure of isolated chromatin. Employing a variety of experimental conditions basically similar results have been obtained from several independent groups although their interpretations of these results have differed. The methods used in X-ray diffraction can be broadly divided into the study of unoriented nucleohistone solution and gel specimens (Luzzati and Nicolaieff,1959,1963; Bram and Ris,1971) and the study of oriented nucleohistone fibres (Wilkins et al.,1959; Pardon et al.,1967; Richards and Pardon,1970). The former groups also considered both the presence of a continuous scatter profile and also the series of diffraction maxima that are superimposed upon this profile. The latter groups largely ignored the scatter profiles, putting emphasis on the diffraction maxima despite their comparatively low intensity.

Wilkins and his co-workers found that nucleohistone fibres, which had been oriented by slight stretching, showed reflections at 105, 55, 35, 27, and 22 A. These same reflections were also observed when 'chromatin' was reconstituted from DNA and four of the five histones (2A,2B,3 and 4); H1 was dispensible. The same reflections have also been observed in whole nuclei in a variety of buffer conditions either fixed in formaldehyde or unfixed, but only when the pH was between 3.9 and 10.9 (Olins and Olins,1972). These latter workers took the presence of the low angle X-ray reflections at 110, 55, 37, 27, and 22 A as an indication that the native structure of chromatin had been maintained. Wilkins interpreted the X-ray diffraction data in terms of

a supercoil of the DNA duplex with a pitch of 120 A and a diameter of 100 A (Pardon et al.,1967; Richards and Pardon, 1970; Pardon and Wilkins,1972). This value for fibre width is in agreement with the electron microscopy results of thin sections and of the later surface spreading method (see Section 4).

If the fibres were stretched further, those reflections attributed to the supercoils (110,35,22 A) were found to diminish in intensity whereas those reflections known to be from the DNA duplex (55,27 A) became more prominent. On relaxation, the fibres again showed all the reflections. It is not clear how this result is consistent with a supercoil-type model; it is, however, consistent with the subunit model of chromatin structure (see Section 7). That the reflections always occurred at the same radius, although intensities varied, suggests that the nucleohistone fibre possesses some discrete configuration. There seems to be no gradual transition from one ordered state to another, but rather an abrupt loss of the configuration that gives rise to the 110 A reflections - an all or none situation. This loss of configuration, however, is reversible.

There are some discrepancies between the results of the Wilkins' group and those of others (Luzzati and Nicolaieff,1959,1963; Bram and Ris,1971) (for discussion see Huberman,1973, and Pardon et al.,1973). Recently, however, Pardon, Richards and Cotter (1973) have extended their earlier studies of X-ray measurements on oriented nucleohistone gels to a much wider concentration range (2% to 100%) and to a consideration of both diffraction and scattering. They claim that the best model to explain

their results remains the supercoil model.

In conclusion then, the X-ray diffraction measurements on both oriented fibres of nucleohistone gels and whole nuclei, unfixed or fixed in formaldehyde, produce reflections at approximately 110, 55, 35, 27, and 22 A, which can be interpreted as indicating some kind of regular repeat in chromatin occurring approximately every 100 A.

Section 4. Studies Using Electron Microscopy.

(a) Early observations.

Ris (1956) used the electron microscope to visualize thin sections of amphibian lampbrush chromosomes and the pachytene chromosomes of some insects and plants. From these observations and later ones, Ris and Chandler (1963) suggested that the principal constituent of the chromosome was a fibre approximately 250 A in diameter. Ris (1969) and Ris and Kubai (1970) concluded that this 250 A fibre was the result of two 100 A fibres lying side by side, a conclusion supported by the finding that surface spreading (first applied to chromosomal material by Gall,1963) carried out in the presence of chelating agents resulted in only 100 A fibres; fibres fixed 10-20 sec after exposure to chelating agents appreared to be pairs of 100 A fibres coiled round each other. Griffith and Bonner (quoted in Huberman,1973) using a modified whole-mount technique where specimens were absorbed to the grid without surface spreading, also concluded that the 250 A fibres were double.

Using the thin-sectioning technique, Davies (1968), Davies and Small (1968), and Everid et al. (1970) demonstrated that some order in the packing of chromosome fibres

exists within the nucleus in the heterochromatic regions. This order was inferred from the presence of a series of light and dark areas adjacent to the nuclear membrane seen in thin sections through nuclei of certain cell types. From examinations of the sections at various degrees of tilt in the electron microscope, it was concluded that the bands near the membrane were the result of several relatively well ordered layers of fibres 130 A thick, each fibre being composed of a coil of thinner material 20-40 A thick. These results are interesting since they can be interpreted as supporting both the 'supercoil' model and the'beads on a string' model (see Section 7).

Thus the two methods - thin sectioning and surface spreading - yielded different values for the fibre diameters. Thin sectioning usually showed fibres 80-180 A in diameter (Davies,1968; Davies and Small,1968; Wolfe,1969), while surface spreading predominantly gave fibres 200-300 A in diameter. Some of this discrepancy may have veen due to variations in the spreading method, such as the presence of divalent cations, the use of alcohol for drying and, in chicken erythrocyte nuclei, the presence of haemoglobin (which binds to DNA); all of which would tend to increase the fibre diameter (Solari, 1971).

Some attempt to resolve this disagreement was made by Wolfe (Wolfe and Grim,1967; Wolfe,1968) who found that the thickening of the 110 A fibre to the 250 A fibre occurred at the time of cell lysis. After lysis both surface spreading and thin sectioning showed the thick fibre only. If, however, the cells were fixed with phosphate-buffered formaldehyde before surface spreading, then the final fibre diameter

was 120-140 A. Wolfe and Grim (1968) and Solari (1971) interpreted these results as evidence for acquisition of a thicker protein coat by the fibres as their ionic environment changed at the time of cell lysis. Ris, on the other hand, believed the 100 A fibre obtained from the thin section technique was an artifact of chelation by buffer ions. According to Ris the 250 A fibre is the native fibre being composed of two 100 A fibres lying parallel. Alternatively, the thicker fibre could be generated through irregular coiling or folding of the thinner fibre (Lampert,1971; Woodcock,1973). More recently, though, Bram and Ris (1971) prepared chromatin for electron microscopy by more gentle means maintaining physiological salt concentrations. They observed networks of fibres of between 80 and 120 A in diameter which they termed unit fibres.

Both interphase and metaphase fibres have been seen to have a bumpy appearance when visualised in the electron microscope. Dupraw (1965) observed that the interphase fibres of isolated chromatin from the honeybee were about 230 A in diameter and bumpy in appearance. Treatment of these fibres with trypsin reduced their diameter to as low as 25 A (the approximate diameter of a DNA duplex) thus supporting the conclusion that each fibre consisted of a single coiled duplex of DNA.

Similar results were obtained with human leucocyte metaphase chromosomes by Abuelo and Moore (1969). These workers observed bumpy fibres of mean diameter 240 $\pm$ 50 A. Trypsin or pronase loosened the packing giving rise to some fibres of approximately 25 A diameter, while DNase broke the fibres into short fragments.

(b) More recent observations.

A major advance in the study of biological material in the electron microscope was made by Miller and Beatty (1969) who visualised the transcription of the ribosomal genes in the extrachromosomal nucleoli of amphibian oocytes. Their method essentially involved the swelling of the oocyte nucleus in deionised water followed by centrifugation of the dispersed nuclear contents through a neutral solution of 0.1 M sucrose containing 10% formaldehyde onto a carbon-coated grid; the grid was rinsed in 0.4% Kodak Photo-Flo before drying, stained positively for 1 min with 1% phosophotungstic acid in 50% ethanol at pH 2.5, ethanol rinsed and finally dried with isopentane. Applying this method, with slight variations, to the analysis of interphase chromatin, Woodcock (1973) and Olins and Olins (1974) produced electron micrographs of chromatin with quite a different structure to that hitherto seen. Discrete spherical particles connected by thin fibres were found in linear arrays emerging from lysed nuclei giving the appearance of 'particles on a string'. The spherical particle, labelled ν body by Olins and Olins, was found to be approximately 100 A in diameter by Woodcock, but 60-80 A in diameter by Olins and Olins, while the thin connecting fibre was between 15 and 30 A wide (depending on the staining method), the dimension of a DNA duplex. Woodcock has since revised his estimate of particle diameter to 70 A (Woodcock et al.,1974).

Oudet et al. (1975) have observed spherical particles of 125A(termed nucleosomes), tightly clustered, by lysing nuclei directly onto the electron microscope grid. Fixation of chromatin in 1% formaldehyde prior to centrifugation onto

the grid probably accounts for the smaller diameter of the ν bodies compared to the nucleosomes. Oudet et al. point out that it is possible that the diameter of the nucleosome would be lower than they measured if the binding onto the grids is accompanied by a spreading of the structure. A spreading phenomenon probably accounts for the larger diameter (150 A) of the 'nodular elements' observed by Slayter et al. (1972) of unfixed chromatin preparations sprayed onto microscope grids through a high pressure spray gun. These authors, however, found considerably less spherical particles distributed along thin strands 20-30 A thick than the previous groups. They also observed aggregations and branched elements suggesting that the nucleohistone structure had been disrupted during spraying.

Further evidence for a particulate structure of chromatin has come from a study of the structure of SV40 as it exists in the host cell. Shortly after infection of a permissive cell with SV40 (or polyoma), a pool of supercoiled, closed circular viral DNA molecules accumulates in the nucleus of the infected cell. This viral DNA exists not as naked DNA but as a nucleohistone complex which contains H2A, H2B, H3, and H4 but not H1 (Huang et al.,1972; Frearson and Crawford,1972; Roblin et al.,1971; Lake et al.,1973; Hall et al.,1973; Goldstein et al.,1973; Seebeck and Weil,1974; Fey and Hirt,1974) and which can be isolated intact (White and Eason,1971; Hall et al.,1973). When this complex was visualised in the electron microscope, Griffith (1975) observed a compact structure which, by a ten-fold lowering of the ionic strength, could be relaxed into a 'particles on a string' structure similar to that seen by Woodcock and Olins

and Olins for the chromatin of lysed nuclei. In this case beads were 100-110 A in diameter.

Using an entirely different method of preparing chromatin for electron microscopy, the freeze etching method, Bram (1975a,b) has also observed a closely packed particulate structure for chromatin. He claims that the freeze etching technique, unlike many other methods, does not distort the dimensions of the chromatin beads which are not spherical but oblate spheroids 90 A long by 70 A wide, around which the DNA is wound giving an overall diameter of about 130 A.

Since many laboratories have now reported that chromatin has a particulate structure, it is important to ask why the numerous earlier electron microscope studies failed to show such a structure for either chromatin or chromosomes. Some possible reasons are now discussed.

(c) Possible reasons why other methods of electron microscopy have failed to reveal a particulate structure.

A commonly used technique, the surface spreading method, involves spreading of nucleohistone fibres upon an aqueous hypophase, picking them up on a grid, fixation with formaldehyde, dehydration through a graded series of ethanol concentrations, transfer to amyl acetate, and drying by the critical point method of Anderson (1951). Employing this method, chromatin generally appears as an irregularly folded thread with thickness varying between 100 and 250 A depending on the presence of divalent cations during the spreading procedure. Woodcock (1973) has pointed out that spherical particles were not seen from osmotically

lysed amphibian or avian erythrocyte nuclei if the chromatin was dried form ethanol or amyl acetate. Furthermore, Pooley et al. (1974) have examined the influence of drying nucleohistone fibres from ethanol or amyl acetate on the low angle X-ray reflections. They found that preparations dried from either ethanol or amyl acetate did not show the characteristic dry pattern (ie. 76 and 38 A reflections) and did not readily exhibit the low angle reflections of native chromatin when rewetted. Pooley et al. concluded that while drying from water retains some structure, drying from organic solvents, either by vacuum evaporation or the critical point method, retains much less. Olins et al. (1975) concluded that drying chromatin from organic solvents produces drastic structural perturbations.

Another reason why the particulate structure of nucleohistone fibres was not observed before has been suggested by Oudet et al. (1975). Isolated chromatin preparations containing H1 are difficult to disperse. Previous investigators never removed H1 from their isolated chromatin preparations and hence the possibility of aggregative artifacts was very real. Other reasons have been discussed by Wolfe and Grim (1967), Wolfe (1968) and Solari (1971), and are mentioned above.

Section 5. Properties of Histones.

(a) Conservation of sequence.

DeLange et al. (1969a,b) first discovered the striking conservation of amino acid sequences for some of the histones. This conservation is greatest with H4 and least with H1. By comparing the amino acid sequences of H4

from pea and calf, DeLange and Smith (1971) have calculated that its rate of evolution must be about 0.06 per 100 residues per 10^8 years, thus making H4 by far the most stable protein known at present. That histones 2A, 2B, 3 and 4 all have quite conserved amino acid sequences (for a review see Lewin,1974) suggests that their function(s) is(are) quite similar in plants, fish and mammals. It also argues against histones, with the possible exception of H1, having a specific role in control of gene expression. Of course, it is possible that post-synthetic modification of histones (phosphorylation,acetylation,methylation,ADP ribosylation) plus microheterogeneity of sequence as found by Zweidler (unpublished), may play a role in tissue specificity.

One obvious function for histones is to pack the large amounts of DNA present in the eukaryotic nucleus into a more manageable form. Estimates of DNA concentration within the nucleus range from 200-400 mg/ml (Olins and Olins, 1972).

(b) Asymmetry of sequence.

Consideration of the amino acid sequence of the histones shows that there is an asymmetry in the distribution of polar and apolar residues. For example, the N-terminal regions of both H3 and H4 contain a high proportion of basic amino acids while being almost completely deficient in apolar residues. From the ~~neutron~~ nuclear magnetic resonance studies of Bradbury and co-workers (Bradbury and Rattle, 1972; Bradbury et al.,1973) it is known that the basic regions of histones are involved in histone-DNA interactions, while the hydrophobic regions are involved in histone-histone interactions. This has been confirmed by

trypsin digestion of isolated chromatin (Simpson,1972; Weintraub and VanLente,1974).

(c) Histone-histone interactions.

The propensity for histones to aggregate with themselves has long been recognised, usually as an irritating sideline to their investigation. However, in recent years evidence has accumulated favouring the notion that specific interaction between certain of the histone species does occur and these may have relevance to the *in vivo* state. Histones H3 and H4 were found to form a strong complex with one another in a 1:1 ratio (D'Anna and Isenberg, 1973,1974a,b; Kornberg and Thomas,1974; Geoghegan *et al.*, Roark *et al.*,1974; Hyde and Walker,1975). In addition, various oligomers of H2A and H2B have been found also in a 1:1 ratio (Skandrani *et al.*,1972; Kelly,1973; Kornberg and Thomas,1974; Hyde and Walker,1975). These histone-histone interactions do not seem to be a consequence of the denaturing solvents used for their extraction, as similar oligomers of histones have been found using both the mild extraction procedures of van der Westhuyzen and von Holt (1971) where the histones are removed by raising the ionic strength and displacement with protamine (Kornberg and Thomas,1974; Geoghegan *et al.*,1974; Roark *et al.*,1974), or by the more conventional acid extraction procedures (D'Anna and Isenberg,1974b). Thus it seems more likely that the tendency to form specific histone oligomers is an inherent property of the histone molecule. This is supported by the finding of Bartley and Chalkley (1972) that histones H2A and H2B, and histones H3 and H4 were eluted coincidently from isolated chromatin by raising the salt concentration.

The simplest explanation to account for both these results is that (H2A and H2B) and (H3 and H4) exist as histone oligomers in chromatin.

Section 6. Studies of Deoxyribonucleases on Isolated Chromatin.

Treatment of isolated chromatin with a variety of deoxyribonucleases (DNases) has been described by several laboratories in attempts to elucidate some interactions between DNA and both histones and non-histone chromosomal proteins (Mirsky,1971; Clark and Felsenfeld,1971,1974; Pederson,1972;Li et al.,1972; Schmidt et al.,1972; Billings and Bonner,1972; Mirsky et al.,1972; Rill and van Holde,1973; Itzhaki,1974). Compared to free DNA, the rate of digestion of the isolated chromatin is, expectedly, slower and may not proceed to completion. Estimates of the amount of 'free' DNA (or DNA accessible to nuclease action) in chromatin vary according to the type of nuclease used, the way in which the chromatin is prepared and the conditions under which it is digested. Clark and Felsenfeld (1971,1974) using both DNase I and staphylococcal nuclease (now usually called micrococcal nuclease) found that a limit digest was achieved when about 50% of the DNA was released from sheared chromatin as acid-soluble material. This work has been criticised on several grounds by Mirsky (1971), Li et al.(1972) and Pederson (1972), where the amount of DNA accessible to digestion with nucleases was found to vary from 20% to 100% depending on the conditions employed.

An alternative to using an exogenous DNase was provided by the studies of Hewish and Burgoyne (1973a) who described

a Ca^{++}-Mg^{++} dependent endonuclease present in isolated nuclei from rat and mouse livers. If nuclei were incubated in the appropriate buffer at 37 ° C, the DNA extracted and analysed on a 2.5% polyacrylamide gels, a series of bands was obtained (Hewish and Burgoyne,1973b). When eluted from the gel and re-electrophoresed, these bands maintained their original mobilities showing that the different bands were discrete species and not loose concatenates. Furthermore, these bands were all multiples of the smallest band, the monomer, which had a molecular weight of 120,000-150,000 daltons, or approximately 200 nucleotide pairs in length (Burgoyne et al.,1974). When this Ca^{++}-Mg^{++} dependent endonuclease was isolated, incubated with DNA and the products analysed on a polyacrylamide gel as before, only a smear was obtained similar to that obtained with DNase I (Hewish and Burgoyne,1973b). DNase I incubated with isolated nuclei also only produced a smear. From these results Hewish and Burgoyne concluded that the discreet bands arose through restricted endonucleolytic action on the nuclear DNA caused by a regular distribution of chromosomal proteins on the DNA. This indicated that 'chromatin has some simple, basic repeating substructure with a repetitive spacing of sites that are potentially accessible to the Ca^{++}-Mg^{++} endonuclease; However, some of the sites are sterically blocked by superstructure proteins. Thus the Ca^{++}-Mg^{++} endonuclease would produce fragments which were all equal to, or greater than, the basic substructure repeat distance' (approximately 200 nucleotide pairs),' and the larger ones would all be multiples of the smaller ones.'

More recently Noll (1974a) has shown that the Ca^{++} -

Mg^{++} endonuclease can be replaced by micrococcal nuclease, a DNase II-like endonuclease with an amino acid composition not unlike histones (Taniuchi et al.,1967a,b). A similar series of DNA fragments was obtained where the monomer was 205 ± 15 np as sized against ϕX174 restriction fragments. Since then other workers have repeated these experiments with micrococcal nuclease on isolated chromatin and nuclei, and also found that DNase II can be used (Rill and van Holde, 1973; Honda et al.,1974; Oudet et al.,1975; Oosterhof et al., 1975; Corden et al.,1975; and results presented herein).

Section 7. The Subunit Structure of Chromatin.

(a) Summary of the evidence that lead to the hypothesis.

A subunit model for chromatin structure has been predicted based on the experimental results discussed above. In summary:

1. X-ray diffraction studies on both oriented nucleohistone fibres and whole nuclei revealed reflections at 110, 55, 37, 27, 22, and 18 A, which suggest that there is some repeating unit every 110 A. The same reflections are found with or without H1.
2. Electron microscopy of fixed chromatin spilling from lysed nuclei has shown parallel arrays of spherical particles (ν bodies, beads, nucleosomes) of diameter 70-130 A depending on the method of preparation for examination, and which are usually connected by thin filaments 15-30 A wide.
3. Histones dissociated from isolated chromatin, usually by non-denaturing methods, tend to form self-aggregates, the most common being dimers (H3 + H4),

the tetramers $(H3 + H4)_2$, and the dimers (H2A + H2B).

4. Nuclease digestion of nuclei, either using an endogenous nuclease present in rat and mouse liver nuclei, or exogenous micrococcal nuclease, or DNase II, has produced a series of DNA fragments all multiples of around 200 np in length.

From these results and the results of crosslinking of histones both in solution and within isolated chromatin (to be discussed below) Kornberg (1974) put forward a model for the organisation of histones and DNA, the essential features of which are:

1. Chromatin is comprised of repeating units connected by the DNA thus forming a 'flexibly jointed chain'.

2. Each unit consists of two each of histones H2A, H2B, H3 and H4 and approximately 200 np of DNA.

3. Stoichiometrically there seems to be one molecule of H1 per subunit but this histone does not seem to have an indispensible role in the structure of the subunit (see below).

Olins and Olins (1974) had earlier suggested that a similar stoichiometry of histones and DNA may exist for their ν bodies (i.e. 2 each of the histones plus DNA) except that the presence of H1 in this structure was considered.

(b) Support for a repeating chromatin subunit model.

Apart from the evidence used to formulate such a model and which has already been discussed, there are several other lines of evidence that give support to the globular model for chromatin structure and these will now be considered.

(i) Neutron diffraction studies on isolated chromatin.

Small angle neutron scatter techniques have been recently applied to the study of chromatin structure by Baldwin et al.(1975). Neutron scatter has the advantage over the X-ray scatter in that, owing to the large difference between the average neutron atomic scattering factors per atom for H_2O (-0.06×10^{-12}cm) and D_2O ($+0.63 \times 10^{-12}$cm), the scattering from all biological materials can be contrast-matched. By plotting the mean neutron atomic scattering amplitude against percentage D_2O in the D_2O + H_2O mixture, it was computed that the neutron scatter from DNA was expected to be contrast-matched at about 60% D_2O while that of the histones at about 37.5% D_2O. Thus by varying the D_2O/H_2O ratio it was possible to selectively investigate which particular chromatin component caused the particular low angle reflections observed. Significantly, at around 30% D_2O the prominent rings present were at 55 and 27 A with a low ring at 37 A and no ring at 110 A. This immediately indicated that the 110 A low angle ring seen by both X-ray and neutron diffraction was not due to the DNA as Wilkins had supposed (Wilkins et al.,1959; Zubay and Wilkins,1962; Pardon et al.,1967; Pardon and Wilkins,1972) but due to the histones. It also eliminated a supercoil type model based on DNA helices. Baldwin et al. concluded that the histones gave rise to the reflections at 110 and 37 A while the DNA caused the reflections at 55 and 27 A. Furthermore, the 110 A reflection most likely represented the inter-particle distance of the proposed chromatin subunits. Although not proven then, Baldwin et

al. suggested that the histones are present as a globular core around which the DNA is wrapped. Again the neutron reflections at 110, 55, 37, and 27 A were obtained with or without H1 as was found with X-ray diffraction. These authors favour an extra-subunit location for H1 based also on the finding of Bram et al. (1974) that removal of H1 from isolated chromatin reduces the radius of gyration of this chromatin.

(ii) The SV40 (polyoma) nucleohistone complex.

As mentioned in Section 4(b) SV40 (and polyoma) exists in the infected permissive cell not as free DNA but associated with the host histone molecules H2A, H2B, H3 and H4 as a nucleohistone complex which, when visualised in the electron microscope, appears as a compact loop 110 A wide with contour length 2100 A (Griffith,1975). By a ten-fold lowering of the ionic strength, Griffith found that this compact structure could be relaxed into a 'flexible string of 21 beads each 110 A in diameter ... joined by bridges roughly 20 A in diameter and 130 A long'. This beaded structure is very similar to that seen for chromatin fibres (see Section 4(b)). (There is some disagreement a-bout exactly how many beads can be seen on the SV40 DNA. Germond et al.(1975) claim they find up to 24 beads.)

Implicit in the Kornberg model is a well-defined packing ratio per subunit. If 200 np are to be packed into a structure approximately 100 A long, then this must mean that the packing ratio is 6.8:1 (200 np is about 680 A assuming the B configuration for DNA). The SV40 nucleo-histone complex has a contour length of 2070 $\pm$ 125 A, while SV40 DNA (relaxed) is 14,800 $\pm$ 370 A long. Thus

the compression is 7 ± 0.5 : 1, in excellent agreement with that predicted from Kornberg's model.

Furthermore, if the polyoma nucleohistone complex is treated with nucleases, the DNA is digested to fragments approximately 200 np in length (Louie,1974), again supporting the notion that the globular structures present in the viral nucleohistone complex are comparable to the chromatin subunits (ν bodies,beads, nucleosomes).

(iii) Reconstitution of nucleohistone fibres.

By various techniques it has been possible to reconstitute deoxyribonucleoprotein complexes from DNA and the five histones into structures that have properties indistinguishable from the 'native' state. Such criteria as circular dichroism, X-ray diffraction, infrared spectroscopy, electron microscopy, thermal denaturation and nuclease digestion have shown that prokaryotic, phage and viral as well as eukaryotic DNAs can be associated with histones into identical structures (Pardon and Richards, 1970; Baldwin et al.,1973; Pardon et al.,1973; Kornberg and Thomas,1974; Axel et al.,1974; Olins et al.,1975; Oudet et al.,1975); these studies again show that H1 is dispensible. That prokaryotic and phage DNAs can substitute for eukaryotic DNAs, argues against sequence specificity being involved in the formation of chromatin subunits since these DNAs are not normally complexed with histones.

Chambon and his co-workers (Oudet et al.,1975) have reconstituted nucleohistone from both λ and adenovirus-2 DNAs, as well as eukaryotic DNA, with H2A, H2B, H3 and H4. All form nucleosomes of approximately 127 A in diameter. These workers point out that their results clearly indicate

that nucleosomes are self-assembling particles and the reconstituted particles are identical to the *in vivo* particles in their size, histone content and the amount of DNA (c. 195 np) contained per particle.

(iv) Isolation of subunits.

Several groups have reported the isolation of chromatin fragments. These were obtained either by nuclease digestion methods (Rill and van Holde,1973; Sahasrabuddhe and van Holde,1974; Noll,1974; Oosterhof *et al.*,1975; Oudet *et al.*,1975) or by shearing isolated chromatin (Senior *et al.*,1975).

Van Holde and his coworkers digested isolated sheared chromatin with micrococcal nuclease according to the procedure of Clark and Felsenfeld (1971). By precipitation of the nuclease-resistant fragments in 0.15 M salt, they collected a nucleoprotein fraction - their PS fraction - which was fairly homogeneous with a protein/DNA ratio of 1.85 and a sedimentation coefficient of about 12 S.

Using milder digestion conditions, Noll (1974) separated the reaction products of a micrococcal nuclease digestion of isolated chromatin by sedimentation through an isokinetic sucrose gradient. A series of seven peaks were resolved, the slowest sedimenting one containing a DNA fragment equivalent to the monomer fragment (i.e. approximately 200 np; see Section 6); the rest contained DNA fragments in multiples of this value, namely peak 2 contained the dimer fragment of approximately 400 np, peak 3 the trimer fragment of 600 np and so on. Noll showed that the monomer subunit particle had a sedimentation coefficient of 11 S. In a comparable study, Oudet *et al.*(1975) obtained a value

between 10 and 12 S for their isolated chromatin subunit monomers. Oosterhof et al.(1975) used DNase II to generate chromatin subunits, the monomer of which had a sedimentation coefficient of 11 S.

An alternative method of isolation of chromatin fragments is that described by Senior, Olins and Olins (1975). Formaldehyde-fixed chicken erythrocyte nuclei were sonicated and the resulting fragments separated on a linear 5-30% sucrose gradient. Electron microscopic examination of the slowly sedimenting region of the single broad peak revealed a concentration of monomer chromatin fragments - monomer bodies - which could be further enriched by recycling through another sucrose gradient. Monomer ν bodies had a molecular weight of 141,000 daltons, a protein/DNA ratio of 1.22 and contained a DNA fragment of 210 n.p.

A comparison of the methods and properties of the isolated chromatin fragments is compiled in Table 2. It can be seen that although there is approximate agreement in the sedimentation coefficients, considerable differences exist in the protein/DNA ratios, the size of the particles and the length of the DNA fragment contained within those particles. Attempts to resolve these discrepancies have been made. Noll (1974a) and Noll et al.(1975) point to the effects of shear when preparing isolated chromatin. I shall return to the apparent discrepancy over the size of the DNA fragment within the chromatin subunit in the General Discussion. Nevertheless, the fact that both nuclease and sonication can break chromatin into defined fragments supports the hypothesis of a repeating chromatin substructure. In particular, the 'beads on a string' model might be expect-

Table 2 Comparison of the Properties of Isolated Subunits

Reference	Method Used	State of Chromatin	H1 Present Initially	Protein/ DNA Ratio	Sedimentation Value (S)	DNA Length (n.p.)
a	micrococcal nuclease	sheared	+	1.5	12	110
b	micrococcal nuclease	unsheared	+	1.3	11.2±0.4	205
c	DNase II	sheared	+		11	120
d	micrococcal nuclease	unsheared	-	0.97	10-12	195
e	shear			1.22		210
f	micrococcal nuclease	unsheared	+			140
g	micrococcal nuclease	unsheared	+			185
h	micrococcal nuclease	unsheared (preincubated)	+		11	130
i	micrococcal nuclease or DNase II	unsheared	+		11	190

a Sahasrabuddhe and van Holde (1974)

b Noll (1974)

c Oosterhof, Hozier and Rill (1975)

d Oudet, Gross-Bellard and Chambon (1975)

e Senior, Olins and Olins (1975)

f McGhee and Kimmel (1975)

g Sollner-Webb and Felsenfeld (1975)

h Rill, Oosterhof, Hozier and Nelson (1975)

i Woodcock, Sweetman and Frado (1976b)

ed to contain weak points in the interbead regions and it is these weak regions that are cleaved by nucleases or broken by sonication. In addition, these monomer subunits (PS particles, ν bodies, nucleosomes, beads) have been visualised in the electron microscope (van Holde et al.,1974; Senior et al.,1975; Oudet et al.,1975; Kornberg,1975). These isolated subunits vary in diameter between 70 and 130 A according to the method of preparation for electron microscopy (see Section 4).

(v) DNA replication studies.

Indirect support for the repeating subunit model comes from the work of Kriegstein and Hogness (1974) on DNA replication in Drosophila melanogaster. They found that the discontinuous synthesis of DNA proceeds in approximately 200 nucleotide steps, and also that the rate limiting step in chromosome replication is not the actual polymerisation reaction but a process involving the histones. These results are readily accomodated by the Kornberg model where the DNA contained in each subunit is replicated first, followed by assembly of the subunit with two molecules each of histones H2A, H2B, H3 and H4.

Section 8. The Nature of the Subunit.

(a) Crosslinking of histones in chromatin.

Of all the results on nucleohistone structure this one has recently produced the most controversy.

Formaldehyde has been used to fix both DNP and nuclei for electron microscopical examination (Section 4). Recently Chalkley and Hunter (1975) reported that the two aldehydes, formaldehyde and glutaraldehyde, differ markedly in their

reactions with nucleohistone complexes. Formaldehyde was found to form reversible covalent bonds between histones and DNA (Jackson and Chalkley,1974) whereas glutaraldehyde fixation was essentially irreversible; both aldehydes, though, formed irreversible complexes with free histones. Glutaraldehyde fixation of nucleohistone initially produced polymers of H1 followed by oligomers of the remaining histones; formaldehyde did not produce histone polymers. From their results Chalkley and Hunter came to the following conclusions:

(i) Based on the formaldehyde fixation studies, all five histone fractions possess the capacity to interact with the bases of DNA.

(ii) Based on the glutaraldehyde data, extended overlapping arrays of all histone fractions are present.

(iii) H1 histones are often arranged contiguously with few other histones interspersed.

(iv) H2B is arranged so that it is next to H3, H2A or H4 with roughly equal frequency.

These workers did not find any enrichment for discrete dimets of (H2A + H2B) or tetramers of $(H3 + H4)_2$ amongst their oligomers as suggested by the results of histone-histone aggregation in solution (see Section 5(c)) although they point out that their results do not exclude the presence of such dimers and tetramers.

Chalkley (1975) has now used three different imidoesters as crosslinking reagents, and with one of them, dimethylsuberimidate, has shown that polymers of H1 and oligomers of H2A and H2B can be recovered from isolated

crosslinked chromatin.

Hyde and Walker (1975) studied the crosslinking of histones in nucleohistone complexes with formaldehyde at various ionic strengths. They found that at low ionic strengths (0.07 mM sodium phosphate, pH 6.8) dimers of (H3 + H4) were the major products while at higher ionic strengths (0.7 M to 2 M NaCl) polymers of these histones were found. Hyde and Walker feel that these results do not support a unique role for the $(H3 + H4)_2$ tetramer in chromatin structure.

Van Lente et al. (1975) have crosslinked both isolated avian erythrocyte chromatin and nuclei with formaldehyde and glutaraldehyde, and obtained different results from either Chalkley and Hunter or Hyde and Walker. Their nucleohistone, prepared by the method of Clark and Felsenfeld (1971), was treated with increasing concentrations of neutralised formaldehyde in 0.2 mM EDTA from 0.005% to 5%; the products of fixation were analysed directly by electrophoresis on SDS-polyacrylamide gels. Two major products were found, X-1 and X-2, plus some histone polymers that did not enter the gels. By iodination of the eluted X-1 and X-2 bands followed by tryptic fingerprinting, definitive identification of the histones H2B and H4 in X-1 and H2B and H2A (with trace amounts of H3 and H4) in X-2 was possible. Consideration of molecular weights suggested X-1 and X-2 were dimers of histones. Van Lente et al. claim that X-1 and X-2 dimers never constitute more than 15% of the total histone content. In contrast to Jackson and Chalkley (1974) they did not find that formaldehyde fixation is reversible. Van Lente et al. conclude that the

'low yield of specific resolvable histone cross-linked products prevents any general statements about the universality of histone association in chromatin'. Except that three of the six possible heterotypic interactions can occur in chromatin, namely H2B + H4, H2B + H2A, and H3 + H4. Using a different crosslinking reagent, tetranitromethane, Martinson and McCarthy (1975) found a dimer of H2B + H4, and with a carbodiimide Bonner and Pollard (1975) identified a dimer of H3 + H4.

Thus from these types of investigation, no clear support for the hypothesis that each subunit contains two dimers of (H2A + H2B) and a tetramer of $(H3 + H4)_2$ has emerged. Due to the disparity between groups, however, there is no clear evidence to the contrary.

In possibly the most definitive study of crosslinking of histones in isolated chromatin, Thomas and Kornberg (1975) have provided convincing evidence for the chromatin subunit being an octamer of two each of histones 2A, 2B, 3 and 4 plus 200 n.p. of DNA.

Thomas and Kornberg used the di-imidoester crosslinking reagent dimethylsuberimidate according to the method of Davies and Stark (1970). They first showed that crosslinking histones in the isolated chromatin monomer did not significantly perturb its structure. Their 'native' chromatin fragments (of average DNA size 1600 n.p.) were prepared by the limited micrococcal nuclease digestion method of Noll et al.(1975). If crosslinking of this DNP was done at pH 8 and at an ionic strength of 0.2, and the histones extracted and analysed on SDS-polyacrylamide gels, a regular series of bands

resolvable up to about 13 were found. Knowing that the four histones have different molecular weights, if all possible multimers had occurred, then only a smear would have been found on the gel. Instead the regular pattern observed indicated some repeating unit. In contrast to the continuous series of bands obtained at pH 8, if the DNP was crosslinked at pH 9 then no bands were visible after the 8-mer, until faint bands appeared at the 16-mer and 24-mer positions. Using succinimidyl propionate (Lomant's reagent) as crosslinking reagent at pH 8 gave an identical pattern to that found with dimethylsuberimidate. But at pH 9, Lomant's reagent only produced bands at the 8-mer, 16-mer and 24-mer, with no bands less than the 8-mer. These results suggested that there were strong associations between a set of 8 histones, but weak associations between each octamer set since at pH 9 these inter-octamer interactions were usually broken.

If the dimer region of the histones obtained by cross-linking DNP with dimethylsuberimidate was further analysed by longer polyacrylamide gels, then the following dimers were identified: H3 + H3, H3 + H4, H2A + H2B, H2B + H4 and H2A + H4. It was found that H2A and H2B were cross-linked at the same rate and at a higher rate than H3 and H4.

Possibly only for technical reasons, it was not possible to form an octamer of histones in solution (see below). But by dissociating the octamer of histones from the DNA in an ionic strength of 2 and pH 9 according to Ohlenbusch et al. (1967), Thomas and Kornberg were able to obtain a comparable effect. If DNP was crosslinked with

dimethylsuberimidate under these conditions at a DNP concentration of 2 mg/ml, the histones extracted and analysed on gels as before, only two bands were visible, one corresponding to the mobility of an octamer, the other to H1. If a similar experiment was done except that the DNP was at 0.1 mg/ml, then an additional hexamer band and a dimer band were obtained. When these bands were analysed further it was found that whereas both the octamer and hexamer contained all four histones (H2A, 2B, 3 and 4), the octamer contained them in equal proportions while the hexamer had more H3 and 4 than H2A and H2B; the dimer contained H2A and H2B. It seemed then that at low DNP concentrations the octamer begins to dissociate by losing H2A and H2B. These results strongly support the hypothesis of Kornberg (1974) that each chromatin subunit contains an octamer of two each of histones 2A, 2B, 3 and 4 plus 200 n.p. of DNA.

Recently Weintraub, Palter and van Lente (1975) have reported that in 2 M NaCl they can isolate a tetramer of H2A, H2B, H3 and H4, a so-called heterotypic tetramer since it contains both slightly lys rich and arg rich histones, and which they believe is the basic repeating unit of chromatin. Evidence for this claim comes from the treatment of both isolated chromatin and extracted histones (in 2 M NaCl) with trypsin, and also by iodination with $^{125}I_2$.

Trypsin digests about 30 amino acids from the basic N-terminus of all the histones except H1, leaving defined trypsin-resistant histone peptides of between 70 and 90 residues in length (Weintraub and van Lente,1974); H1 (and H5 where it occurs) is degraded to very small peptides

again indicating a different location for these histones. Indistinguishable patterns were obtained by analysis on SDS-polyacrylamide gels of the trypsin-resistant fragments isolated from DNP or histones extracted by 2 M NaCl, although the rate of digestion of the latter was 20 times faster than of the former. Iodination of both and characterisation of the labeled residues again yielded indistinguishable results.

Furthermore, this heterotypic tetramer complex of H2A, H2B, H3 and H4 could be reassociated from histones extracted from DNP either by acid or by the more gentle dissociation in high ionic strength. It has a molecular weight of about 60,000 daltons and is therefore claimed to be a tetramer and not an octamer. Weintraub et al.(1975) suggest that two of these tetramers become associated with 200 n.p. of DNA in chromatin to form the nucleosome. However, their results do not eliminate the possibility that they are looking at a dissociating octamer.

(b) Location of the DNA within the subunit.

Conclusive evidence that the DNA is on the outside of the chromatin subunit has come from the studies of Bradbury and coworkers (Bradbury,1975). By separately measuring the contribution of the DNA component and the protein component to the radius of gyration of isolated chromatin subunits, they have found a higher value for the DNA than for the protein. This must mean that the DNA is around the outside of the subunit. Similar results were obtained by Pardon et al.(1975).

(c) Internal configuration of the DNA within the subunit.

Precisely how the DNA is wound around the

putative subunit remains an intriguing question. One approach to an elucidation of this problem is that of Noll (1974b). As was described in Section 6, micrococcal nuclease and DNase II are thought to cleave between the chromatin subunits. If DNase I is used, the background is very high although the 200 n.p. repeats are still visible (Noll, 1974a). But if the products of DNase I digestion of isolated chromatin are analysed on the denaturing formamide polyacrylamide gels of Staynov et al.(1972), then a series of bands is again obtained, this time in multiples of 10 nucleotides from 10 to 220 nucleotides in length. As digestion proceeds, the longer fragments are converted to shorter ones, suggesting that the cleavage sites of each DNA strand occur at every 10 nucleotides. The pattern is characteristic and reproducible, with a more intense band at the equivalent of 80 nucleotides and weaker ones at 60, 100, and 130 nucleotides. These results show that within the chromatin subunit itself there exists a much shorter repeat. They also indicate that each subunit must have this repetitive structural element to generate the uniform series of bands; in other words the chromatin subunits must be very similar, if not identical, in structure. In addition, since the DNA is so accessible to nuclease attack, Noll suggests that this is further evidence for the DNA being situated on the outside of the subunit.

These results and consideration of molecular models have led Crick and Klug (1975) to put formward the hypothesis that the DNA is not folded 'smoothly' around the histone core but is kinked at regular intervals. To fold a DNA helix - itself approximately 20 A in diameter - round

a core with a radius of curvature of 30-50 A, would require, so Crick and Klug believe, a considerable amount of energy. In addition, bending a helix destroys its symmetry. One alternative to the smooth folding would be to have fairly long straight regions of DNA duplex interspersed with a kink of 95-100° at regular intervals. The results of Noll (1974b) indicated that these straight duplex regions might be 10 np in length, but more recent results with micrococcal nuclease (Noll and Kornberg, unpublished: quoted by Crick and Klug) have indicated a repeat every 20 np. Thus in each 100 A subunit there would be 10 straight stretches of 20 np kinked through an angle of 95-100.

Germond et al. (1975), studying the structure of the chromatin subunit using the SV40 nucleohistone complex as a model system, have concluded that the introduction of one sununit (nucleosome) to DNA causes an unwinding of the helix by one turn. They suggest that 'their results are better accounted for by a model of nucleosome structure in which the DNA would be folded (kinked) rather than by models in which the DNA would be regularly wound in a superhelical form.'

(d) Location of H1.

As yet no definitive evidence exists on the precise position of H1 in the repeating nucleohistone fibre except that it probably has an extra-subunit location. As has been discussed above, this histone is certainly unnecessary in the reconstitution of the nucleohistone subunits and it can be removed from isolated chromatin without altering such parameters as the nuclease digestion patterns, (although see the General Discussion), X-ray or neutron

diffraction patterns, or the beaded structures as visualised in the electron microscope. Indeed, the only physical effect of removing H1 seems to be to lower the radius of gyration of the DNP (Bram et al.,1974).

Some properties of H1 may be functionally important. For example, H1 has a higher affinity for supercoiled DNA than for relaxed DNA (Vogel and Singer,1975a,b).

Recently an attempt has been made to visualise in the electron microscope the distribution of H1 molecules along the DNA after first removing the other four histones (Hayashi, 1975). The published electron micrographs, however, are not very convincing due to the small size of the H1 molecules relative to the thickened DNA fibre. Probably a more convincing way would be to increase the effective size of the H1 molecules by reacting the H1 - DNA complex with a ferritin labelled antibody raised against H1 (N. Davidson, personal communication). Although H1 exchange has probably not occurred in these experiments, histone migration along the DNA cannot be ruled out.

I shall return briefly to the location of H1 in the General Discussion.

(e) Conclusions.

It can be seen that rapid progress has been made in the study of the actual structure of the chromatin subunit. Obviously what is wanted is an X-ray crystallographic study of monomer subunits, but most attempts so far to isolate crystals have failed. A recent report by Bakayev et al. (1975) claims to have achieved this goal.

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So far in this Introduction I have only discussed the structure of interphase chromatin ignoring the formidable problem of how these interphase fibres condense at mitosis to form the more compact and ostensibly ordered metaphase chromosomes. Although several hypotheses have been put forward to explain such a nucleohistone condensation (for example, phosphorylation of H1 just prior to prophase may be the trigger for the condensation; Bradbury et al.,1974), the process at present is not understood. The nature of the metaphase chromosome has been studied in the electron microscope (for reviews see Dupraw,1970; Ris and Kubai, 1970; Stubblefield,1973; Lewin,1974) and recently by neutron diffraction by Bradbury's group in Portsmouth, but so far no compelling evidence has emerged concerning its higher order structure.

——oOo——

Another omission has been that I have concentrated only on the structural features of chromatin, saying nothing about the control of gene expression and how this might be achieved within a repeating nucleohistone subunit fibre. Also I have intentionally excluded a discussion of the copious literature on attempted separations of chromatin into 'active' and 'inactive' fractions. This will be partly rectified in the General Discussion. Moreover, I have purposely neglected to mention how much of the nuclear DNA is packed into nucleohistone subunits. Some obvious questions are: Assuming that sequences exist in eukaryotic DNA that have similar or equivalent

functions to the operators, promotors etc. of bacteria and phages, then can specific DNA sequences be recognised by a protein molecule (or any other type of compound) if the DNA is present as a nucleohistone subunit fibre? Is all the DNA in the nucleus present as chromatin subunits, even DNA which is being transcribed, or are some classes of DNA packed in another way? Are the histones complexed with the same DNA sequences at every round of replication (in other words are the histones put down in the same register each time), or is the assembly of the subunit a 'random' process? Are there any unpaired regions of DNA in chromatin? (Such single-stranded regions could conceivably be used in recognition by proteins.) Is there any preferential localisation of methylated nucleosides in chromatin?

This thesis will attempt to answer some of these points, but first I should like to briefly summarise the evidence for the presence of single-stranded regions in chromatin, and finally to say something about DNA methylation.

Section 9. Single-Stranded DNA Regions in Chromatin.

There has been much discussion on this topic since Gierer (1966) published his hypothesis that DNA might form clover-leaf structures which might be important in recombination, protein-DNA recognition etc., and Crick (1971) published his hypothesis that some control regions may in fact be unpaired structures.

The experiments designed to probe for single-stranded DNA (ssDNA) have been mainly immunochemical

methods (Razavi,1967; Levy and Simpson,1973). For example, Levy and Simpson (1973) prepared antisera to ssDNA by injecting rabbits with a complex of heat denatured calf thymus DNA and methylated bovine serum albumin. The antisera produced was found to be specific for ssDNA based on the fact that monovalent antigens, the four monodeoxyribonucleotides and extensive DNase digests of DNA all inhibit its interaction with ssDNA. By constructing quantitative complement fixation curves for antibody reaction with ssDNA, Levy and Simpson showed that there could be only a maximum of 0.01% single-stranded regions in chromatin. This 'low value' was not due to the inhibition of antibody binding by chromosomal proteins for several reasons: the addition of histones did not affect the binding of the antibody to ssDNA; antibodies specific for native DNA react with chromatin (Stoller,1970); and ssDNA of fd bacteriophage can be detected using the antibody with intact virions. Levy and Simpson consider that most of this 0.01% comes from the antibody reacting with ends of DNA in chromatin. They also conclude that 0.01% is significantly below the 0.05% figure that Crick (1971) arrived at for the amount of ssDNA necessary for control sequences. However, since the calf genome is approximately 3.10^9 n.p. then 0.01% represents 3.10^5 n.p. of unpaired DNA. Since control sequences may be quite short (e.g. the lac operator is 24 n.p. in length; Gilbert and Maxam,1973), say 10-30 n.p., then 0.01% could represent about 10-30,000 different sequences, values that may be of the order of the number of genes in animal cells (Ohta and Kimura,1971; Ohno,1971).

A more recent estimate of the amount of unpaired DNA within interphase chromatin has been made by Mirzabekov and Melnikova (1974) using dimethylsulphate to label the DNA. This compound methylates the amino group of adenine at the 6 position only when it is not involved in hydrogen bonding, or in other words, when it is present in single-stranded regions. (Dimethylsulphate methylates other bases as well but these can be identified and subtracted from the estimates.) From the amounts of methylation of adenine, these workers calculate that the amount of unpaired DNA in chromatin is less than 0.5%.

Unpaired regions in chromatin may be the type of DNA at which initiation of transcription and/or replication occurs. Recent results from Groner et al. (1975) indicate that RNA polymerase may bind to chromatin in regions of single-strandedness.

Section 10. DNA Methylation.

It should be stated at the outset that there is no definitive function for DNA methylation in eukaryotes. In bacteria, however, the phenomenon of host-controlled restriction and modification (Arber,1965; Arber and Linn,1969; Arber,1971) provide methylation with a now well documented role (see Meselson et al.,1972). The possibility that DNA methylation in eukaryotes is concerned with gene control, X chromosome inactivation, differentiation etc., has been discussed (see Riggs,1975; Holiday and Pugh,1975; and references within).

(a) The DNA bases methylated.

In bacteria the most common methylated base is

6-methylaminopurine (6-MAP) followed by 5-methylcytosine (5-MeCyt) (Vanyushin et al.,1968). The situation in eukaryotes is simpler with 5-MeCyt the predominant - and perhaps only - methylated base (Hotchkiss,1948; Dunn and Smith,1958; Scarano et al.,1965; Brown and Attardi,1965; Winocour et al.,1965; Burdon,1966; Vanyushin et al.,1970; Kappler,1970). Attempts to discover 6-MAP in higher eukaryotes have generally been unsuccessful (Dunn and Smith, 1958; Brown and Attardi,1965; Sheid et al.,1968; Grippo et al., 1968; Kalousek and Morris,1969). Unger and Venner (1966) reported the existence of 6-MAP and some N^6methyl derivatives of guanine in human sperm DNA, but Vanyushin et al. (1970) were unable to repeat these observations. Also Culp et al. (1970) reported that HeLa cell DNA contained methylated bases other than 5-MeCyt. 6-MAP has been found in lower eukaryotes such as Tetrahymena (Gorovsky et al.,1973).

The frequency of occurrence of 5-MeCyt in animals is about 1% (Vanyushin,1970), in plants 4-7% (Shapiro and Chargaff,1960; Tewari and Wildman,1966).

(b) Non-random distribution of 5-MeCyt in eukaryotes.

It has long been recognised that 5-MeCyt does not occur at random throughout the eukaryotic genome, but occurs preferentially - and sometimes predominantly - as part of the doublet C_pG (Sinsheimer,1954,1955; Shapiro and Chargaff,1960; Lehman,1960; Doskocil and Sorm,1962; Spencer and Chargaff,1963; Hall and Sinsheimer,1963; Doskocil and Sormova,1965a,b,c; Grippo et al.,1968). This doublet itself is abnormal in that it is underrepresented (Subak-Sharpe,1967; Morrison et al.,1967).

(c) The methylation reaction.

Both in prokaryotes and eukaryotes methylation occurs at the polynucleotide level (Borek and Srinivosan, 1966; Srinivosan and Borek,1966; Gold et al.,1966; Sheid et al.,1968) by specific enzymes, the DNA methylases, which transfer the methyl group from S-adenosyl-L-methionine to the DNA base (Gold et al.,1964; Scarano et al.,1965). DNA methylases have been purified from bacteria (Gold and Hurwitz,1964) and mammalian cells (Burdon et al.,1967; Sheid et al.,1968; Kalousek and Morris,1969; Morris and Pih,1971; Drahovsky and Morris,1971a,b) where in the latter case they are normally found in the chromatin fraction of the cell.

(d) Methylation follows DNA synthesis.

In bacteria methylation follows DNA replication very closely (Billen,1968; Lark,1968a,b; Whitfield and Billen,1972). Although in eukaryotic cells, methylation also follows replication, precisely when it does so is in dispute. Kappler (1970) found the interval between the two in a mouse adrenal cell line was only about 2 min, whereas Burdon and Adams (1969) found that methylation lagged behind synthesis by approximately 1 h; Adams and Hogarth (1973) showed that this also occurs in isolated L cell nuclei. Geraci et al. (1974) found that in isolated HeLa nuclei DNA methylation and synthesis were coincident, and mimicked the in vivo state.

(e) Variations in amounts of 5-MeCyt.

Within a species the amount of 5-MeCyt in the genome has been found to vary. Adams (1973) found that no methylation occurred during sea urchin embryogenesis

until gastrulation. Kappler (1971) has shown tissue variations in amount of 5-MeCyt in chick embryos where brain eye > gut > liver > smooth muscle (heart) > striated muscle (thigh) > skin > kidney > chorioallantoic membrane, 4.2% down to 3.6% (5-MeCyt percentage of cytosine residues). And human sperm DNA is undermethylated compared to other tissues (Vanyushin et al.,1970).

Satellite DNA sequences have been shown to be slightly enriched for 5-MeCyt in the kangaroo rat (Fry et al.,1973) and the mouse (Salomon et al.,1969). Also mitochondrial DNA was shown by Nass (1973) to be less methylated than the nuclear DNA.

Lastly, there have been reports of abnormal amounts of methylated bases in the DNA of malignant cells. Patients with acute lymphoblastic leucaemia were found to have elevated quantities of 5-MeCyt compared to normal lymphocytes of patients with infectious mononuclosis (Desai et al.,1971).

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The initial impetus for the work described in this thesis came from an observation made in this laboratory by Dr. J.M.R.Hatfield that 5-methylcytosine residues may not be distributed randomly in the DNA of chromatin, but may be preferentially localised in unpaired DNA regions. Beforethis result could be rigorously tested, the single-stranded DNA regions themselves had first to be characterised and this is presented in Chapter 4. This is followed in Chapter 5 by the actual testing of the initial observation. While this work was in progress the Kornberg

model for the subunit structure of chromatin was published, and since there seemed to be a numerical correlation between the amount of 5-methylcytosine in the genome and the amount of DNA assumed to present within the subunits, it was decided to investigate this further; this work is described in Chapter 6. The remainder of the thesis is concerned with some aspects of this model for chromatin structure: How widespread in nature is a repeating nucleohistone structure? (Chapter 7). How much of the nuclear DNA and what types of DNA sequences are packaged in this type of structure? In particular, are the simple sequences present? (Chapter 8). Are the ribosomal genes present? (Chapter 8). And perhaps most interestingly, are genes that are being transcribed present in the repeating subunit structure, or are they packed in a different way from the non-transcribed sequences? (Chapter 9).

The thesis opens with a chapter devoted to the more general methods and the more unusual materials used throughout this work (Chapter 2). Chapter 3, at first glance perhaps unrelated to the rest, describes a simple and accurate assay for the detection of mycoplasma contamination in tissue culture cell lines. As mycoplasma infection repeatedly caused spurious results, and as standard culture assays for them were repeatedly inaccurate, it was important to know when cells were infected. The thesis concludes with a general discussion.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

CHAPTER TWO

Section 1. Materials.

Where possible, all chemicals were analytical grade and most of ~~which~~ them were purchased from BDH Chemicals Ltd., Poole, Dorset.

N.crassa endonuclease was purchased from Boehringer-Mannheim, The Boehringer Corporation (London) Ltd., and deoxyribonuclease I, snake venom phosphodiesterase and micrococcal nuclease from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Ribonuclease A, diethyl-pyrocarbonate, agarose (electrophoresis grade), ethidium bromide and sodium dodecyl sulphate were obtained from the Sigma London Chemical Company Ltd., Kingston-upon-Thames, Surrey. Acrylamide and methylene-bis-acrylamide were bought from Eastman Kodak Ltd., Rochester, New York, U.S.A.

Foetal bovine serum was from Flow Laboratories Ltd., Irvine, and the media used were obtained from either Gibco-Biocult Ltd., Paisely, or from Wellcome Reagents Ltd., Beckenham.

Photographic films, papers, developers and fixers used were from various manufacturers: Kodak Ltd., London; Ilford Ltd., Essex; and May and Baker Ltd., Dagenham.

Nitrocellulose filters were purchased from Millipore Corporation, Bedford, Massachussetts, U.S.A.

Radioisotopically labelled compounds were all obtained from The Radiochemical Centre, Amersham.

Section 2. Cells and tissue culture methods.

The following cell lines were used in this work: a human amnion cell line (FLA;obtained from the Western General Hospital, Edinburgh), HeLa cells (clone D98/AH2;from Professor H.Harris, Sir William Dunn School of Pathology, Oxford), mouse L929 cells (from Flow Laboratories, Irvine), kangaroo rat (*Dipodomys ordii*) cells (from Dr. C.J.Bostock, MRC Clinical and Population Cytogenetics Unit, Edinburgh), *Xenopus leavis* kidney cells (from Dr. U.E.Loening, Department of Zoology, Edinburgh), *Drosophila melanogaster* Schneider line 3 (from Ms M.Izquierdo, Department of Genetics, Edinburgh).

FLA, HeLa, L, kangaroo rat, and Xenopus cells were grown in an enriched 199 medium. *Drosophila* cells were grown in Schneider's modified nedium (Gibco-Biocult, Paisley). Both *Xenopus* and *Drosophila* cells were grown at 25°C; the rest were grown at 37°C.

Tissue culture methods were standard (e.g. Paul,1975).

Section 3. Isolation of nuclei from cell lines.

The following method used in this laboratory was adapted from various pbulished methods (see Bonner *et al.*,1968b; Busch,1972; Roodyn,1972), in particular Vaughan *et al.* (1967) Schildkraut and Maio (1968) and Rickwood *et al.* (1974). Cells were harvested by trypsinisation (0.05% trypsin in Dulbecco-EDTA), poured into 25 ml universal bottles on ice containing 1-2 ml serum and collected by centrifugation at 500 g for 10 min. All steps after the initial harvesting were performed between 0 and 4°C. After washing twice in Hank's solution, the cells were washed once in 0.01 M

Tris-HCl, pH 7.4, 2 mM $CaCl_2$ and resuspended in at least 10 volumes of the same buffer. Approximately 30 min was allowed for the cells to swell after which time the cells were transferred to a 40 ml Dounce homogeniser and lysed by about 10 up-and-down strokes of the tight-fitting B pestle. The contents were centrifuged at 500 g for 10 min, the supernatant removed by careful aspiration and the pelleted nuclei washed once in the Tris-$CaCl_2$ buffer. Cytoplasmic contaminants were removed by resuspending the nuclei in 1% Triton X-100, 0.25 M sucrose, 0.003 M $CaCl_2$, 0.01 M Tris-HCl, pH 7.4. The nuclei were collected by centrifugation at 1000 g for 10 min, washed several times in 0.01 M Tris-HCl, pH 7.4, 0.001 M $CaCl_2$, and resuspended in a small volume of the same buffer. Earlier experiments used 0.01 M sodium phosphate, pH 7.7, 0.002 M $MgCl_2$ in place of the Tris-$CaCl_2$ buffer. Indistinquishable results were obtained with either method. It was not necessary for most experiments described to pellet the isolated nuclei through 2 M sucrose.

Section 4. Peparation of DNP.

DNP was always prepared from purified nuclei, isolated as described above. All operations were carried out at 0°C. Nuclei were lysed by sequential resuspension in 0.01 M Tris-HCl, pH 7.4, 0.005 M Tris-HCl, pH 7.4, 0.001 M Tris-HCl, pH 7.4; twice in each concentration; the nuclei were left in each concentration of Tris for 30 min and then collected by centrifugation at 500 g for 10 min. Gentle resuspension of the nuclear pellet was achieved either with a pipette or by one or two strokes in a Dounce homogeniser (A pestle)

or by passing through a 19 guage needle. The supernatant from the low speed centrifugations were spun at 12,000 g for 20 min, the pellets combined and redissolved overnight in either water or 0.001 M Tris-HCl, pH 7.4. The opaque solution was the isolated chromatin preparation used throughout.

Section 5. Digestion of DNP with N. crassa endonuclease.

Isolated chromatin prepared in either 1 mM Tris-HCl, pH 7.4 or distilled water was made 0.01 M Tris-HCl, pH 7.4, 0.001 M $MgCl_2$, and 0.2 M NaCl. If the additions were made slowly in the order given and with shaking, then the precipitate that formed was fibrous and did not inhibit digestion with the endonuclease or with DNase I. For the initial part of this work N.crassa endonuclease preparations were prepared according to published methods (Linn and Lehman, 1965; Linn,1967) and ~~was a~~ were generous gifts from Dr. Gerard Roizes, but for later work commercial preparations were used; indistinquishable results were obtained with either preparation. The enzyme was added to the concentrations described in the text and incubated for various times. If a measure of TCA-soluble radioactivity was to be ~~measured~~ made then the reaction was stopped simply by addition of ice-cold TCA to 5%.

Section 6. Isolation of DNA from N.crassa endonuclease digested DNP.

This was done as described by Elgin and Bonner (1970). Histones were removed first by extracting the DNP with 0.4 N H_2SO_4 at 4°C for 30 min. The precipitate of

non-histone protein-DNA complex was collected by centrifugation at 10,000 g for 10 min at 4°C, the pellet washed once with 0.4 N H_2SO_4 and once with 0.01 M Tris-HCl, pH 7.4. The non-histone proteins were dissociated by dissolving in 0.05 M Tris-HCl, pH 7.4, 1% SDS overnight at 37°C, and dialysis overnight at 37°C against the same buffer. DNA was pelleted by centrifugation at 105,000 g for 18 h at 25°C in a 3x3 ml MSE rotor, the pellet washed in a small quantity of distilled water and redissolved overnight at 4°C. DNA produced by such a procedure had a 280/260 value of 1.5.

The histones were precipitated by addition of 4 vol of ethanol and leaving at -20°C overnight. The non-histone proteins were precipitated by addition of 4 vol of ethanol and redissoved in 0.05 M Tris-HCl, pH 7.4, 1% SDS, and 4 M urea.

Section 7. Isolation of DNA from micrococcal nuclease digested nuclei.

Several methods were used. Early experiments followed basically the procedure of Hewish and Burgoyne (1973b) except that the tetra sodium salt of EDTA was used. Reactions were terminated by addition of 0.1 M EDTA, pH 10.5, to 50 mM, 10% SDS to 1%, solid NaCl to 1 M, the mixture was vortex stirred briefly followed by addition of 78% aqueous phenol. The phenol extractions followed by several chloroform-octan-2-ol (24:1,v/v) extractions produced two phases with little or no interphase. The DNA samples could then be concentrated by precipitation with 2.5 vol of ethanol at -20°C overnight, or by extensive dialysis against 10^{-5} M EDTA (Na_4) at 4°C followed by concentration dialysis

against polyethylene glycol (PEG).

For quick, analytical results with small amounts of nuclei, the following method based on that described by Cryer et al. (1973) was found to be satisfactory. After digestion, 0.2 vol of 5% SDS, 5% sodium lauryl sarcosinate, 5% sodium deoxycholate, 50 mM EDTA (Na_4) was added to lyse the nuclei and dissociate the proteins from the DNA. An aliquot of this mixture could be electrophoresed directly on agarose slab gels.

Other quick methods were tried, such as brief acid extraction before addition of detergents, but these were not satisfactory as the DNA fragments were degraded.

Later experiments were processed as follows. After digestion with micrococcal nuclease the reaction was terminated by addition of 2 ml of 0.15 M NaCl, 0.1 M EDTA, pH 10.5, 0.1 vol of 10% SDS, and pronase to 20 µg/ml. The samples were incubated at 37°C for about 4 h, phenol - chloroform extracted (see Section 8) twice, chloroform-octan-2-ol (24:1 v/v) extracted once, and the DNA precipitated by addition of 0.1 vol of 1 M NaCl and 2.5 vol ethanol and leaving at -20°C overnight. The precipitate was collected by centrifugation at 10,000 g for 30 min at -15°C and redissolved in distilled water. Often a second ethanol precipitation was carried out. It was found that dialysis against 10^{-5} M EDTA, pH 10.5, after the organic extractions did not make a significant difference to the purity of the final DNA fragments.

Section 8. Preparation of DNA from cell lines.

The medium was decanted and the cells washed once with

approximately 10 ml of a balanced salt solution (e.g. Hank's, Earle's). Cells were trypsinised (0.25% trypsin in Dulbecco-EDTA) until they became rounded, removed from the surface by squirting with a pipette and poured into centrifuge tubes (on ice) containing 1-2 ml of serum to prevent cell aggregation. Centrifugation at 1000 g for 5 min pelleted the cells, which were washed twice with ice-cold Hank's solution. The washed cellular pellet was resuspended at room temperature in 0.15 M NaCl, 0.1 M EDTA, pH 10.5 (approximately 5 ml per 0.5 ml of packed cells) and lysed by addition of 0.1 vol of 10% SDS. One tenth volume of pronase (heat treated according to Hotta and Stern (1965); 1 mg/ml) was added and the mixture incubated at 37°C for 4 h with intermittant shaking. An equal volume of phenol, or, in the more recent experiments, phenol-chloroform mixture (1:1,v/v), was added, the contents cooled to 4°C and the tube inverted repeatedly for about 15 min. Centrifugation at 3000 g for 10 min at 4°C separated the phases; the lower, organic phase was removed with a glass syringe and needle, the organic solvents replaced and the aqueous phase re-extracted as before. After a third extraction with $CHCl_3$-octan-2-ol (24:1 v/v), the DNA was wound out on a heat sterilised glass rod by addition of 2.5 vol of absolute ethanol. The DNA was briefly air dried before dissolving in a small volume (1-2 ml) of sterile water at 4°C overnight. One tenth volume of 10xSSC was then added, and the RNA contaminants removed by incubation with RNase A (50 µg/ml) for 1 hr at 37°C. This was followed by addition of 0.1 vol of 3 M sodium acetate, 20 mM EDTA, pH 7 and the solution extracted with an equal volume of phenol-$CHCl_3$-octanol as before. Repeated further

extractions with $CHCl_3$-octanol were carried out until no interphase remained. DNA was then selectively wound out by slow addition of propan-2-ol (0.6 vol), briefly air dried and dissolved in a small volume of sterile water.

Section 9. Hydrolysis of DNA.

DNA samples (specific activity 2000 cpm/µg; 5000 cpm) were placed in thick walled (1.5 mm) Pyrex tubes (0.8 cm external diameter x 14 cm) sealed at one end. If the sample volume was greater than 50 µl, then it was evaporated to dryness by vacuum desiccation; otherwise 150 µl of 88% formic acid was added to the tube directly. The tube was sealed by drawing out the glass in the bunsen flame, allowing the glass to cool and finally sealing the tapered glass in the flame. Use of this method and leaving a large volume of air in the tube, eliminated the necessity of freezing the liquid prior to sealing. Hydrolysis was accomplished by placing the sealed tube in an oven at 175°C for 40 min. After the tube had cooled to room temperature the liquid was frozen by placing it in an acetone-dry ice bath, the tube scratched with a file and carefully popped open. (Wrapping the tube at this stage in a towel was done in case of the glass shattering). The liquid was evaporated to dryness in a vacuum desiccator and the residue taken up in a small volume (20-50 µl) of 0.1 N HCl.

Section 10. Thin Layer Chromatography.

Samples from the hydrolysate were spotted in 1-2 µl lots, drying onto a precoated 20 x 20 cm plastic thin-layer chromatography sheet (MN-Polygram, Cellulose MN 300). This

was followed by overlaying the radioactive spot with a mixture of unlabelled uracil, thymine, cytosine, 5-methylcytosine, adenine and guanine (c. 50 µmole/ml of each; 2 µl applied) as carriers. Separation of the bases was effected by two dimensional chromatography as described by Randerath (1965); the first dimension in methanol:conc.HCl:water (70:20:10 by vol) takes approximately 2 h, the second in butan-1-ol:methanol:6 N ammonium hydroxide (60:20:20 by vol) approximately 2.5 h. Between dimensions the chromatogram was dried in a draft of cold air from a hair dryer for 5 min followed by warm air for 5 min, and the solvent front was removed to permit the second dimension solvent front to run in a straight line.

Location of the spots was done by viewing under ultraviolet light, and the spots cut out. Radioactivity was estimated in each spot by directly counting the piece in toluene PPO-POPOP scintillator. This method was found to be approximately 20% efficient for counting ^{3}H. Only a slight increase in efficiency was found by scraping off the cellulose and/or counting in Triton X-100 based scintillant.

Section 11. Gel electrophoresis.

(a) 2.5% polyacrylamide tube gels.

Tube gels (0.6 cm x 11 cm) were prepared acording to Loening (1967) but containing 0.2% SDS in the buffers (Loening,1969; Lockard and Lingrel,1972). RNA samples (approximatly 40 µg) were mixed with 10% glycerol, 1 mM EDTA, 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, loaded under buffer, and electrophoresed at 2 mA/gel for 6 h at room temperature. Gels were stained with 0.2% methylene blue dissolved in 0.2 M

sodium acetate, 0.2 M acetic acid and destained by diffusion in the same buffer without methylene blue. Gels were photographed onto FP4 film through an orange filter.

E buffer consisted of 0.036 M Tris base, 0.03 M NaH_2PO_4, 0.001 M EDTA, pH 7.9.

(b) 10% polyacrylamide SDS tube gels.

These were done as described by Panyim and Chalkley (1971).

(c) 3%-6% polyacrylamide slab gels.

The method used was that of K.W.Gross (personal communication). Gels were cast between glass plates; the 6% gel was 10 cm wide by 12 cm long by 1 mm thick, the 3% gel was 10 cm wide 2 cm long by 1 mm thick. Spacers were made from soft PVC plastic (a gift from Ken Gross); the use of this plastic obviated the requirement for silicon grease providing the 6% gel was poured almost horizontally. The following solutions were mixed together:

24% acrylamide, 0.64% bis-acrylamide in H_2O	5 ml
1 x E buffer (Loening,1967)	2 ml
H_2O	12.5 ml
10% ammonium persulphate	0.1 ml

The mixture was degassed for 1 min at room temperature followed by addition of:

20% SDS	0.2 ml
TEMED	0.025 ml

This mixture was poured carefully into the assembled gel apparatus and a piece of Perspex inserted to form a flat surface. Polymerisation usually took 30 min at about 20°C, but was usually left for several hours. After this time the Perspex piece was withdrawn, the 6% gel surface washed

twice with H_2O and dried with tissue paper. It was then ready for the 3% gel which was made as follows:

24% acrylamide, 0.64% bis-acrylamide in H_2O	1.25 ml
1 x E buffer	1.0 ml
H_2O	7.5 ml
10% ammonium persulphate	0.05 ml

This mixture was degassed for 1 min at room temperature followed by addition of:

20% SDS	0.1 ml
TEMED	0.025 ml

This solution was then poured carefully over the 6% gel, a slot former inserted and polymerisation allowed to proceed.

The running buffer consisted of 0.2% SDS in one-tenth E buffer. The sample buffer was 85% glycerol, 0.7% SDS, 0.35 mM EDTA, pH 7.5 and a small amount of bromophenol blue dye. For loading, 2 parts of the sample buffer was mixed with 5 parts of an RNA solution in H_2O, the solution mixed well and heated to 60°C for 2 min immediately before loading to reduce aggregation of the RNA. The gel was run at 37°C, and the buffer reservoirs were circulated continuously. Voltage was 250 volts with the current 3.5 mA, and the running time 12 h.

At the completion of electrophoresis the gels were subjected to fluorography as described in Section 13.

(d) Agarose slab gel electrophorsis.

Agarose gels were run in an apparatus similar to that used by DeWachter and Fiers (1971) for polyacrylamide slab gels. Gels 19 cm x 19 cm x 0.3 cm were cast between glass plates, a fifteen slot former (0.65 cm wide, 0.75 cm deep, 0.1 cm thick) acting as the bottom while the gel

was poured upside down. Silicone grease and clips were used to prevent leakage. Agarose (from 0.6% to 2%) was dissolved in E buffer (Loening,1967) by gentle refluxing for about 10 min, after which the liquid was cooled to 65°C by swirling the flask under running tap water, and poured slowly between the plates. Gels usually took about 30 min to set.

Samples (20-40 µl) were loaded in 5-10% polyethylene glycol under E buffer and electrophoresis was usually at 2 volts/cm for 15-16 h at room temperature. Having 0.2% SDS in the running buffer did not improve resolution. The reservoirs contained 1.5 litre of E buffer which was circulated throughout the run. At the completion of electrophoresis, the gel was soaked in ethidium bromide (0.5 µg/ml) in E buffer in the dark for 30 min at room temperature. The DNA fluorescence was photographed under ultra-violet light onto FP4 film through a red filter.

(e) Agarose tube gel electrophoresis.

Agarose gels (1%-2%) were made in Perspex tubes (24.5 cm long by 0.9 cm diameter) using plasticine to hold the tubes upright during preparation. When the agarose had set, cheesecloth was attached to the other end of the tubes with medical adhesive plaster and the tubes inverted. Up to 100 µl samples containing up to 100 µg of DNA could be loaded onto these gels. Electrophoresis in E buffer was at 1 volt/cm for 18 h at room temperature.

Section 12. Method for transferring DNA fragments from agarose gels to nitrocellulose filters.

The method was that of Southern (1975b). Either slab gels or tube gels could be used. With slab gels the

longitudinal strip containing the DNA fragments was cut from the agarose slab; with tube gels a gel holder, described by Southern, was used.

After staining and photographing the gel, the DNA was denatured *in situ* by immersing the strip or tube in 0.5 M NaOH, 1.5 M NaCl for 15 min in the case of the strip, or for 30 min for the tube gel, at room temperature with occasional inversion. This was followed by immersion in a neutralising solution of 1 M Tris-HCl, 3 M NaCl, pH 5, for 15 min (strip) or 90 min (tube), at 4°C again with occasional inversion. During the neutralising step a piece of nitrocellulose filter (a little wider than the dimensions of the agarose gel) was wetted in distilled water. The DNA was eluted from the agarose gel onto the nitrocellulose filter in 20xSSC at room temperature overnight, using the system described in Southern (1975b). The filter was washed in 2xSSC, blotted between filter papers and dried overnight under vacuum at room temperature. The DNA was baked onto the filter by subjecting it to 80°C under vacuum for 2 h. This filter was then ready for hybridisation.

Section 13. Fluorography.

(a) Of polyacrylamide slab gels.

This was done essentially as described by Bonner and Laskey (1974). After electrophoresis, the gel was soaked in two changes of dimethylsulphoxide (DMSO) for 30 min each at room temperature to remove the water from the gel. It was then soaked for 4 h in approximately 4 vol of 20% 2,5-diphenyloxazole (PPO, w/v) in dimethylsulphoxide. This was followed by soaking in distilled water for 2 h to

remove the DMSO and precipitate the PPO in the gel. Several changes of water were used. At this stage the gel could either be dried down and then fluorographed (see below); or be fluorographed wet as follows according to the method of K.W.Gross (personal communication). The gel was placed on a glass plate wrapped in 'Seran wrap' or 'Cling film', and both wrapped lightly together in the plastic film. A piece of Kodak RP Royal X-omat film, flash sensitised by the method of Laskey and Mills (1975), was placed over it, followed by a piece of cardboard and another piece of glass. The two glass plates were taped together, wrapped in aluminium foil, wrapped in black plastic and put at -70°C for exposure. The advantage in this method is that the RNA can be recovered more easily from a wet gel than by rehydrating a dried gel. Its disadvantage is in its lowered sensitivity.

To dry down the gel standard methods were used, namely, the gel was dried onto a piece of filter paper under vacuum and with heating on a steam bath. Once dry, the gel was fluorographed on flash sensitised Kodak RP Royal X-omat film as before, except that black Perspex plates were used instead of glass plates.

(b) Of nitrocellulose filters.

The washed and dried filter from the hybridisation reaction was immersed in a solution of 20% PPO in toluene (w/v), drained by touching the corners of the filter onto filter paper, and finally dried thoroughly in a stream of air. Fluorography was as described for the dried polyacrylamide gel, except that X-ray film was placed on both sides of the nitrocellulose filter.

(c) Development and fixing.

This was done in DX-80 developer for 10 min at room temperature followed by fixing for 15 min in Perfix (May and Baker Ltd.,Dagenham,England) fixer and washing for 30 min in running water.

Section 14. Sucrose gradient centrifugation.

These were linear gradients from 15% to 30% sucrose containing 0.5% SDS. To form the gradients the step method was used as little joy had been had with gradient mixers. The following concentrations of sucrose were made in buffer R: 15%, 20%, 25%, and 30%. Buffer R consisted of 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, 0.5% SDS, pH 7.4. All solutions were treated with diethyl pyrocarbonate (DEP). Standard RNase-free methods were used throughout, and these are described in Chapter 9, Section 1. Gradients were 12.5 ml in 14 ml polycarbonate tubes; 3.1 ml of each concentration were layered carefully on top of each other. Equilibration was for at least 24 h and linearity was checked by refractive index measurements.

RNA samples (100-200 µl) dissolved in buffer R ~~was~~ were loaded onto each gradient and centrifuged in an MSE 6x14 ml titanium rotor at 26,000 rpm at 22°C for 18 h. These conditions adequately separated the 18 S and 28 S ribosomal RNA species. Gradients were fractionated simply by puncturing the bottom of the tube and collecting 10 drop fractions in sterile tubes. RNA could be located either by optical density readings at 260 nm, or by radioactivity measurements. When the latter was used, aliquots (5 µl) were spotted onto GF/A filters, dried, precipitated with 10% TCA, followed by washing in 5% TCA, ethanol-ether

(1:1, v/v), ether, all at 0°C, and finally drying for 15 min at 100°C.

CHAPTER THREE

A METHOD FOR THE DETECTION OF MYCOPLASMA CONTAMINANTS.

CHAPTER THREE

During the course of the work described in this thesis using cell lines, it became apparent that aberrant results were obtained and which were found to be due to the redistribution of nucleoside labels. When these cell lines were tested for mycoplasma contamination using methods that culture the mycoplasma (see Ludovici and Holmgren,1972), negative results were often obtained. However, when DNA isolated from sister cultures was centrifuged in analytical CsCl gradients, a new band of density approximately 1.68 g/ml was found, indicative of mycoplasma contamination. Thus methods of mycoplasma detection involving culturing of the organism seemed to be inaccurate. For experiments using radioactively labelled precursors of nucleic acids it was essential that our cell lines were free from mycoplasma as it had been shown that the host's metabolism is radically altered in their presence (Levine et al.,1975; Perez et al., 1972; Schneider et al.,1974). This meant that a reliable, and preferably quick and simple, method for detection of the presence of mycoplasma was essential.

There are many published methods for the detection of mycoplasmas in cultured eukaryotic cells (for reviews see Ludovici and Holmgren,1972; Levine,1974; and references within), most of which are complex, time consuming and often inaccurate giving false-negative results. Recently a method was published (Russell et al.,1975) that involved detection of cytoplasmic DNA-containing cell contaminants by staining their DNA with either 4'-6-diamidino-2-phenylindole or Hoechst 33258 and viewing by fluorescence microscopy. This method, as are several others, was based on the fact that

mycoplasmas and some DNA animal viruses (e.g. vaccinia virus) replicate in the cytoplasm. However, one drawback of this procedure is that cells must be grown on coverslips. In the method described below a small culture vessel (e.g. a 6 oz medical flat) routinely used for sub-culture is adequate for the assay. An answer can be obtained within two hours, which makes it possible to check for the presence of mycoplasma in sister cultures before each experiment, as suggested by Levine(1974).

The method described is a simpler and more sensitive double-label modification of the one described by Schneider et al.(1974) and depends on the ability of mycoplasmas to synthesize the enzyme pyrimidine phosphorylase (Levine,1972) which both hydrolyses uridine (and thymidine) to uracil (and thymine), and can 'salvage' pyrimidines to nucleosides by the reverse reaction; this means that exogenous uracil can be incorporated into RNA in mycoplasma infected cells but not in uninfected cells (Levine,1972; Perez et al.,1972) where this enzyme is in the main absent. (Low levels do occur in some cell lines; a list is provided by Levine,1974).

Section 1. The method.

A fifty times concentrated mixture of ^{3}H-uridine and ^{14}C-uracil was added to a rapidly growing, subconfluent bottle of cells to final levels of 1 μCi/ml and 0.1 μCi/ml respectively, without changing the medium. This bottle was allowed to incorporate label for 1 h, after which time the medium was poured off and the attached cells washed twice with a balanced salt solution (e.g. Earle's, Hank's, phosphate buffered saline). Five millilitres of 0.1% SDS (w/v)

was then added and, after leaving for 5 min to allow complete cell lysis, the viscous solution was poured into 10 ml of 20% TCA (w/v) at 0°C. A further 5 ml of 0.1% SDS was used to rinse the culture vessel, and this was also added to the 20% TCA. The mixture was left for 20 min at 0°C before filtration onto a Whatman GF/A or GF/C filter, which was washed sequentially with 20 ml of ice-cold 10% TCA, 10 ml of ethanol-ether (1:1, by vol) and 10 ml ether. Finally the filter was dried in an oven at 100°C for 5 min, added to a vial containing PPO-POPOP toluene scintillant and counted in a Packard liquid scintillation spectrometer; the settings were such that ^{3}H and ^{14}C could be differentiated e.g. ^{3}H: window 50-400, gain 90%, ^{14}C: window 350-1000, gain 10%. With these settings ^{3}H is counted at 47% relative efficiency of counts, and ^{14}C at 35%; spillover of ^{14}C channel into ^{3}H channel is 15% of the corrected ^{14}C cpm, while there is no spillover of ^{3}H cpm into the ^{14}C channel. Background values were obtained by stopping the incorporation at time zero (i.e. immediately after addition of label), and processing exactly as above.

Section 2. Results and discussion.

If cell lines are contaminated with mycoplasma then, as mentioned above, the host's metabolism is altered. For example, mycoplasma synthesise pyrimidine phosphorylase. An important consequence of this is that the use of thymidine or uridine either to label cellular nucleic acids, or in the case of thymidine, to synchronise cells, becomes impractical since these compounds are degraded by the mycoplasma contaminants. The presence of pyrimidine phosphorylase

however, can be exploited in convenient assay for the presence of mycoplasma.

Schneider et al. (1974) labelled separate cultures with either ^{3}H-uridine or ^{3}H-uracil, purified total cellular RNA from both, and determined their specific radioactivities. Thus the ratio of the specific radioactivities of the RNA labelled wtih ^{3}H-uridine to the RNA labelled with ^{3}H-uracil gave an indication of elevated levels of uridine phosphorylase and hence of mycoplasma contamination. This method has now both been simplified and made more sensitive by using uridine and uracil labelled with different radioisotopes in the same culture vessel, and also speeded up by labelling for much shorter periods. Using two radioisotopes obviates the necessity of determining the precise amounts of RNA present.

Cells were labelled with a mixture of ^{3}H-uridine and ^{14}C-uracil and the incorporation into RNA measured as described in Section 1. Table 3 shows results from a typical experiment using mouse L cells. Isolation of total cellular DNA followed by analytical CsCl gradient centrifugation (shown for FLA cell DNA in Figure 1) confirmed the presence of mycoplasma DNA (density c. 1.68 g/ml). Furthermore, treatment of mycoplasma infected cells with kanamycin (200 µg/ml) for seven days suppressed the infection but did not eliminate it. This suggests that incorporation of ^{14}C-uracil into RNA indicates the presence of some cell culture contamination.

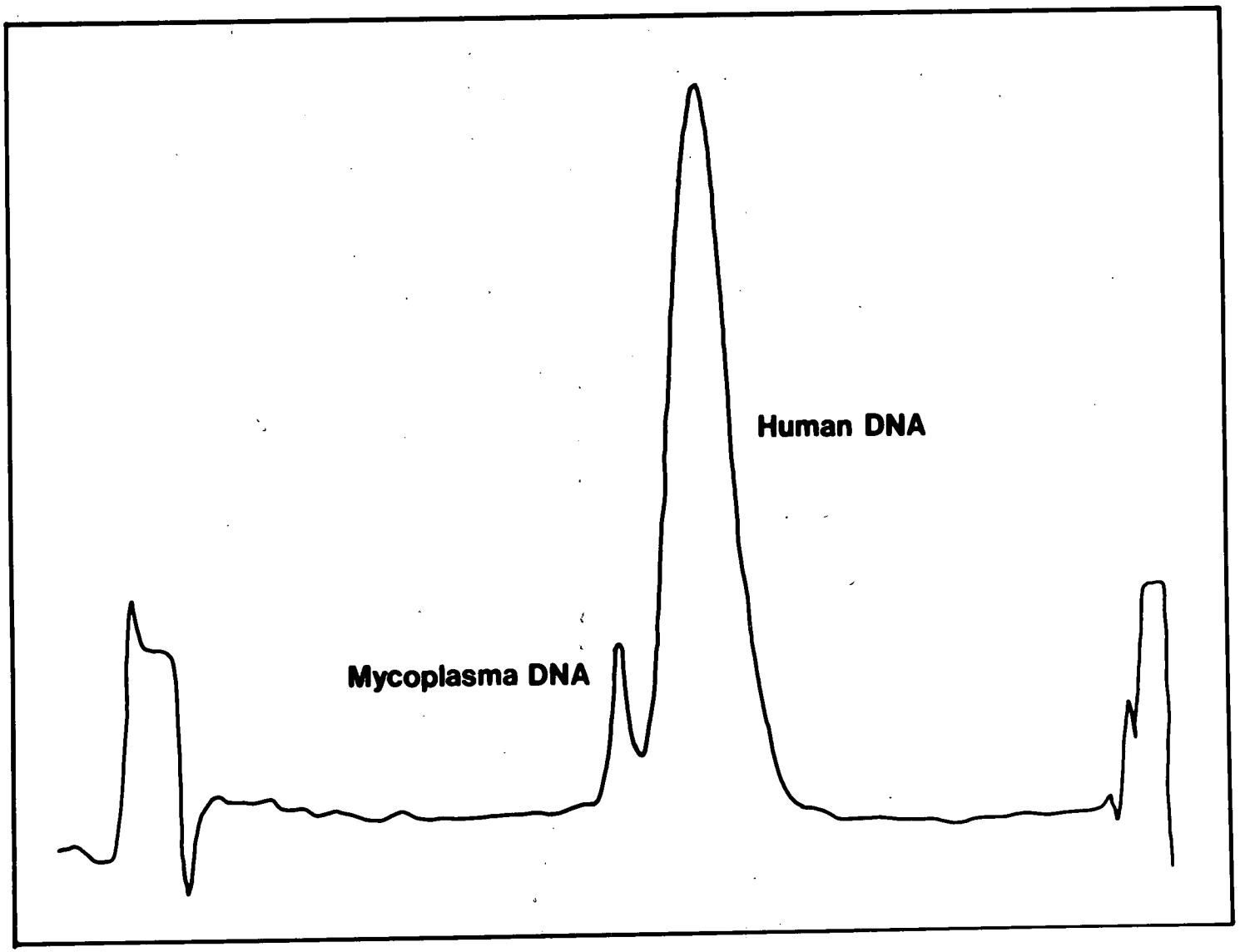
Since the medium used (modified 199) contains uracil (2.68 µM) but not uridine, it was necessary to check if the addition of cold uridine (to 3 µM and 30 µM) had any effect

Table 3

A comparison of ^{3}H-uridine and ^{14}C-uracil incorporation in healthy, mycoplasma-infected, and kanamycin-treated infected mouse L cells.

Condition of cells	Corrected TCA-insoluble radioactivity (cpm)	
	^{3}H-uridine	^{14}C-uracil
Healthy cells	81,540	7
Infected cells	3,050	5,285
Kanamycin (200 µg/ml) treated for 7 days	7,488	1,522

Figure 1. Analytical CsCl Gradient Centrifugation of Human DNA Isolated from Mycoplasma-infected FLA Cells.


Human DNA
Mycoplasma DNA

on either ^{3}H-uridine or ^{14}C-uracil incorporation. As expected, addition of cold uridine decreased ^{3}H-uridine incorporation, but ^{14}C-uracil incorporation was unaffected. Also, using a medium lacking uracil (F10) slightly increased ^{14}C-uracil incorporation in mycoplasma-infected cells.

So far this method has been successfully applied to the following cell types: to human lymphocyte suspension cultures; to monolayer culture - human HEP, D98/AH2 and FLA cells, mouse L and 3T3 cells, kangaroo rat (Dipodomys ordii) cells, marsupial (Sminthopsis crassicaudata) cells and Xenopus laevis kidney cells; and to several human-mouse somatic hybrid cells. Drosophila Schneider line 3 cells were found to incorporate ^{14}C-uracil seemingly in disagreement with the other cell lines. However, it has since been learned that cells from both Drosophila embryos and adults (Akai et al.,1967; Rae and Green,1968), as well as Schneider's cell lines 1, 2, and 3 (Williamson and Kernaghan,1972), all contain virus-like particles, so that this could explain the uracil incorporation. It also points to the usefulness of the method. Mycoplasmas, though, are usually the most common and troublesome cell culture contaminants.

CHAPTER FOUR

CHARACTERISATION OF SINGLE-STRANDED DNA REGIONS IN ISOLATED CHROMATIN BY NUCLEASE DIGESTION.

CHAPTER FOUR

This Chapter describes an investigation into the occurrence of single-stranded DNA (ssDNA) regions in isolated chromatin.

The possible importance of unpaired DNA regions has been discussed by Crick (1971) who postulated that such regions, if they occur, might be important as, say, recognition sites for proteins involved in the control of gene expression.

One result that indicated that no single-stranded regions were present in the DNA of interphase chromatin was that of Erlanger et al.(1972) who found that anti-nucleoside antibodies (raised in rabbits by injection of a BSA-conjugated nucleoside and found to be specific for the antigen and denatured DNA) did not react with nuclei unless these nuclei were first subjected to conditions that separated the two DNA strands; reaction with anti-nucleoside antibodies did occur, though, if the nuclei were isolated during S phase where, in the process of DNA replication, single strands would be generated (Klein et al.,1967; Freeman et al.,1971). However, the lack of reaction of the antibodies with non-S phase nuclei, as pointed out by Erlanger et al., may not reflect the absence of short regions of ssDNA in interphase chromatin, but simply the inability of these large molecules (c.180,000 daltons) to penetrate the chromatin. A more sensitive method might be to use an endonuclease specific for ssDNA to digest those parts of the genome present as unpaired sequences. The two most characterised single-strand specific nucleases are the S_1 nuclease from Aspergillus oryzae (Ando,1966;

Sutton,1971; Vogt,1973) and the enzyme from Neurospora crassa (Linn and Lehman,1965a,b; Linn,1967; Rabin and Fraser,1970). For the following studies the latter enzyme was chosen since it was well characterised, available in pure form in the laboratory, and its pH optimum was almost physiological (c.8), whereas that of the S_1 nuclease was not (c.4.5). The N.crassa endonuclease has a molecular weight of about 70,000 daltons, much smaller than the anti-nucleoside antibodies.

Section 1. Isolation of DNP and some of its characteristics.

Isolated chromatin was prepared by lysing purified nuclei into low salt as described in Chapter 2. Care was taken to disrupt the isolated material as little as possible by shear forces so as to preserve a quasi-native structure. Recent evidence (Noll et al.,1975) has confirmed this notion that shearing DNP complexes introduces structural alterations.

Isolated chromatin preparations usually had a 280/260 ratio of 0.7, and a 240/260 ratio of 0.88. Extraction of non-histone chromosomal proteins and histones by the usual methods (e.g. Elgin and Bonner,1970) followed by analysis on 15% polyacrylamide gels according to Panyim and Chalkley (1971) demonstrated protein profiles similar to those already published. Analysis of the DNA is discussed in Section 4 (b).

Section 2. Labelling of cellular DNA.

Since it was important for succeeding experiments to have cells whose DNA was uniformly labelled, it had to be established that no, or insignificant, redistribution of

label from ^{3}H-(methyl)thymidine to another nucleoside occurred during the long incorporation periods required. Uniformly labelled DNA was necessary if the problem of analysing newly-replicated rather than total DNA was to be avoided.

Human amnion (FLA) cells were labelled for 3 days with ^{3}H-(methyl)thymidine (0.1 μCi/ml) in modified 199 medium supplemented with 10% foetal bovine serum, and their DNA isolated and purified as described in Chapter 2. Two methods of base analysis were used: sequential digestion of the DNA with pancreatic DNase I and snake venom phosphodiesterase with separation of the 5'-nucleoside monophosphates by electrophoresis; and formic acid hydrolysis of the DNA with separation of the resulting bases by two dimensional thin layer chromatography. Both methods of analysis confirmed that thymidine was the only labelled nucleoside in DNA after incorporation for 3 days.

Section 3. The specificity of the endonuclease.

The specificity of the endonuclease was tested in two ways: the activity of the N.crassa endonuclease to native, double-stranded DNA, and its activity towards denatured DNA.

Uniformly labelled ^{3}H-thymidine human DNA and ^{32}P-labelled λ DNA (a generous gift of Dr.E.M.Southern) were separately incubated with 5 units/ml of endonuclease in 0.1 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.2 M NaCl at 37°C for 0 and 1 h. Both TCA-soluble and TCA-insoluble radioactivity was measured. It was found that the enzyme lacked activity against both DNA samples, less than 0.01% appearing in the acid-soluble fraction.

When the endonuclease was tested for activity against human DNA that had been heated to 100°C for 10 min and cooled to 37°C prior to the addition of the enzyme, greater than 90% of the radioactivity was rendered acid-soluble. The resistance of a certain proportion of eukaryotic DNA to single-strand specific nucleases is well known and is due to the so-called 'snap-back' fraction (see Schmid et al., 1975).

These experiments confirm that the N.crassa endonuclease is specific for unpaired DNA while only digesting duplex molecules to less than 0.01%; in other words, the enzyme is nearly 10,000 times as active with denatured DNA as native DNA.

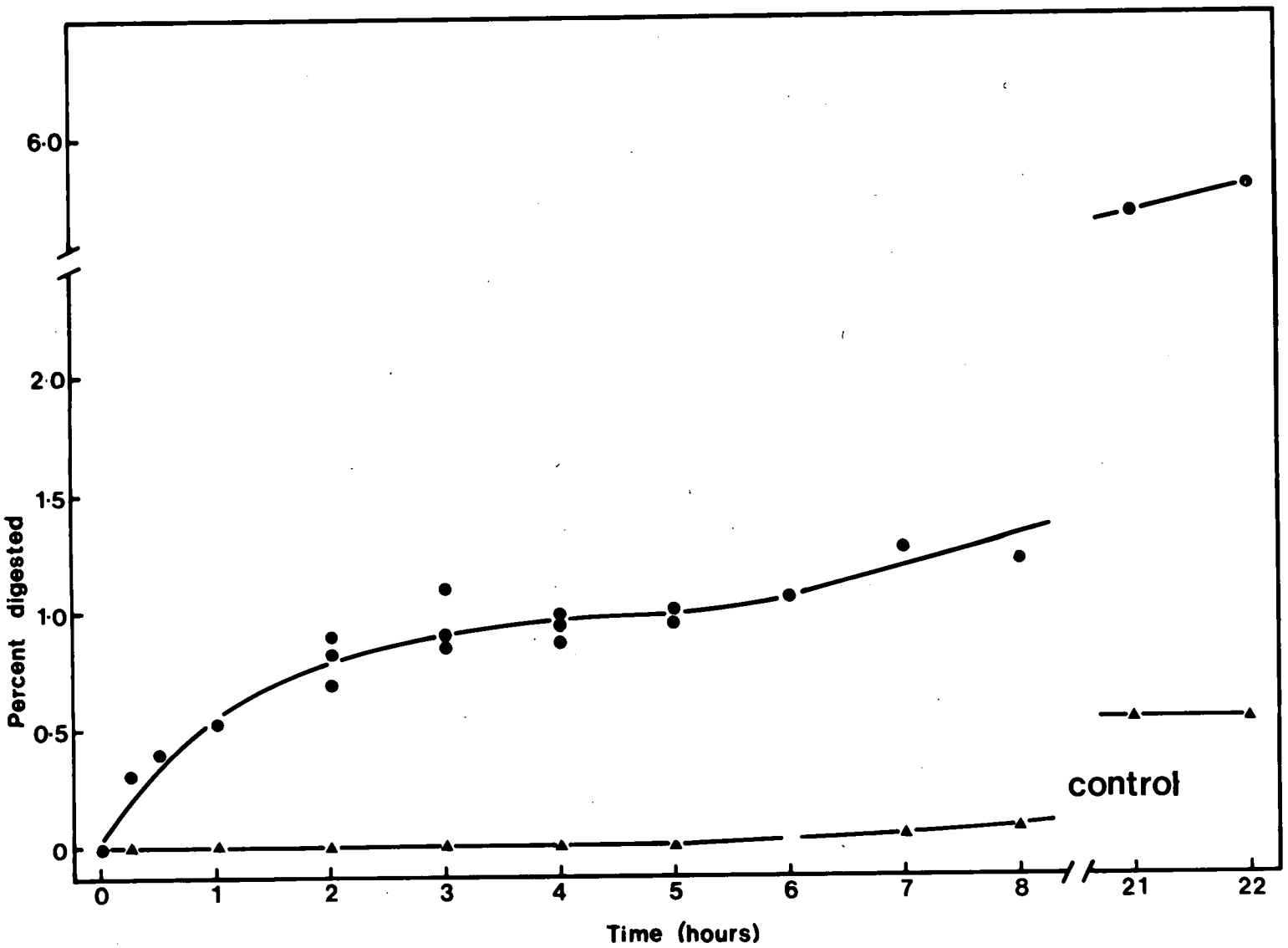
Section 4. Digestion of isolated chromatin with N.crassa endonuclease.

(a) Kinetics of digestion and extent of reaction.

Isolated chromatin was prepared from cells labelled for 3 days with ^{3}H-thymidine (0.1 μCi/ml) as described in Chapter 2. Digestions of the isolated chromatin with the endonuclease (at 2.5 units/ml) was monitored over a 24 h period by measurement of the TCA-soluble and insoluble radioactivity. The results, shown in Figure 2, indicate that there was an increase in the amount of acid-soluble material released from the DNP complex until it levelled off at about 4 h. This was followed by a gradual and continual increase. Control incubations with no enzyme showed no release until about 5 h after which the rate of release increased. If DNase I was used in place of the N. crassa endonuclease, between 90 and 100% of the radioactivity

Figure 2. Time Course of Digestion of Isolated Chromatin with N.crassa Endonuclease.

Isolated chromatin was prepared as described in Chapter 2 and digested with N.crassa endonuclease at 2.5 units/ml in 0.001 M Tris-HCl, pH 7.5, 0.001 M $CaCl_2$ for the times indicated. At each time point samples were withdrawn and the TCA-soluble and TCA-insoluble radioactivities determined.


6·0
2·0
1·5
1·0
0·5
0
Percent digested
0
1
2
3
4
5
6
7
8
21
22
Time (hours)
control

became acid-soluble in an hour at 37°C. These results can be interpreted as follows: the initial acid-soluble release is due to the endonuclease digesting the single-strand regions in the DNP complex; this digestion reaches completion at about 4 h at the enzyme level chosen (2.5 units/ml); the gradual digestion found after 5-6 h incubation could be due to nibbling in from the ends, or to the chromatin-associated nucleases known to be present (Urbanczyk and Studzinski,1974), or to both. In any case, all further incubations were for less than 4 h in an effort to restrict digestion to only the single-stranded regions.

An estimate of the amount of DNA present in isolated chromatin in a single-stranded configuration was obtained from the plateau value of digestion. Results from at least 10 experiments gave a value of 1 $\pm$ 0.5%.

(b) Analysis of DNA from isolated chromatin digested with N.crassa endonuclease.

It was of interest to look at the DNA of isolated chromatin that had been digested with the single-strand specific endonuclease since this would indicate whether single-stranded regions were located at defined intervals, possibly in a regular pattern.

Several methods were tried to obtain DNA of high purity from isolated chromatin; the one finally decided upon was similar to that used by Elgin and Bonner (1970) to dissociate DNP complexes into their constituents and is described in Chapter 2. Briefly, it involves dilute acid extraction of histones, dissociation of the NHP with 1% SDS followed by pelleting the DNA which was then dissolved in H_2O for further analysis.

Sizing of the DNA was accomplished by the method of electrophoresis on agarose gels as described in Chapter 2. DNA (1-2 μg) was loaded onto 0.6% agarose slab gels and electrophoresed in buffer E at 1 volt/cm for 36 h. Ethidium bromide fluorescence was used to locate the DNA. The gel was photographed and microdensitometer tracings were taken from the negative. Figure 3 shows a comparison of whole cell DNA, DNA from isolated chromatin and DNA from endonuclease digested isolated chromatin. It can be seen that the molecular weight distributions for either digested or control samples is both more heterogeneous than whole cell DNA, and is much smaller, presumably reflecting degradation by cellular nucleases during isolation. By comparing the mobility of the DNA samples with T_4, and SV40 DNAs, the following mean values were calculated:

whole cell DNA	$100 \cdot 10^6$ daltons
undigested chromatin	$4 \cdot 10^6$ daltons
endonuclease digested chromatin	$3 \cdot 10^6$ daltons

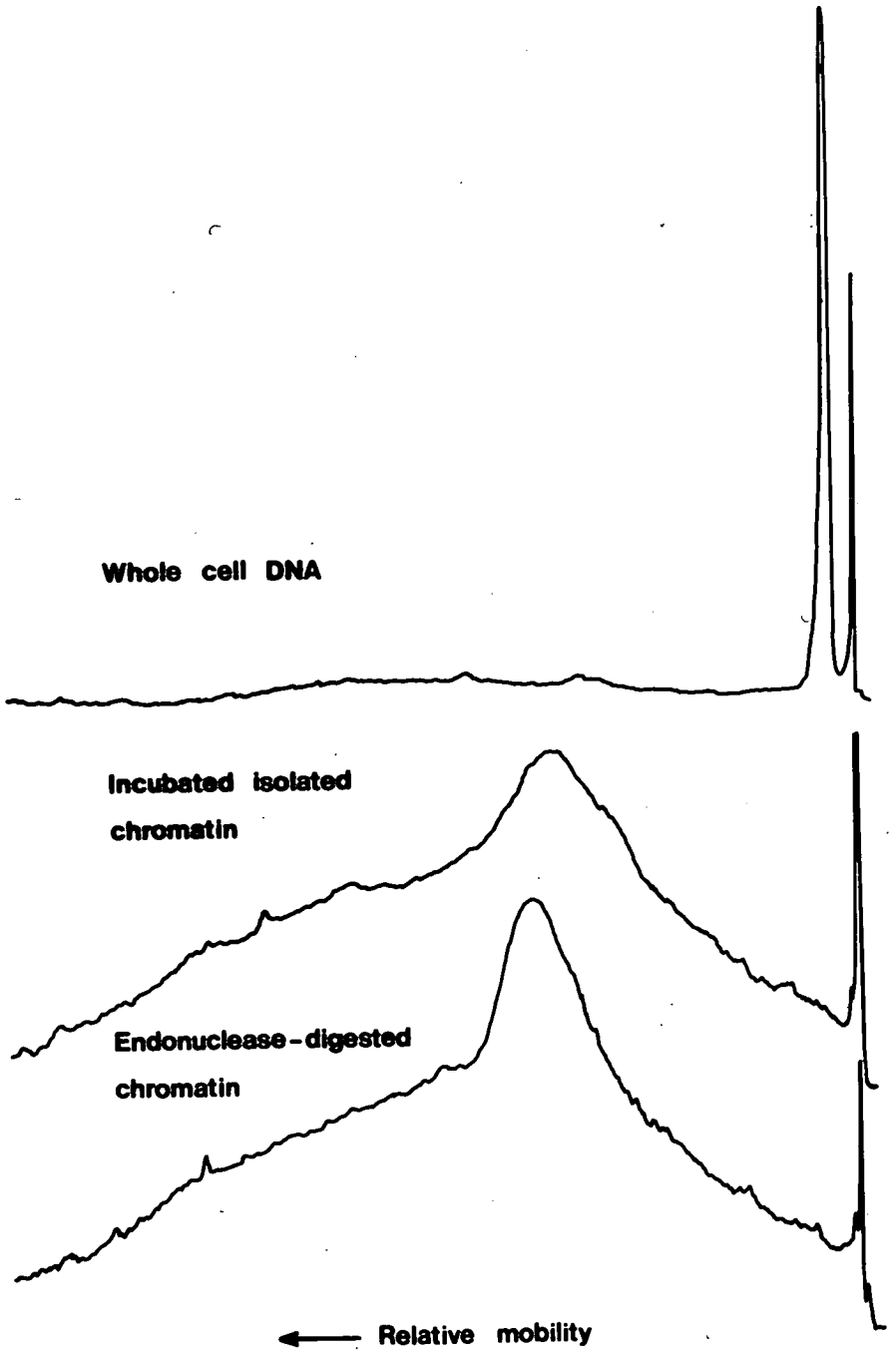
Analysis of the DNA isolated from endonuclease-digested DNP on denaturing formamide polyacrylamide gels may have shown a lower molecular weight distribution than that seen on the non-denaturing gels used if digestion was only from one strand.

Section 5. Conclusions.

From the digestion of isolated chromatin with a single-strand specific endonuclease it can be concluded that a significant, reproducible and unexpectedly large fraction - approximately 1% - of the DNA in chromatin is present in an unpaired state. This value should be compared to the

Figure 3. Analysis by Agarose Gel Electrophoresis of Whole Cell DNA, DNA from Isolated Chromatin and DNA from Isolated Chromatin after Digestion with N.crassa Endonuclease.

DNA samples were prepared as described in Chapter 2 and subjected to electrophoresis on 0.6% agarose slab gels in E buffer at 1 volt/cm for 36 h, after which the gels were stained in ethidium bromide and photographed. Microdensitometer tracings of the negatives are shown. The digestion conditions were 2.5 units/ml for 3 h at 37°C.


Whole cell DNA
Incubated isolated chromatin
Endonuclease-digested chromatin
Relative mobility

value of less than 0.01% obtained by Levy and Simpson (1973) using an immunochemical and complement fixation assay, and less than 0.5% by Mirzabekov and Melnikova (1974) using dimethylsulphate.

The single-stranded DNA fraction of the chromatin was now considered to be well enough characterised to proceed to test the original observation more rigorously, namely, are these regions enriched for 5-methylcytosine?

CHAPTER FIVE

IS THE DISTRIBUTION OF 5-METHYLCYTOSINE CORRELATED WITH THE DISTRIBUTION OF SINGLE-STRANDED DNA REGIONS IN ISOLATED CHROMATIN?

CHAPTER FIVE

Some preliminary experiments in this laboratory had suggested that single-stranded regions of DNA within isolated chromatin - as defined by the N.crassa endonuclease susceptibility - were preferentailly enriched for 5-methylcytosine (5-MeCyt). As there is no known function for DNA methylation in eukaryotes (see Chapter 1,Section 10), if methylation could be correlated with chromatin structure in some way, greater insight into both may be achieved. In this light it was considered worthwhile to investigate this phenomenon more thoroughly.

The original observation made by Dr.J.M.R.Hatfield was that if isolated chromatin was prepared from human cells labelled with ^{3}H-(methyl)methionine and digested with the N.crassa endonuclease, the acid-soluble material released during digestion amounted to between 10 and 15% of that originally present in the chromatin preparation, compared to approximately 1% of the material if ^{3}H-thymidine was used as label. Since the predominant, and perhaps only, methylated base in mammalian DNA is 5-MeCyt and ~~that~~ methylation occurs at the polynucleotide level by donation of the methyl group from S-adenosylmethionine (see Chapter 1,Section 10), then this approximately 10-fold increase in digestible material was assumed to be oligonucleotides and nucleotides containing 5-MeCyt. It was decided to analyse this released material more thoroughly.

Section 1. Experiments using ^{3}H-(methyl)methionine to label cells.

(a) Labelling of cells with ^{3}H-(methyl)methionine and analysis of nucleotides labelled.

FLA cells were grown to sub-confluency in modified 199 medium supplemented with 10% foetal bovine serum (FBS), the medium decanted, the cells washed twice with Hank's balanced salt solution and the medium replaced with methionine-free 199 medium (+10% FBS) to which had been added :

adenosine	50 μM
cytidine	20 μM
guanine	25 μM
thymidine	10 μM
sodium formate	10 μM
methotrexate	100 μM
^{3}H-(methyl)methionine	5 μCi/ml

Incorporation was allowed to continue for 12 h.

In all following experiments, whether cells were labelled with ^{3}H-(methyl)methionine, or ^{14}C-thymidine or ^{3}H-thymidine, DNA was prepared from sister cultures and a base analysis determined, either by enzymatic digestion with separation of the resulting dNMPs by paper electrophoresis, or by formic acid hydrolysis with separation of the resulting bases by two-dimensional thin layer chromatography or both. Paper electrophoresis, however, did not separate dCMP from its 5-methyl derivative, whereas thin layer chromatography separated 5-MeCyt from cytosine.

If the cellular label was ^{3}H-(methyl)methionine then the radioactivity was found to be approximately 90% as dCMP

and 10% as dTMP if analysed electrophoretically, but 90% as 5-MeCyt and 10% as thymine if formic acid hydrolysis was used, showing that the 5 position of cytosine was by far the most highly methylated position. In agreement with others, no 6-methylaminopurine was ever detected (see Chapter 1,Section 10). The appearance of radioactivity as thymine was found to be always present with or without added thymidine and in the presence or absence of methotrexate (amethopterin, an inhibitor of dihydrofolate reductase); it probably arises both through methylation at the nucleotide level and through chemical deamination of 5-MeCyt (to be discussed later). In any case it did not affect the use of ^{3}H-(methyl)methionine to label isolated chromatin in this context.

(b) Digestion of ^{3}H-(methyl)methionine labelled DNP with the N.crassa endonuclease.

DNP was prepared from FLA cells labelled for 12 h with ^{3}H-(methyl)methionine as described above; sister cultures were labelled with ^{14}C-thymidine (0.015 μCi/ml). Digestions were carried out as described in Chapter 2 and were for 4 h, after which time the incubation mixture was precipitated by addition of ice-cold 10% TCA. Centrifugation at 1000 g for 10 min produced a supernatant fraction and a pellet; both were analysed for radioactivity. Control tubes without added enzyme with either ^{3}H-methionine or ^{14}C-thymidine labelled DNP, revealed that no radioactivity was released during the incubation period. The single-strand specific nuclease, however, solubilized 10% of the ^{3}H-methionine counts but only 1% of the ^{14}C-thymidine ones.

(c) Analysis of the endonuclease-digested material.

Since ^{3}H-(methyl)methionine would not only be expected to label the DNA but also be incorporated as an amino acid into proteins, it was essential that the released material be identified as nucleotides. But since the N.crassa endonuclease produces oligonucleotides as well as mononucleotides, it was necessary to firstly convert the acid-soluble, endonuclease-released material to a uniform, easily assayable form. Two methods were tried: formic acid hydrolysis and separation of the bases in the usual way; and DNase I-snake venom phosphodiesterase digestion followed by separation of the dNMPs by thin layer chromatography on PEI-cellulose layers.

(i) Formic acid method.

To avoid the large amounts of acid that would be present in the supernatant if TCA was used as precipitant, the digested material was diluted with 0.01 M EDTA, pH 8.0, and separated from the undigested material by centrifugation at 50,000 g for 2 h at 2°C in an MSE 6x5 ml rotor. The supernatant was evaporated to dryness and hydrolysed with formic acid. On separation of the resulting bases from ^{3}H-methionine labelled DNP and counting the chromatograph under the ultra-violet absorbing regions, no radioactivity was found in any of the bases, despite the fact that 10^{4} cpm were applied to the chromatograph. However, ^{14}C-thymine was found in the hydrolysate from the ^{14}C-thymidine labelled DNP, showing that the method itself was satisfactory. By sectioning the remaining ^{3}H-methionine chromatograph, it was discovered that radioactivity was present in a broad band which ran faster than the bases in

the first dimension. At this stage this material was not investigated further.

(ii) Enzymatic digestion and separation on PEI-cellulose thin layers.

A similar experiment was carried out ~~as to~~ that described above, except that instead of formic acid hydrolysis, the dried, digested material was resuspended in a small volume of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and digested sequentially with DNase I (3 h) and snake venom phosphodiesterase (1 h). An aliquot was then applied to a PEI-cellulose thin layer which was chromatographed firstly in 1 M acetic acid : 3 M LiCl (9:1, v/v), followed by Tris-methanol (1.2 g/litre) in the second dimension according to Randerath and Randerath (1967). This procedure separates dCMP from 5-methylcytidylic acid. Initial attempts with the chromatographs failed and this was subsequently found to be due to variations in commercial PEI-cellulose layers. Thus, when one box was found to work it was used for the remaining experiments.

Again, using this method of analysis, no radioactivity was found under any of the nucleotide markers.

It should be added that if the undigested material was analysed by the formic acid method, radioactivity was found in the bases in a pattern indistinguishable from DNA. Purification of the DNA from isolated, digested chromatin was found to be unnecessary as formic acid hydrolysis of digested DNP gave identical results to purified DNA.

Therefore, it seemed likely that the 10-15% material released by the N.crassa endonuclease was not of nucleotide origin but may have arisen from chromosomal proteins. This was then tested.

(d) Origin of the endonuclease released material.

One way of examining the origin of the ^{3}H-methionine labelled material released during digestion of DNP with N.crassa endonuclease, was to label cells with an amino acid that could not, in the short incubation periods employed, become incorporated into DNA. If labelled material was released during endonuclease digestion of DNP extracted from cells which had been labelled with this amino acid, then this would indicate that it arose from polypeptides. Four cultures of FLA cells (4 Flow bottles each) were labelled for 12 h with ^{3}H-(methyl)methionine (5 μCi/ml), ^{35}S-methionine (5 μCi/ml), ^{3}H-(methyl)thymidine (0.1 μCi/ml), or ^{3}H-valine (5 μCi/ml), and DNP was prepared from them. Each labelled DNP was divided into twelve lots and subjected to one of the following conditions:

pancreatic DNase I (10 μg/ml) at 0°C for 3 h

pancreatic DNase I (10 μg/ml) at 37°C for 3 h

pancreatic RNase A (10 μg/ml) at 37°C for 3 h

proteinase K (200 μg/ml) at 37°C for 30 min,
followed by 0°C for 2.5 h

N.crassa endonuclease (2.5 units/ml) at 37°C for 4 h

0.3 M KOH at 37°C for 4 h

Control incubations were done for each. At the completion of each incubation the reaction was stopped by addition of ice-cold 10% TCA and the precipitates collected by centrifugation at 10,000 g for 20 min at 4°C; TCA-soluble and TCA-insoluble radioactivities were measured and the percent digested shown in Table 4. The expected results were obtained with ^{3}H-thymidine labelled DNP treated with DNase I and the endonuclease. Of significance is the similarity

Table 4

	Percentage Release			
	^{3}H-dThyd	^{3}H-met	^{35}S-met	^{3}H-val
DNase I 0 C	89	2.3	2.0	4.8
DNase I 37 C	100	5.6	3.1	3.9
RNase A	0	2.4	0	0.2
Proteinase K	0	72.3	72.2	58.0
N.crassa endonuclease	1.5	9.8	17.3	14.7
KOH	0	5.3	8.5	8.4

between the ^{3}H-methionine, ^{35}S-methionine and ^{3}H-valine under all conditions (except perhaps RNase where methylated ribonucleotides may have accounted for the slight release with this enzyme on ^{3}H-methionine labelled DNP), particularly with the N.crassa endonuclease and proteinase K. This suggests that proteolytic action may have accounted for the previously observed high percentage release with DNP labelled with ^{3}H-methionine.

Another reason that could account for the release of ^{3}H-methionine upon digestion of DNP with N.crassa endonuclease could be that methionine-labelled proteins, specific for single-stranded DNA regions, were dissociated from the DNP once the unpaired DNA was remove by digestion. These putative ssDNA-binding proteins could have important structural and/or functional roles. That this was probably not true was shown by the fact that the N.crassa endonuclease preparations contained proteolytic activity, either as a contaminating enzyme or a minor enzymatic activity. Cells were labelled with ^{35}S-methionine as before except that proteins were prepared. Incubation with the endonuclease for 30 min at 37°C converted 30% of the proteins to acid-soluble radioactivity. (The N.crassa endonuclease had earlier been checked for proteolytic activity by incubation with bovine serum albumin (BSA). In this case the reaction was terminated with 5% perchloric acid and the absorbance at 280 nm measured. Even after 2 h at 37°C, no ultra-violet absorbing, acid-soluble material was found, which at the time, was taken to indicate the absence of proteolytic contaminants, but in light of the more recent evidence, had to be revised. The discrepancy was obviously

due to the fact that, by using ultra-violet absorbance, only those amino acids such as tyr, phe, trp were measured leaving undetected those amino acids, such as met, which do not absorb in the ultra violet).

These experiments do not eliminate the possibility that a small amount of methionine-labelled radioactivity released after digestion of DNP with N.crassa endonuclease ~~being~~ is due to ssDNA-specific proteins (digested to peptides once released), but by far the majority of released radioactivity could be accounted for by proteolytic activity.

From these experiments it was concluded that the use of ^{3}H-(methyl)methionine to label 5-MeCyt was unsuitable, due to the much higher incorporation into chromosomal proteins overshadowing the minor DNA methylation role of methionine.

Section 2. Experiments using ^{14}C-deoxycytidine to label cells.

A more specific way to follow methylation of cytosine residues in DNA would be to label cells with ^{14}C-deoxycytidine, hydrolyse the isolated DNA to bases, separate these bases by chromatography and compute the percent 5-MeCyt of 5-MeCyt + cytosine, as was first done by Kappler (1970).

(a) Labelling of cells with ^{14}C-deoxycytidine analysis of nucleotides labelled.

It first had to be ascertained that FLA cells could incorporate deoxycytidine into their DNA as well as they could thymidine, and that no redistribution of the label occurs.

FLA cells were labelled for 12 h with ^{14}C-deoxycytidine (uniformly labelled) at 0.015 μCi/ml, the cells harvested and their DNA extracted. Base analysis, using the formic acid method, revealed an unexpected finding: that not all the label appeared as either cytosine or 5-MeCyt, but a significant amount, between 10 and 20%, appeared as thymine. Such an appearance of 'minor thymine' (Scarano et al.,1967; Sneider and Potter,1969; Grippo et al.,1970) has been implicated in the control of differentiation and gene expression (Scarano,1971; Tosi et al.,1972) but is more likely to occur as a consequence of metabolic conversion of deoxycytidine to thymidine via deoxuridine, than from deamination of 5-methylcytosine to thymine at the DNA level (Sneider,1973). Indeed, by adding thymidine (10^{-4} M) to the medium, the amount of conversion of deoxycytidine to thymine was reduced, in agreement with Sneider (1973), but never eliminated entirely. This was found to be due in part to the chemical deamination brought about by formic acid hydrolysis - the 'methodological' conversion of Sneider. That this chemical deamination could not totally account for the appearance of thymine was shown by carrying out an enzymatic digestion of the DNA to nucleotides using DNase I and snake venom phosphodiesterase where a 2-4% conversion of deoxycytidine to thymidine was found. However, knowing about, and being able to account for, the conversion of deoxycytidine to thymidine, made the use of ^{14}C-deoxycytidine feasible to study DNA methylation.

(b) Digestion of ^{14}C-deoxycytidine labelled DNP with the N.crassa endonuclease.

Sister cultures of FLA cells were labelled

either with ^{3}H-thymidine (0.1 μCi/ml) or ^{14}C-deoxycytidine (0.015 μCi/ml) and DNP prepared from both. Digestions were carried out for 0, 1, 2, 3 and 4 h as previously described, the reactions stopped by addition of ice-cold 10% TCA, and the acid-soluble and insoluble radioactivity determined. Expectedly, there was no difference in the kinetics of release with DNP labelled with either ^{3}H-thymidine or ^{14}C-deoxycytidine, and was similar to that shown in Figure 2.

(c) Analysis of the products of endonuclease digestion.

DNP, either labelled with ^{14}C-deoxycytidine or ^{3}H-thymidine, was prepared and digested for 3 h with N.crassa endonuclease. The reaction was stopped by addition of ice-cold 0.01 M EDTA, pH 8 and the digested material separated from the undigested material by centrifugation at 50,000 g for 2 h in an MSE 6x5 ml rotor; the supernatant was concentrated by evaporation. Both supernatant and pellet were formic acid hydrolyzed, the resulting bases analysed by thin layer chromatography and the radioactivity in each determined. Table 5 shows a composite from about twenty actual experiments. In experiment 1 the supernatant fraction was not analyzed. By comparing the M/M+C values for the pellets with the controls in each set of experiments, it can be seen that there is a slight reduction, approximately 30%, after digestion with the N.crassa endonuclease and processing of the material. If this reduction in the amount of 5-MeCyt was due to an actual loss of this base from the DNP after digestion of the single-stranded DNA regions with the endonuclease, then an enrichment in the supernatant fraction over the control value would be expected. This was never found, by whatever method of analysis of the products

Table 5

	Percentage Radioactivity		
	M+C	T	M/M+C
Experiment 1			
Control	77.4	20.7	4.9
Endonuclease-treated pellet	78.0	20.8	3.1
Experiment 2			
Control	76.2	22.9	5.9
Endonuclease-treated pellet	74.4	22.8	4.7
Supernatant	74.1	25.9	5.0
Experiment 3			
Control	86.1	12.2	6.8
Endonuclease-treated pellet	87.7	12.1	4.6
Supernatant	82.1	13.5	5.1

All 'Experiments' were an average of at least two measurements from at least five experiments.

M 5-methylcytosine

C cytosine

T thymine

that was used, although the supernatant (digested) ratio was usually slightly higher than the pellet (undigested) ratio, the reason for which is unknown. Certainly there is no absolute localisation of 5-MeCyt residues in the regions of DNP digestible with the single-strand specific endonuclease, although these experiments do not rule out the possibility that there is slight enrichment.

The high incorporation of thymine in Experiments 1 and 2 was due to the absence of exogenous thymidine in the medium for these series of experiments. For experiment 3, thymidine was added to 10^{-5} M, and this markedly reduced the conversion of deoxcytidine to thymidine. As mentioned previously, higher levels still (10^{-4} M) further reduced the conversion.

One further observation that requires explanation is the lower values for M/M+C in both the pellet and the supernatant fractions compared to the control values. This is not understood but probably is due to the rates of chemical deamination of 5-MeCyt and cytosine being different. Lindahl and Nyberg (1974) have found that the 5-methyl derivative was deaminated four times faster than dCMP by heating at neutral pH; at acid or alkali pHs the rate of deamination increased.

(d) Conclusions.

From the results of both Chapters 4 and 5 it can be said that there exists in DNP a limited amount of single-stranded DNA (c. 1%) that is accessible to, and defined by, digestion with a nuclease specific for unpaired DNA. The hypothesis that these regions contained a high proportion – perhaps all – of 5-methylcytosine residues ~~– perhaps all –~~

has been disproven. If an enrichment does occur then it is small and not detectable by the methods used. In this light it was considered not worthwhile to continue with this work.

In addition, after this work had been completed the 'micrococcal test' of Noll, Thomas and Kornberg (1975) was published; this test provides a criterion for 'native' chromatin preparations (see Chapter 7). It thus seemed prudent to go back and test our chromatin preparations befor and after incubation in the N.crassa endonuclease buffer, with and without enzyme. The results were unambiguous. DNP preparations before being subjected to the endonuclease buffer 'passed' the micrococcal test whereas once the DNP had been subjected to the endonuclease buffer, with or without enzyme, it did not pass, in other words no bands were found. This did not necessarily mean that the chromatin structure had been disrupted out of all recognition, but could be a consequence of the microccocal nuclease not being able to digest precipitated DNP fibres whereas the N. crassa endonuclease and DNase I could.

Having decided that there was no significant enrichment for 5-methylcytosine residues in the unpaired regions of the DNA within isolated chromatin, it was decided, for the reasons outlined in the next chapter, to see if there was any specific localisation of 5-MeCyt within the chromatin subunit structure. This work is described in the following chapter.

CHAPTER SIX

IS THE DISTRIBUTION OF 5-METHYLCYTOSINE CORRELATED WITH THE DISTRIBUTION OF CHROMATIN SUBUNITS?

CHAPTER SIX

While the previous work was in progress there appeared a paper in the literature by Kornberg (1974) describing his hypothesis on the subunit structure of chromatin; this is discussed in Chapter 1. Furthermore, the work of Noll (1974a) on the use of micrococcal nuclease made it possible to test the hypothesis of whether 5-MeCyt distribution in the DNA was connected with the assembly of the chromatin subunits. A relationship between 5-MeCyt and subunit structure was thought worth testing owing to the following properties of both: Each subunit contains about 200 n.p. of DNA. The frequency of occurence of 5-MeCyt is about 1%, that is one 5-MeCyt residue per 100 residues or 50 n.p. But since 5-MeCyt predominantly occurs as part of the independent (see Subak-Sharpe (1969) for explanation of independent and dependent doublets) doublet C_pG then 1% in fact means one 5-MeCyt occurs per 100 n.p. This could therefore mean that there are 2 or 4 5-MeCyt residues/subunit, and these may roughly define the limits of each subunit; in other words be at the beginning and end of each subunit. If this is true, then conversion of the chromatin to monomer subunits by digestion with micrococcal nuclease might be expected to release most of the 5-MeCyt residues from the DNA. Firstly, however, it had to be ascertained how much material was released during micrococcal nuclease digestion of nuclei.

Section 1. Amount of DNA accessible to micrococcal nuclease.

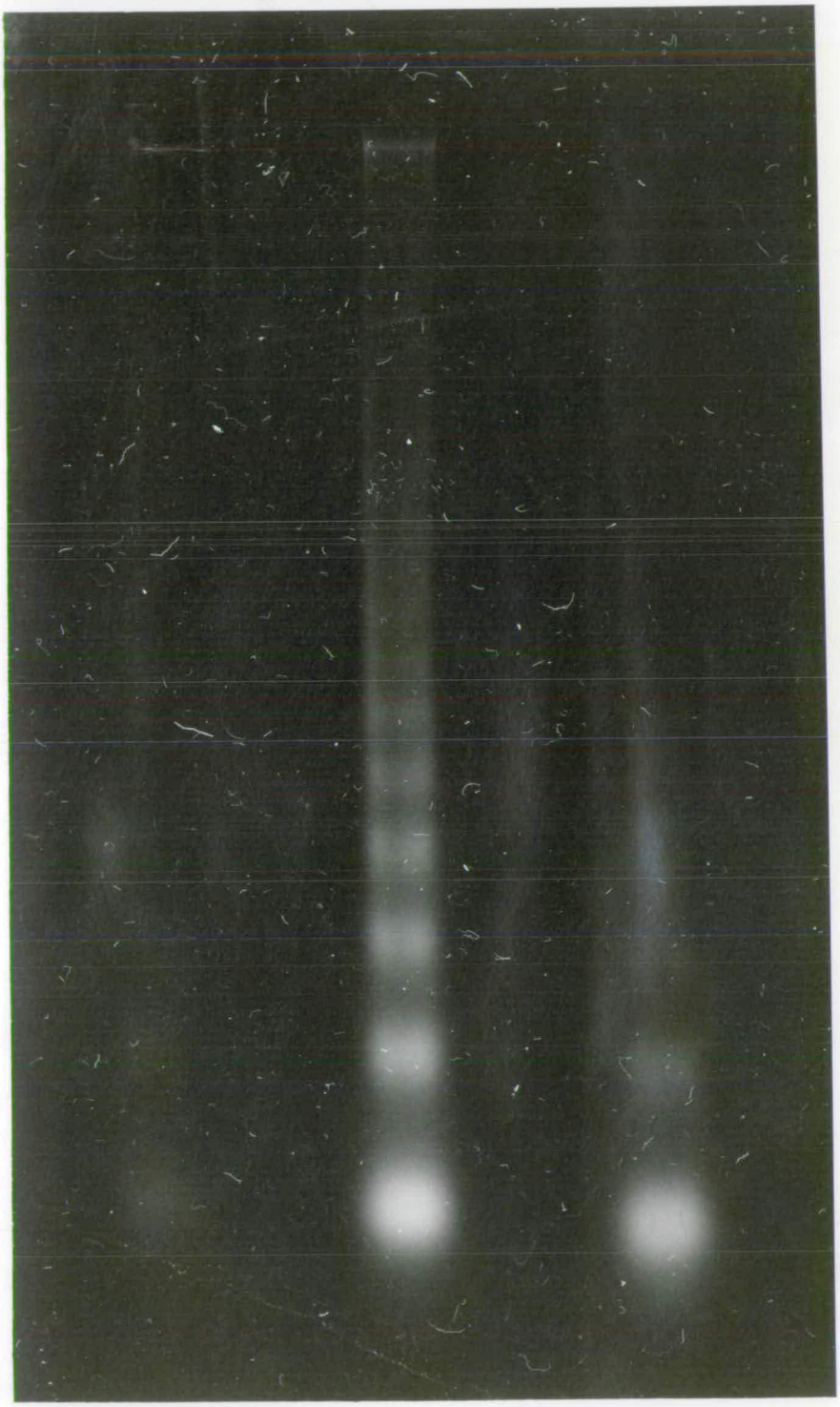
FLA cells were labelled for 3 days with ^{3}H-thymidine

(0.1 μCi/ml) and their nuclei prepared. The final nuclear pellet was resuspended in 3 ml of 10 mM Tris, pH 7.5, 1 mM $CaCl_2$ at approximately $5 \cdot 10^6$ nuclei/ml followed by digestion at 37°C with micrococcal nuclease at 0, 5, 100 units/ml for 6 min. At the completion of the incubation, duplicate 0.1 ml samples were withdrawn and each expelled into 1 ml of ice-cold 10% TCA, while the remaining 0.8 ml was made 50 mM in EDTA (pH10.5), 1 M in NaCl, 1% in SDS, briefly vortex mixed and the DNA extracted as described in Chapter 2. Both TCA-soluble and TCA-insoluble radioactivity was determined and checked against the radioactivity present in an aliquot taken from the nuclear suspension. Figure 4 shows the analysis of DNA fragments on 2% agarose gels and also the percentage TCA-insoluble radioactivity in each sample. Even when the monomer fragment is the predominant digestion product, only 18% of the nuclear DNA had been converted to TCA-soluble material (Figure 4c), whereas when many multimers were present (Figure 4b) this value was 12%. Thus it appears that about 20% of the nuclear DNA is accessible to nuclease digestion, the rest appearing as discrete fragments, in agreement with others (Noll,1974a; Honda et al.,1974).

Section 2. Distribution of 5-methylcytosine residues in chromatin.

FLA cells were labelled for 24 h with ^{14}C-deoxycytidine (0.015 μCi/ml) and their nuclei prepared as described in Section 1. Nuclei were digested with micrococcal nuclease at 0, 10, 100 and 500 units/ml for 6 min at 37°C, after which the DNA was prepared as described in Chapter 2; both the method with pronase and without pronase gave comparable results. The digestion pattern is shown in

Figure 4. 2% Agarose Gel Electrophoresis of DNA Fragments Isolated from ^{3}H-thymidine Labelled FLA Nuclei Digested with Micrococcal Nuclease.
After incubation at 37°C for 6 min, aliquots (0.1 ml) were withdrawn and both TCA-soluble and TCA-insoluble radioactivities measured. The remaining mixture was made 50 mM in EDTA, pH 10.5, 1 M in NaCl, 1% in SDS, briefly vortex mixed and the DNA extracted as described in Chapter 2; the percentage TCA-soluble radioactivities are shown. (a) 0 units/ml; (b) 5 units/ml; (c) 100 units/ml.



a b c

0% 12% 18%

Figure 4 from which it can be seen that in the higher levels of nuclease most of the DNA had been converted to monomer fragments. If aliquots of this material were hydrolysed with formic acid as described in Chapter 2, Section 9, the resulting bases separated by thin layer chromatography and the relative radioactivities of 5-methylcytosine and cytosine determined, then the results shown in Table 6 are found. It is clear that even in the most extensive digestion there is no preferential loss of 5-MeCyt residues from the DNA. In fact there appears to be an opposite effect, namely that 5-MeCyt residues are more resistant to micrococcal nuclease digestionin the chromatin, or that 5-MeCyt residues are probably located in the subunit rather than in the inter-subunit regions.

Section 3. Conclusion.

The aim of the experiments described in this chapter was to test the hypothesis that the distribution of 5-MeCyt residues in the DNA was in anyway connected with the distribution of chromatin subunits. One possibility has been eliminated, that is these residues exist primarily in the inter-subunit regions of the nucleohistone structure. A constant ratio of 5-MeCyt to 5-MeCyt pluscytosine would havemeant that the distribution was random and therefore the two things were unconnected. But since this ratio increases slightly as micrococcal nuclease digestion proceeds, the distribution of 5-MeCyt is not entirely random within the chromatin structure, although exactly what the connection is, if any, was not investigated (but see the General Discussion). In retrospect,it would seem unlikely that

Figure 5. 2% Agarose Gel Electrophoresis of the DNA Fragments Isolated from ^{14}C-deoxycytidine Labelled FLA Nuclei Digested with Micrococcal Nuclease. Conditions of incubation and method of DNA extraction are the same as for Figure 4. (a) 10 units/ml; (b) 100 units/ml; (c) 500 units/ml. The undigested sample is not shown.

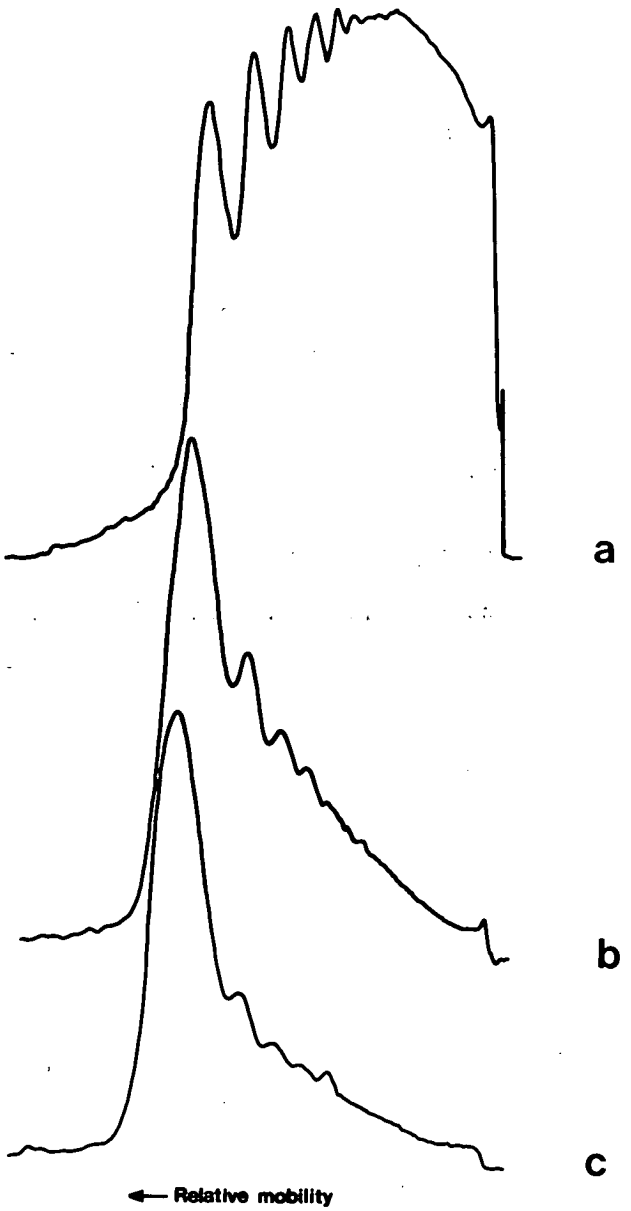

a
b
c
Relative mobility

Table 6

Digestion conditions for nuclei	$\frac{\text{5-methylcytosine}}{\text{5-methylcytosine + cytosine}}$
0 units/ml	3.6%
10 units/ml	4.2%
100 units/ml	5.2%
500 units/ml	6.6%

there is a connection between DNA methylation and formation of chromatin subunits due to the higher frequency of occurrence of 5-MeCyt in satellite DNA than in main band DNA (Fry et al.,1973; Salomon et al.,1969).

CHAPTER SEVEN

A BRIEF PHYLOGENETIC SURVEY OF THE PREVALENCE OF CHROMATIN SUBUNIT STRUCTURE.

CHAPTER SEVEN

As the work described in Chapter 6 was begun, a repeating substructure had been described for chromatin isolated from rodents (Hewish and Burgoyne,1973b; Kornberg, 1974; Noll,1974a), birds (Olins and Olins,1974) and fish (Honda et al.1974). Therefore, it was of interest to see if such a nucleohistone subunit structure existed in other animals of both higher and lower orders and in plants, as was expected. The following were considered representative: yeast (Sacchromyces cervisiae), a protozoan (Tetrahymena pyriformis), a plant (barley seed embryos), an echinoderm (sea urchins), an insect (Drosophila melanogaster), an amphibian (Xenopus laevis), a marsupial mammal (Sminthopsis crassicaudata), and the following placental mammals - mouse, kangaroo rat, and human. Where possible cell lines were used, as isolation of nuclei from whole tissues is more difficult. Fortunately cell lines existed for human (FLA, D98/AH2), mouse (L929), kangaroo rat, marsupial (Sc9), Drosophila (Schneider line 3) and Xenopus (kidney cell line) and these were tried first. Barley seeds were the plant material, and yeast and Tetrahymena were grown under standard conditions. The work on sea urchins is described separately in Chapter 9.

Section 1. Isolation of nuclei.

(a) From tissue culture lines.

It turned out that the method described in Chapter 2 for FLA cells was applicable to all cell lines except Drosophila, where the high pH method of Wray et al.

(1972) as modified by Hanson and Hearst (1973) was used. Schneider line 3 cells were grown in Schneider's Drosophila medium supplemented with 20% foetal bovine serum at 25°C. The harvested cells were washed twice at room temperature in 3-4 vol of 0.05 M sucrose, 0.33 M 2-methyl,2,4-pentanediol (hexylene glycol), 0.0013 M $CaCl_2$ and 0.001 M cyclohexylamino propane sulphonic acid (CAPS) titrated to pH 10 by addition of solid $Ca(OH)_2$. After washing, the cell pellet was resuspended in one-half vol of buffer, cooled to 0°C, and an equal vol of cold buffer containing 1.5% Nonidet P-40 was added to lyse the cells. Dounce homogenisation (15 up-and-down strokes with a B pestle) produced an homogenate containing interphase nuclei, metaphase chromosomes and cellular debris, from which the nuclei were selectively removed by centrifugation at 1000 g for 15 min. The resulting nuclear pellet was washed twice in the buffer and digested in the same buffer. (For the experiment described here the nuclear pellet was a generous gift from Ms P.Mounts.)

(b) From barley seed embryos.

Nuclei were prepared from barley seeds by the unpublished method and with the guidance of Dr.A.J.Trewavas, Department of Botany, University of Edinburgh.

Barley seeds (20 g) were soaked in 50% sulphuric acid (v/v) for 2 h at room temperature to soften the seed coat. The seeds were then washed extensively in tap water, using a Buchner funnel, to remove the acid. Firm rubbing of the seeds between the fingers was necessary to dislodge the husks which could then be washed away with the tap water. The seeds were soaked in distilled water overnight

at 4°C after which time the embryos could be easily removed using a spatula. From 20 g of seeds almost a gram of embryos (wet weight) was obtained. All succeeding operations were carried out between 0 and 4°C. The embryos were suspended in 10 ml of 0.3 M sucrose, 0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$ and homogenized in a mechanical homogeniser of the Potter-Elvehjem type with a loose fitting pestle. Twenty passes were necessary to produce an homogenate which was filtered through two layers of buffer-saturated Miracloth (layered flat on a funnel) and washed three times with the same buffer. The filtrate contained about 60% of the nuclei. A second homogenisation of the residue in the same buffer using a tight fitting pestle disrupted the remaining cells producing a second nuclear suspension which was again filtered through two layers of buffer-saturated Miracloth and the residue washed twice with buffer. The two nuclear suspensions were combined, made 1% in Triton X-100 and centrifuged at 275 g for 6 min at 4°C to pellet the nuclei. At this stage protein storage bodies were the major contaminant. The supernatants were decanted, the pellets resuspended in the 0.3 M sucrose buffer (1.25 ml per tube) and the combined nuclear suspensions pelletted through a 1 M sucrose cushion (in the same buffer) at 1000 g for 10 min. After decanting the supernatant, the pellet containing the nuclei were resuspended in 20 ml of 0.01 M Tris-HCl, pH 7.5, 0.001 M $CaCl_2$, centrifuged at 1000 g for 5 min and the pellet resuspended in 0.01 M Tris-HCl, pH 7.5, 0.001 $CaCl_2$ ready for digestion.

(c) From Tetrahymena pyriformis.

Tetrahymena were cultured in 2% proteose

peptone using standard methods such as those described in Everhart (1972). Nuclei (both macronuclei and micronuclei) were isolated according to the method of Lee and Byfield (1970) as modified by Engberg and Pearlman (1972) and resuspended in their nuclei buffer (0.01 M Tris-HCl, pH 7.4, 0.002 M $CaCl_2$, 0.0015 M $MgCl_2$, 0.1 M sucrose) for digestion with micrococcal nuclease.

(d) From yeast.

Nuclei were isolated by lysing spheroplasts with Triton X-100 as described by Mitchison et al. (1973). The lysed spheroplasts were kindly prepared by Dr.R.S.S. Fraser, Department of Zoology. The nuclei were pelleted by centrifugation at 1000 g for 20 min at 4°C, washed twice in 0.01 M Tris-HCl, pH 7.4, 0.001 M $CaCl_2$, and digested in the same buffer.

Section 2. Digestion of nuclei with micrococcal nuclease.

Nuclei at approximately $5 \cdot 10^4$ per ml were digested with increasing concentrations of micrococcal nuclease for 6 min at 37°C, the reaction stopped by addition of EDTA Na_4 (to 50 mM), NaCl (to 1 M) and SDS (to 1%,w/v), and the DNA fragments purified and analysed by agarose gel electrophoresis as described in Chapter 2. All enzyme concentrations used are given in the figure legends. Figure 6 (a-d) shows a comparison of the DNA fragments obtained after nuclei prepared from human (HeLa) cells, marsupial (Sc9) cells, barley embryos and kangaroo rat cells, respectively, were digested with micrococcal nuclease. Only partial digests are shown for comparison. Figure 7 (a-c) shows the results of similar experiments with mouse (L) cells, Xenopus kidney

Figure 6. Analysis by Electrophoresis on a 1.8% Agarose Gel of the DNA Fragments Isolated from Micrococcal Nuclease Digested Nuclei from Human (HeLa) Cells, Marsupial (Sc9) Cells, Barley Embryos, and Kangaroo Rat Cells.

Nuclei were prepared either as described in Chapter 2 (tissue culture lines) or in Section 1 of this chapter (barley embryos). Digestions were for 5 min at 37°C at the following enzyme concentrations:

(a)	human cells	20 units/ml
(b)	marsupial cells	20 units/ml
(c)	barley embryo cells	50 units/ml
(d)	kangaroo rat cells	50 units/ml

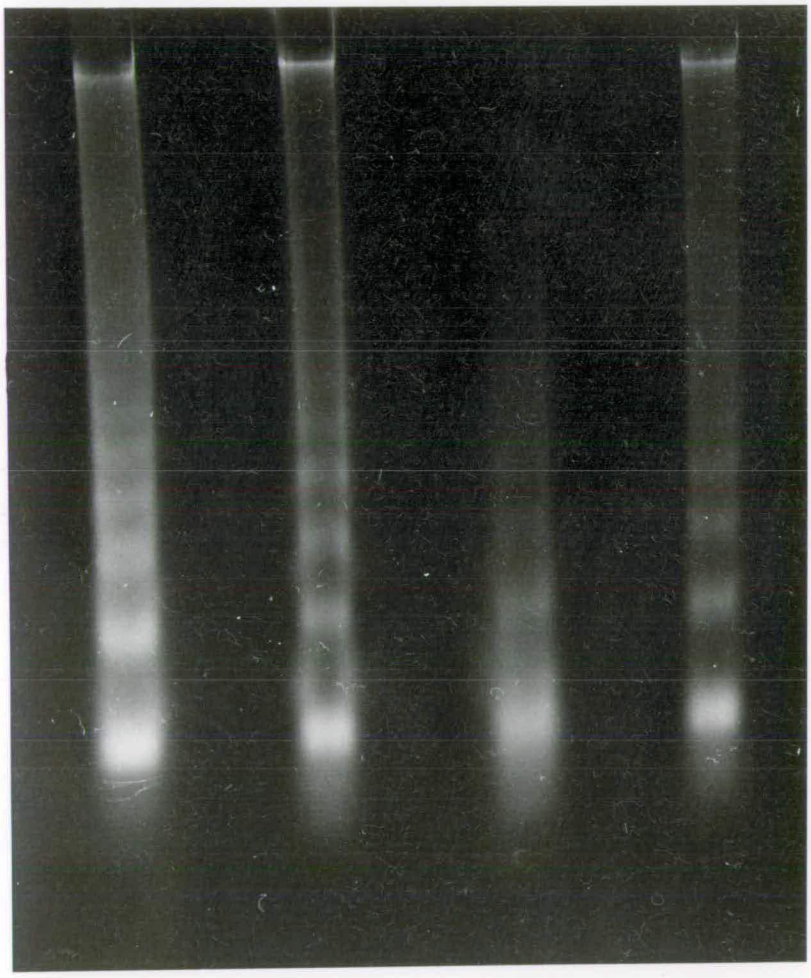

a
b
c
d

Figure 7. Analysis of the DNA Fragments from Mouse L Cell Nuclei, Xenopus Kidney Cell Nuclei and HeLa Cell Nuclei After Digestion with Micrococcal Nuclease. Digestions and purification of the DNA were done as described for Figure 6.

(a)	L cell	0 units/ml
(b)	L cell	10 units/ml
(c)	L cell	50 units/ml
(d)	L cell	100 units/ml
(e)	Xenopus cell	0 units/ml
(f)	Xenopus cell	10 units/ml
(g)	Xenopus cell	50 units/ml
(h)	Xenopus cell	100 units/ml
(i)	HeLa cell	50 units/ml

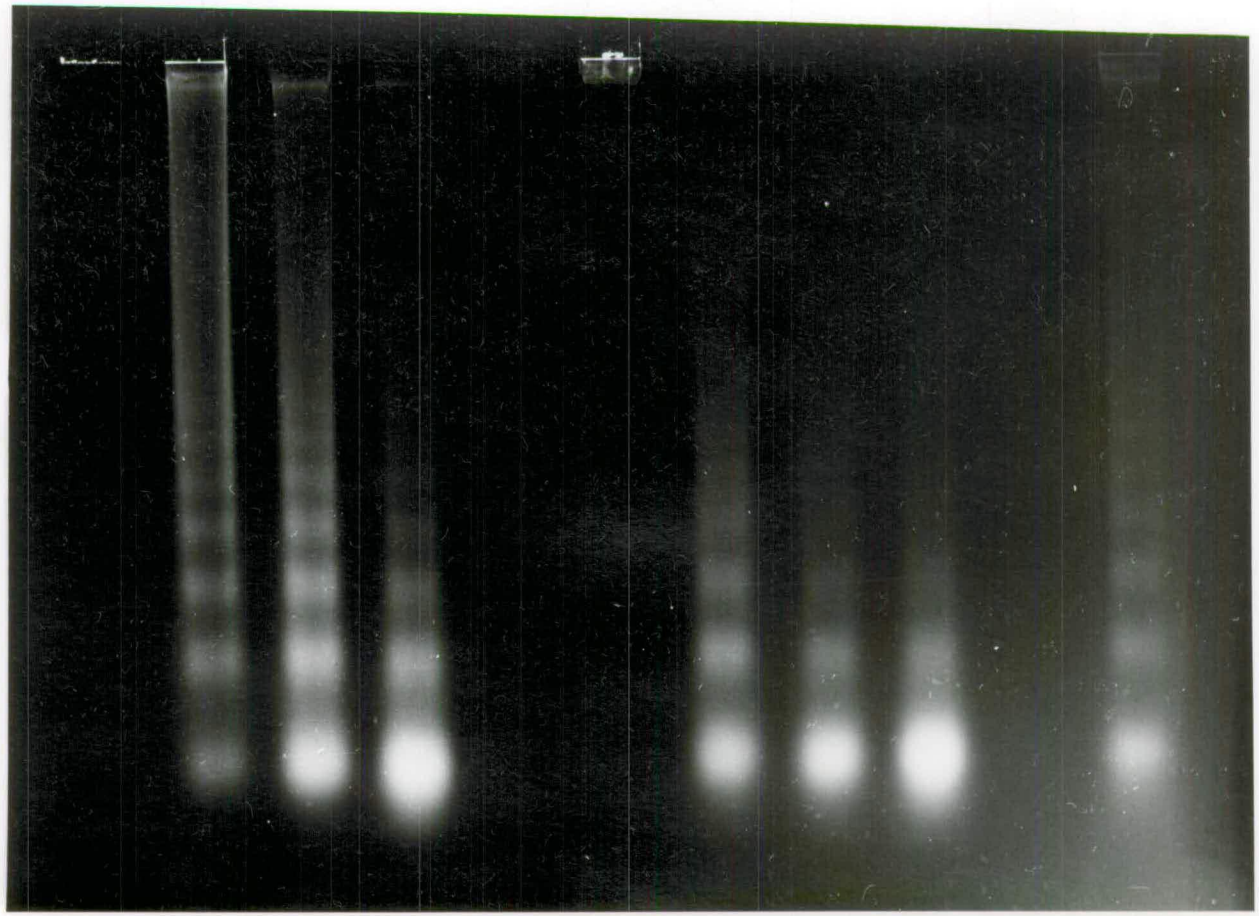

a
b
c
d
e
f
g
h
i

Figure 8. Micrococcal Nuclease Digestion of Drosophila Cell Nuclei.

Nuclei were isolated as described in Section 1 and digested for 5 min at either 0°C or 37°C at various enzyme concentrations. (a) 0 units/ml at 0°C; (b) 0 units/ml at 37°C; (c) 50 units/ml at 37°C; (d) 100 units/ml at 37°C.


d
c
b
a

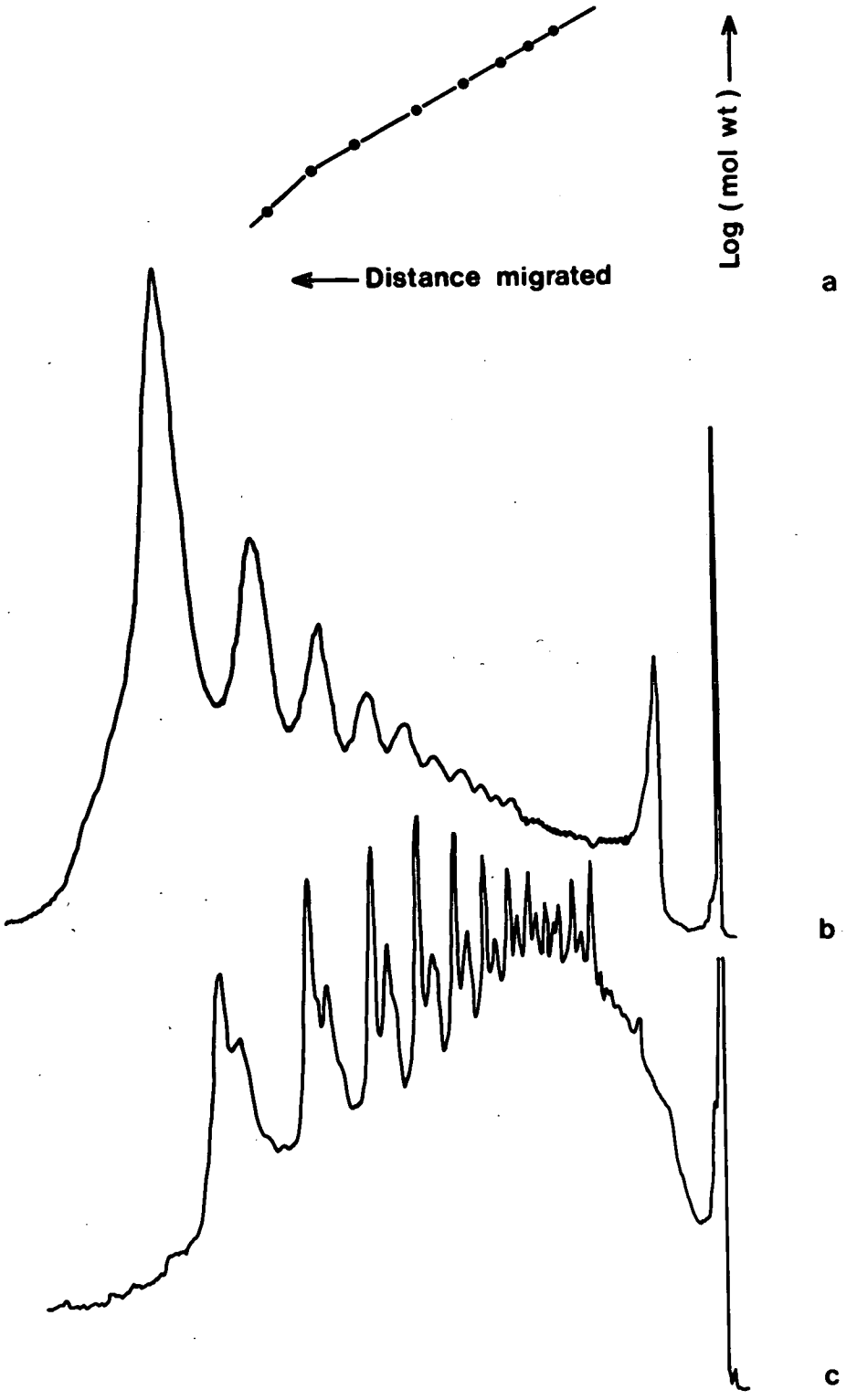
cells and human (HeLa) cells. And Figure 8 shows Drosophila cell nuclei subjected to increasing concentrations of nuclease.

An estimate of the size of the fragments was obtained by comparing the electrophoretic mobility of the DNA fragments arising through micrococcal nuclease digestion of nuclei to the electrophoretic mobility of DNA fragments produced by restriction endonuclease digestion of mouse satellite DNA (Figure 9). $EcoR_{II}$ cleaves mouse satellite into a regular series of bands, the smallest of which is 240 $\pm$ 20 n.p. in length (Southern,1975a). Thus, knowing that electrophoretic mobility in agarose gels is inversely proportional to the log of the molecular weight, a value of 170 $\pm$ 30 n.p. was calculated for the DNA fragment contained in the chromatin subunit monomer; from the larger DNA fragments, slightly higher values were calculated for the monomer, for example the 7-mer gave a value of 185 $\pm$ 15 n.p. for the monomer. The lower value is probably due to exonucleolytic attack. Such values are in agreement with other published values (Noll,1974a; Oudet et al.,1975).

The most striking feature of the results shown in Figures 6-8 is the similarity of both the pattern obtained and the size distribution of the DNA fragments. This argues in favour of the subunit model for nucleohistone being essentially universal in type, although minor tissue or species variations not detected by nuclease digestion (a rather imprecise method) may of course exist. This nucleohistone structure remains intact at pH 10 as demonstrated by the fact that Drosophila nuclei prepared and digested at this pH yield similar repeating structures.

Figure 9. Calculation of the Size of the DNA Fragments Purified from HeLa Nuclei Digested with Micrococcal Nuclease.

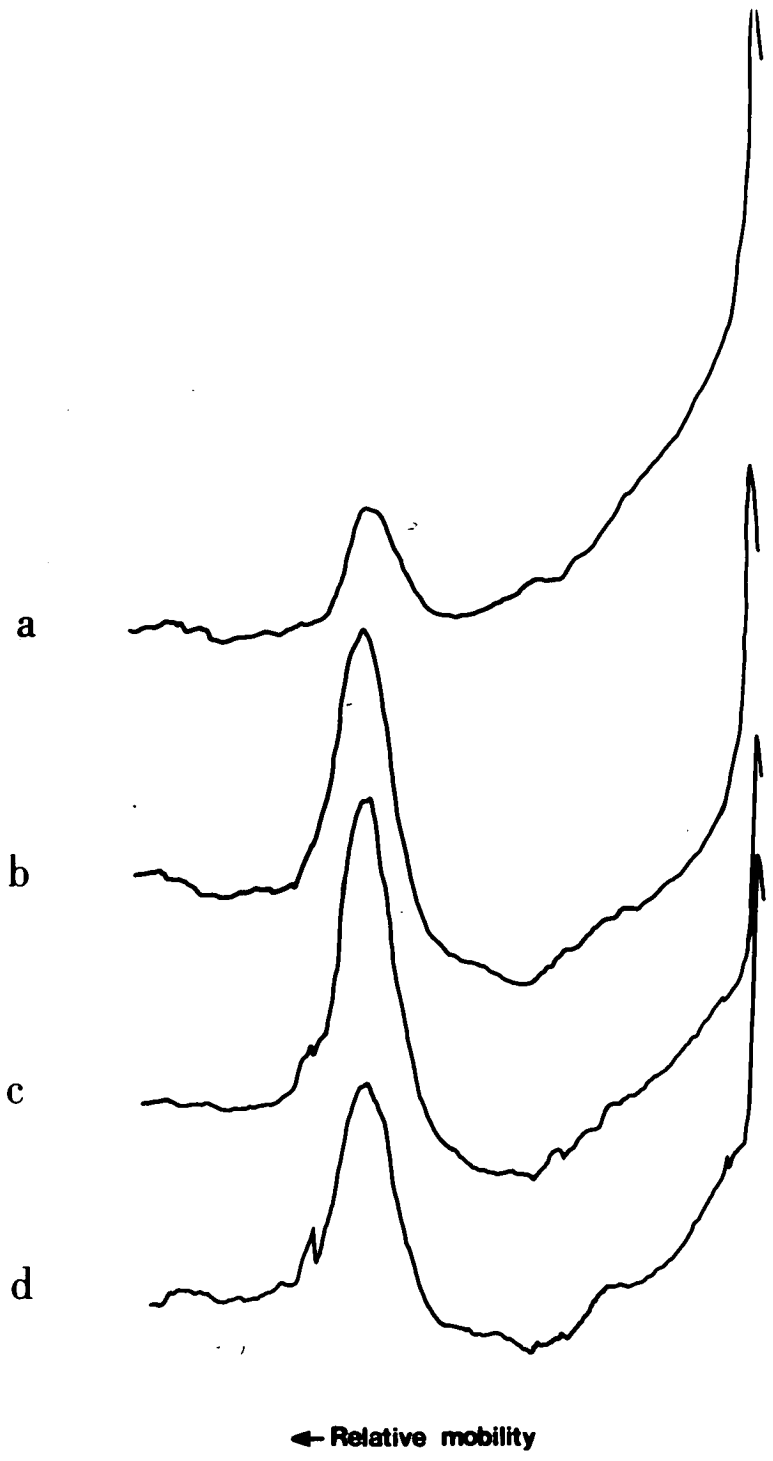
(a) shows a plot of log molecular weight against distance migrated, obtained from the gel shown in (b) which is a partial digest of HeLa nuclei (20 units/ml of micrococcal nuclease for 5 min at 37°C). Sizes were calculated from a partial digest of purified mouse satellite DNA digested with Endo R.Eco RII (c) where the monomer has been estimated as 240 ± 20 n.p. in length (Southern,1975a).


Log (mol wt)
Distance migrated
a
b
c

Yeast nuclei, either with exogenous micrococcal nuclease or without added enzyme, produced only a monomer band of around 170 n.p. more heterogeneous than found in the higher eukaryotes (Figure 10). Subsequently Lohr and van Holde (1975) showed that nuclei or chromatin prepared by the method of Wintersberger et al. (1973) yielded bands but of different sizes (approximately 140 n.p., 300 n.p., 450 n.p.) than those obtained with higher eukaryotes. This could be related to the fact that yeast is ~~difficient~~ deficient in H1 and possibly H3 (Wintersberger et al.,1973; Franco et al.,1974), or to proteolytic activity rapidly degrading histones in the chromatin once the cells have been lysed or during lysis. (This proteolytic activity may account for the hitherto failure to detect H1 or H3.) In any case it was decided not to continue work on yeast.

Tetrahymena nuclei with or without digestion with micrococcal nuclease, yielded a smear when the DNA was analyzed on agarose gels. At high levels of micrococcal nuclease, heterogeneous DNA fragments of approximately 200 n.p. were obtained. Superimposed upon this were very faint bands (only visible on the negative, hence not shown) which may be due to either residual native chromatin structure, the rest having been disrupted during nuclear isolation, or less likely perhaps, to the macronuclear DNA (amitotic replication) being packed in a different structure than the micronuclear DNA (mitotic replication). This intriguing possibility could not be followed due to lack of time. Recently, Gorovsky and Keevert (1975a) have shown that H1 is absent in the mitotically dividing, transcriptionally inactive micronucleus, whereas H1 is present in the amitotically

Figure 10. Micrococcal Nuclease Digestion of Yeast Nuclei. Nuclei were prepared as described in Section 1 and digested at (a) 0 units/ml, (b) 20 units/ml, (c) 50 units/ml, (d) 100 units/ml, for 5 min at 37°C.


a
b
c
d
← Relative mobility

dividing, transcriptionally active macronucleus.

Section 3. Conclusions.

From the results presented in this chapter it can be seen that the presence of a repeating structure in chromatin, as defined by nuclease digestion, is a widespread phenomenon, probably occurring in all organisms where a nucleohistone complex is the genetic material, at least in all higher eukaryotes. It has now been shown to occur in the following organisms: plants (this chapter; McGhee and Engel,1975), sea urchins (Chapter 9); fish (Honda et al.,1974); insects (this chapter); amphibia (this chapter); birds (Olins and Olins, 1974; Oudet et al.,1975; Olins et al.,1975); marsupial mammals (this chapter); and placental mammals (Hewish and Burgoyne,1973b; Burgoyne et al., 1974; Kornberg and Thomas, 1974; Noll,1974a; Baldwin et al.,1975; Oosterhof et al.,1975; Olins et al.,1975; and this chapter). The results with the unicellular eukaryotes are less clear cut at present although these too will probably turn out to have similar repeating structures once better methods of nuclei preparation are devised.

One interesting fact of the results presented in this chapter is the finding that a genetically inert nucleus - the barley embryo - yields a similar pattern of chromatin fragments as a genetically active nucleus - the tissue culture nucleus. This opens up the important question of what types of DNA sequences are packed in the repeating substructure. Work in the following chapter is designed to investigate this question.

CHAPTER EIGHT

THE DISTRIBUTION OF SATELLITE DNA AND RIBOSOMAL DNA WITHIN INTERPHASE CHROMATIN.

CHAPTER EIGHT

From the results presented in Chapter 7 it appears likely that a repeating chromatin substructure (Hewish and Burgoyne,1973b; Kornberg,1974) exists in every organism that contains a nucleohistone complex as its genetic material. It was shown in the last chapter (in agreement with Noll,1974a) that digestion of nuclei with micrococcal nuclease cleaved the DNA at regularly spaced vulnerable sites releasing a maximum of 20% of the DNA as TCA-soluble material. This 20% acid-soluble material presumably arises, at least in part, by digestion of the intersubunit nucleotides. That 80% or more of the nuclear DNA is protected from nuclease digestion suggests that all kinetic classes of DNA are represented in ~~this structure~~ the subunits. However, it is possible that some types of DNA sequences are more accessible to nuclease digestion than others (thus appearing in the 10-20% acid-soluble fraction) and it is necessary, therefore, to identify and measure specific sequences in the fragments of DNA extracted from nuclei digested with micrococcal nuclease. This chapter is concerned with an examination of the distribution of satellite DNA and ribosomal DNA among the fragments produced from interphase chromatin.

Section 1. The distribution of satellite DNA.

To obtain some preliminary evidence on whether satellite DNA was packed in the repeating chromatin structure it was decided to look at a cell line from the kangaroo rat, Dipodomys ordii, where satellite DNA in this species represents approximately 60% of the nuclear DNA (Hatch and Mazrimas,

1970). If on digestion of kangaroo rat nuclei with micrococcal nuclease only about 20% of the DNA was converted to acid-soluble material, then this must mean that at least some satellite sequences were present in the chromatin subunits.

(a) Digestion of kangaroo rat nuclei with micrococcal nuclease.

For a comparison the following cell lines were used: mouse L929 cells (8% satellite; Waring and Britten, 1966; Bond et al.,1967; Flamm et al.,1967), human FLA cells (6% satellite; Corneo et al.,1968,1970,1971; Jones et al., 1970) and kangaroo rat (60% satellite; Hatch and Mazrimas, 1970). All three cell lines were labelled for 3 days with ^{3}H-thymidine (0.1 μCi/ml), their nuclei isolated as described in Chapter 2, and digested with micrococcal nuclease at 150 units/ml for 6 min at 37°C. At the completion of digestion, two 0.1 ml aliquots were removed from each digestion and precipitated by addition of ice-cold 5% TCA (1 ml). From the remainder DNA was purified and analysed by electrophoresis on a 1.8% agarose slab gel as described in Chapter 2. This analysis (not shown) confirmed that the monomer DNA fragment (180 ± 30 n.p.) was the predominant product of the digestion. Both TCA-soluble and TCA-insoluble radioactivity was determined and a comparison between the three species shown in Table 7. Since kangaroo rat contains 60% satellite while 82% of the nuclear DNA occurs as the monomer fragment after micrococcal nuclease digestion, this must mean that most of the satellite DNA sequences of this species are packed in a repeating chromatin subunit structure. This cannot yet be said for the mouse (8% satellite) and therefore a direct method of analysis was sought.

Table 7

The percentage radioactivity remaining in 'monomer' DNA fragments after digestion with micrococcal nuclease

Organism	% Satellite	% in monomer	Number of estimates
Kangaroo rat	56	82	4
Mouse	8	83	5
Human	6	80	8

(b) Analysis of mouse satellite DNA in the DNA fragments produced by micrococcal nuclease digestion of nuclei.

(i) Using the filter transfer method.

The work described in this part was done in collaboration with Drs. R.J.Shmookler-Reis and P.R. Musich.

A more direct method was considered to be RNA/DNA hybridisation on nirocellulose filters. This could be accomplished by partially digesting L cell nuclei with micrococcal nuclease, purifying the DNA fragments and separating them by agarose gel electrophoresis. Using the transfer procedure developed by Southern(1975b) and which is described in Chapter 2, DNA fragments can be transferred from agarose gels onto nitrocellulose filters producing a filter replica. This is accomplished by first denaturing the DNA fragments in situ in alkali, neutralising, and eluting the single-stranded fragments out of the gel onto nitrocellulose filters on which they bind (Gillespie and Spiegleman,1965). Once, produced, this filter-replica can be used in standard filter hybridisation reactions.

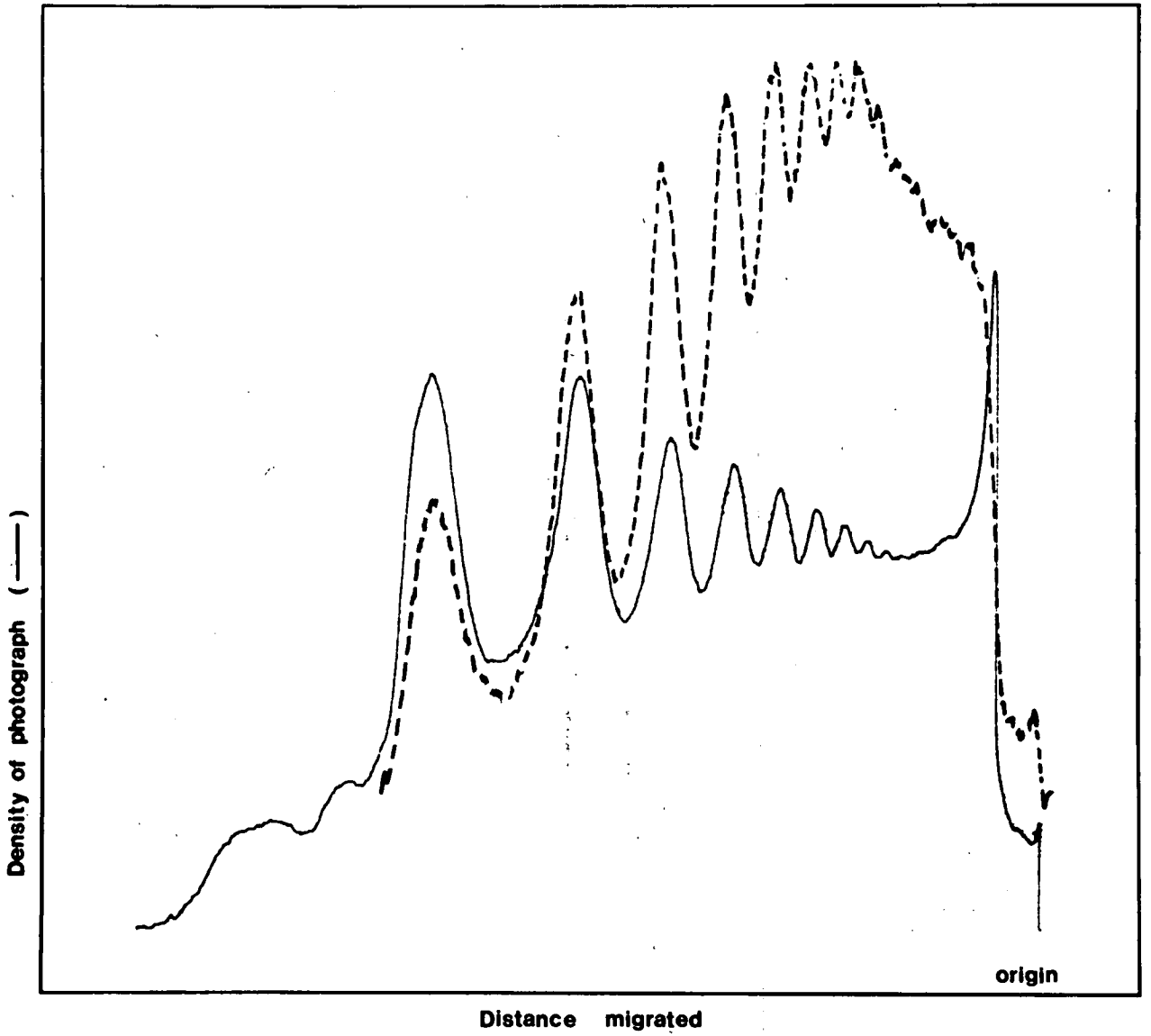
Unlablelled L cell nuclei were prepared, digested with micrococcal nuclease and their DNA purified and separated on 1.8% agarose gels as described in Chapter 2. After the DNA bands were stained with ethidium bromide and photographed, they were transferred to a nitrocellulose filter. Instead of using labelled mouse satellite DNA for the hybridisation, it was thought preferable for technical reasons to use complementary RNA (cRNA) transcribed from purified satellite using E.coli RNA polymerase in the presence of ^{32}P-UTP as

described by Biro et al. (1975): firstly, a higher specific activity is possible with satellite cRNA than with in vivo labelled satellite DNA; and secondly, since the hybridising nucleic acid would be single-stranded DNA, these molecules could also bind to the nitrocellulose filter themselves obliterating the hyridisation reaction. This latter problem has been overcome by Denhardt (1966) who found that by treating the filter with albumin before the hybridisation eliminated the non-specific binding of single-stranded DNA to the nitrocellulose filter. The satellite DNA had been purified on Ag-Cs_2SO_4 gradients by Dr.R.J.Shmookler-Reis. ^{32}P-satellite cRNA was hybridised to the filter in 5xSSC containing 0.2% SDS at 70°C to a Rot value (Bishop,1972a) of $5 \cdot 10^{-3}$ mole nucleotides.sec/litre; such a Rot value would only permit repeated sequences to hybridise. The filter was washed five times with 25 ml of 5xSSC at room temperature; after drying overnight at 37°C the filter was autoradiographed on Kodak RP Royal X-omat film. Microdensitometer tracings of both autoradiograph and the photograph of the original gel are shown in Figure 11; scintillation counting of the sectioned nitrocellulose filter confirmed the linear relationship between autoradiographic exposure and radioactivity bound.

From Figure 11 several points are immediately evident. There is a gradual decrease in radioactive intensity, relative to ethidium bromide fluorescence, as the molecular weight of the fragments decreases, particularly apparent below the pentamer. This is discussed further below. There is a lack of hybridisation of satellite ^{32}P-cRNA near the origin of the gel; this is known to be due to the high molecular

Figure 11. Hybridisation of Satellite ^{32}P-cRNA Across a 1.8% Agarose Gel of DNA Isolated from a Partial Micrococcal Nuclease Digestion of Mouse L Cell Nuclei.

Nuclei were prepared from mouse L cells and digested with micrococcal nuclease at 30 units/ml for 6 min at 37°C. DNA was extracted as described in Chapter 2, and electrophoresis carried out on a 1.8% agarose slab gel in E buffer at 2 volts/cm for 24 h at room temperature. The gel was then stained with ethidium bromide (0.5 µg/ml) for 30 min in the dark and photographed under ultra-violet light with a red filter using Ilford FP4 film. Negatives were scanned in a Joyce-Loebl microdensitometer. After photography, the DNA was transferred from the gel to a nitrocellulose filter (Southern,1975b). ^{32}P-cRNA, prepared by transcribing mouse satellite DNA with E.coli RNA polymerase (Biro et al.,1975), was hybridised to this filter in 5xSSC, 0.2% SDS at 70°C to a Rot value of 5.10^{-3} mole nuc. sec/ litre. The filter was washed 5 times in 5xSSC at 70°C, once in 2xSSC, followed by RNase A treatment (20 µg/ml) at 37°C for 30 min, and finally 4 more washings in 2xSSC. It was dried and autoradiographed. Densitometer tacings of the original gel and autoradiograph are shown.


Density of photograph (——)
Density of autoradiograph (----)
origin
Distance migrated

weight DNA containing satellite sequences forming networks, which are not transferred from the agarose gel (Southern, 1975b). Lastly, since the radioactive profile follows the ethidium bromide fluorescence profile (ignoring for the moment the question of amplitude), this must mean that mouse satellite sequences are packed in the repeating chromatin subunit structure, confirming the kangaroo rat results by a more direct method.

The gradual decrease in radioactive intensity compared to the fluorescence intensity of the fragments smaller than the pentamer in Figure 11, could be due to several technical reasons. It has been found that there is a decrease in both the rate and extent of hybridisation with low molecular weight DNA (E.M.Southern, personal communication). Furthermore, low molecular weight hybrids may be preferentially lost from nitrocellulose filters during hybridisation (Melli and Bishop,1970). Either or both of these effects could adequately account for the apparent reduced hybridisation found with the lower molecular weight DNA fragments. It is, however, also possible that the lower molecular weight fragments are actually deficient in satellite DNA. This deficiency could arise if heterochromatin, which contains satellite DNA (Jones,1970; Pardue and Gall,1970), is more resistant to micrococcal nuclease than euchromatin. If this is true, then the higher molecular weight fragments would be expected to be enriched in satellite DNA. This has been shown to be the case in the kangaroo rat, Dipodomys ordii, where analytical CsCl gradient centrifugation has demonstrated that DNA from higher multiples (6-mer, 10-mer, and the DNA excluded from the agarose gel) obtained from a

partial nuclear digestion with micrococcal nuclease consists mainly of satellite and not main band DNA (C.J. Bostock, personal communication).

At the time it was thought that this latter type of experiment would be less convincing with mouse which has only 8% satellite (Waring and Britten,1966; Bond et al., 1967; Flamm et al.,1966) and for this reason alternative RNA/DNA solution hybridisation was attempted.

(ii) DNA excess hybridisation in solution.

DNA excess reactions using labelled tracer RNA can be used to ascertain the reiteration frequency in the genome of the hybridising RNA (Melli et al., 1971; Bishop,1972a,b). If the hybridising RNA is ^{32}P-cRNA transcribed from purified satellite DNA, then the reiteration frequency in the genome will be effectively that of satellite DNA. The slower rate of hybridisation compared to the rate of renaturation (Bishop,1972b) will not be important in this case since direct comparisons will be made rather than absolute values calculated. Thus by hybridising satellite ^{32}P-cRNA to an excess of monomer DNA fragments (180 ± 30 n.p.) produced by micrococcal nuclease digestion of mouse L cell nuclei an estimate of the reiteration frequency of satellite DNA can be made. If the monomer DNA fragments are obtained from a 'complete' micrococcal nuclease digest of nuclei, then these fragments should contain all types of DNA sequences assuming no type of sequence is preferentially digested. (A complete digest is where approximately 80% of the nuclear DNA is converted to fragments 180 ± 30 n.p. in length.) If, on the other hand, the monomer fragments are obtained from a partial micrococcal nuclease

digest, then these fragments would be expected to be enriched in those types of DNA sequences present in the chromatin in structures more readily digestible with micrococcal nuclease; conversely, those types of sequences present in a more resistant type of chromatin structure would be expected to be deficient in the monomer fragments of a partial digest.

To obtain enough monomer DNA fragments to be able to carry out the above experiment, larger, preparative 1.5% agarose gels (3 cm in diameter, 30 cm in length) were used. With these gels about 500 µg of DNA can be loaded on each gel. The potential gradient was 1 volt/cm and the gels were run for about 16 h by which time the orange G marker was three quarters down the gel. The DNA was located by staining the gel in ethidium bromide (0.5 µg/ml) and viewing under the ultra-violet light. It was then possible to excise the bands required using a scalpel. To recover the DNA from the agarose gel slice, the sodium perchlorate method was used followed by absorbing the DNA onto hydroxyapatite (HAP).

To disperse the agarose, the gel was squirted through a sterile plastic syringe without a needle into a tube; the syringe was washed out with 5 M sodium perchlorate. An equal volume of 5 M sodium perchlorate, 2 mM sodium phosphate, pH 6.8 was added to the agarose, the tube stoppered and put into a water bath at 60°C. When the agarose had dissolved, an equal volume of water was added followed by approximately 1 g of HAP (prepared according to the method of Pakroppa & Mueller (1974) and washed extensively with 0.05 M sodium phosphate, pH 6.8 for equilibration and

then at 60°C with the same buffer). The tube was inverted several times and left at 60°C for 15 min. The HAP was washed twice with 2 vol of 0.05 M sodium phosphate, pH 6.8, by resuspending and centrifugation at 500 g for 5 min at 60°C. Each supernatant was assayed for ultra-violet absorbing material, but only a small and insignificant amount of DNA washed off at this stage. Elution was accomplished with one addition of 0.3 M sodium phosphate, pH 6.8 (6 ml) followed by a second elution of the same buffer (4 ml). Both eluates were combined and put at 0°C. Residual agarose was removed by centrifugation at 3000 g for 20 min at 4°C. The supernatant was dialysed overnight at 4°C against 0.1 mM EDTA with one change of buffer, made 0.2 M in NaCl and ethanol precipitated overnight at -20°C. The DNA precipitate was collected by centrifugation at 10,000 g for 30 min at -15°C in a Sorvall RC2B refrigerated centrifuge, dissolved in sterile water and the DNA concentration determined by ultra-violet absorption.

For these experiments the ^{32}P-cRNA transcript was a generous gift of P.A.Biro and was purified by phenol extraction and ethanol precipitation as described by Biro et al. (1975).

Cot values were calculated from the formula Cot = g/ml x t/5500 and covered the range from $5 \cdot 10^{-4}$ to 1. Hybridisations were carried out in 2xSSC with 0.2% SDS at 65°C and done in sealed capillary tubes. All solutions had been treated with diethylpyrocarbonate. At the completion of the Cot point, each tube was cooled quickly to 0°C and then diluted into 10 ml of 2xSSC at 0°C. These tubes were stored on ice until all points had been completed. To determine

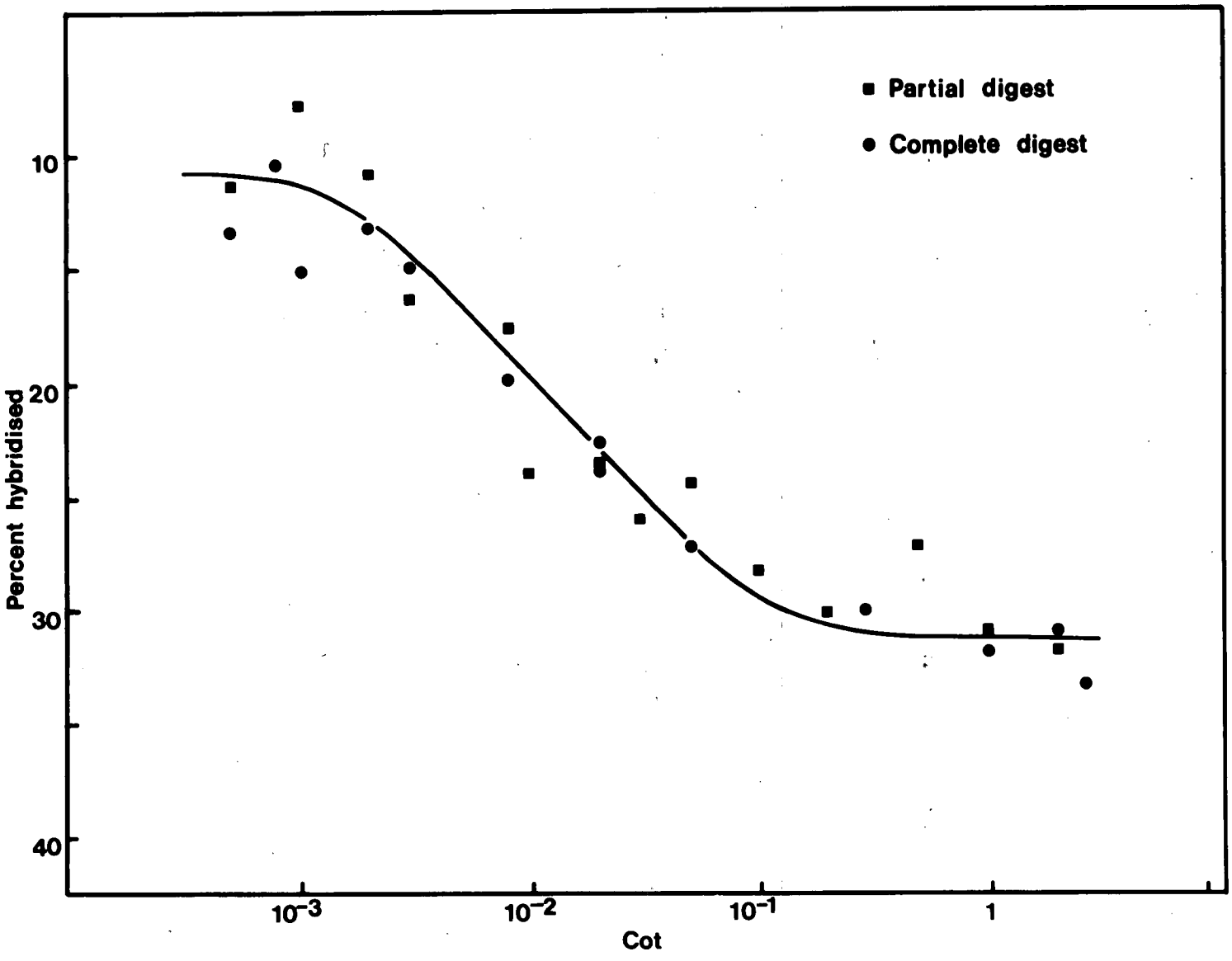
the fraction of the RNA that had hybridised each tube was divided into two 5 ml aliquots: to one was added bovine serum albumin to 50 µg total followed by one fifth volume of 50% TCA; to the other was added RNase A to 20 µg/ml and incubated at 37°C for 30 min bebore addition of serum albumin (to 50 µg) and precipitation with TCA. All precipitates were collected on Whatman GF/C filters washed with 5% TCA (20 ml), ethanol:ether (1:1,v/v;10 ml) ether (10 ml) and finally dried in an oven at 100°C for 10 min before counting in a liquid scintillation spectrometer. All washing solutions were kept on ice.

The results shown in Figure 12 are plotted in the standard way with percentage hybrid on the ordinate and Cot along the abscissa; the data have not been normalised. What is apparant from Figure 12 is the lack of significant difference between the $Cot_{\frac{1}{2}}$ values for satellite sequences between the monomer DNA fragments derived from a partial micrococcal nuclease digest and from a complete digest. Given that a one to two fold difference may not have been detected by this method, it seems that there is no marked deficiency of satellite sequences in the monomer of a partial digest compared to the monomer of a complete digest. This argues against heterchromatin being much more resistant to micrococcal nuclease than euchromatin.

If RNA excess hybridisation is used then the initial rate of reaction will be dependent upon the proportion of complementary satellite DNA sequences in the DNA if a constant amount is used. Similarly by this method no differences were found between the monomer DNA fragments obtained from a complete digest and those from a partial digest.

Figure 12. DNA Excess Hybridisation of Mouse Satellite ^{32}P-cRNA to the Monomer DNA Fragments Produced from either a Partial Nuclease Digestion or a 'Complete' Nuclease Digestion of Mouse L Cell Nuclei.

DNA was purified by agarose tube gel electrophoresis and extracted from the gels by a modification of the method of Fukes and Thomas (1970) which is described in the text. Cot values were calculated from the approximate formula Cot = g/ml x t/5500.


Partial digest
Complete digest
Percent hybridised
10
20
30
40
10^{-3}
10^{-2}
10^{-1}
1
Cot

The conclusion, then, must be that if there is a difference in the accessibility of satellite DNA in heterochromatin to micrococcal nuclease, then the methods used are not sensitive enough to detect it.

Section 2. The distribution of ribosomal DNA.

The work described in this part was done in collaboration with Dr.R.J.Shmookler-Reis.

The method described in Section 1 (b) for the detection of satellite DNA sequences in the DNA fragments produced by micrococcal nuclease digestion of isolated nuclei can also be applied to the detection of other repeated sequences, for example the ribosomal genes which are repeated about 500 times per haploid mammalian genome (Brown and Weber,1968; Jeanteur and Attardi,1969).

(a) Preparation of ^{32}P-rRNA from L cells.

L cells were labelled with ^{32}P-orthophosphate (100 μCi/ml) for 2 days and harvested as described in Chapter 2. The cells were washed twice with Earle's balanced salt solution at 4°C and lysed by addition of 15 ml of 0.15 M sodium acetate, pH 5.9, 0.5% SDS buffer followed by an equal volume of phenol-cresol mixture (phenol 500 g; m-cresol 70 g; 8-hydroxyquinoline 0.5 g; distilled 200 ml). After two phenol-cresol extractions the aqueous layer was transferred to a clean tube and extracted with phenol-cresol once more. The RNA was precipitated by addition of ethanol (2 vol) and leaving overnight at -20°C. The precipitate, which was collected by centrifugation at 10,000 g for 30 min at -15°C, contained RNA, DNA, protein and glycogen. To remove the DNA, glycogen, and some protein, this precipitate was

extracted three times with ice-cold 3 M sodium acetate, pH 5.9, according to the method of Hastings and Kirby (1966). This procedure removed tRNA while leaving rRNA as a precipitate which was washed twice with cold 75% aqueous ethanol, and finally dried overnght in a vacuum desiccator.

To check the purity of the rRNA, the precipitate was dissolved in 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.4, 0.5% SDS (w/v), and electrophoresed on 2.5% polyacrylamide gels as described in Chapter 2. Figure 12 shows the result of a gel stained with toluidine blue. It is clear that the predominant RNA species are the 18 S and 28 S rRNAs. Thus the ^{32}P-rRNA preparation was considered pure enough to use in the filter hybridisation analysis.

(b) Analysis of ribosomal DNA in the DNA fragments produced by micrococcal nuclease digestion of nuclei.

A partial micrococcal nuclease digest of unlabelled L cell nuclei was prepared, and the resulting DNA fragments purified and separated by electrophoresis on 1.8% agarose slab gels as described in Chapter 2, using a three slot former, each slot 5.8 cm long by 0.5 cm deep by 0.1 cm thick. These fragments were transferred to a nitrocellulose filter in the usual way, and the filter dried and baked. The use of a larger slot former meant that the filter could be sectioned longitudinally producing several replicate strips. To one filter was hybridised satellite ^{32}P-cRNA in 5xSSC containing 0.2% SDS at 70°C to a Rot value of 5.10^{-3} mole nucleotides.sec/litre as described in Section 1 (b). To another filter was hybridised the ^{32}P-rRNA also in 5xSSC with 0.2% SDS at 70°C but this time to a Rot value of 2.5 mole nucleotides.sec/litre. After the

Figure 13. 2.5% Polyacrylamide Gel Electrophoresis of ^{32}P-ribosomal RNA.

Mouse L cells were labelled for 2 days with ^{32}P-orthophosphate (100 μCi/ml). Ribosomal RNA was purified according to Hastings and Kirby (1966), and approximately 40 μg analysed by electrophoresis on a 2.5% polyacrylamide gel. The gel was stained with toluidine blue and photographed using an orange filter and FP4 film. Uneven illumination accounts for the darkly staining region in the middle of the gel.

hybridisations both filters were washed sequentially in 5xSSC, in 2xSSC, treated with RNase A, washed in 2xSSC and finally dried, as described above. Both filters were autoradiographed on Kodak RP Royal X-omat film. Both autoradiographs were scanned on a Joyce-Loebl microdensitometer and their profiles shown in Figure 14.

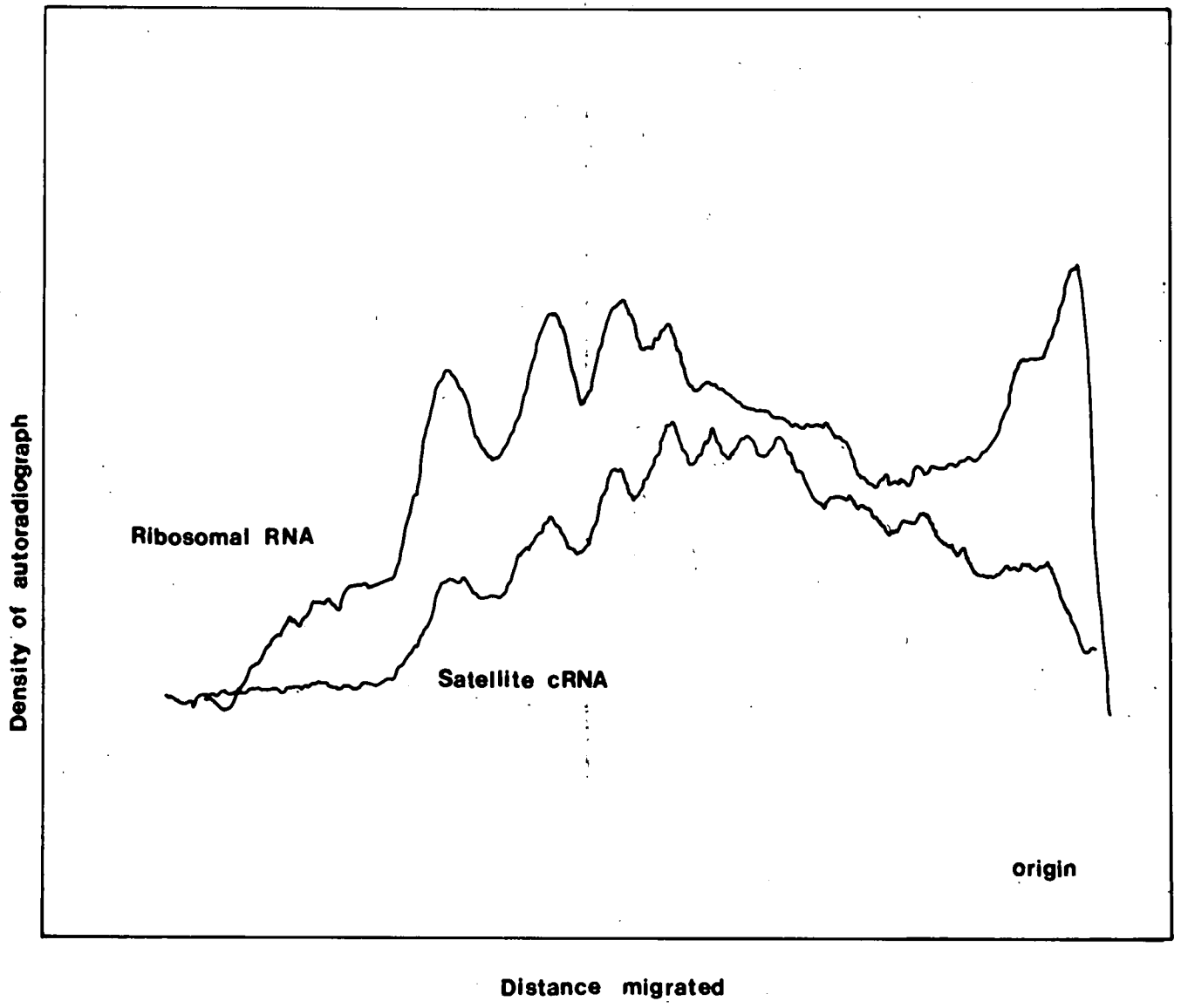
The most important feature of Figure 14 is the coincidence of the peaks for both satellite cRNA and ribosomal RNA. This must mean that both satellite DNA and ribosomal DNA are packed into similar repeating nucleohistone structures, and this in turn means that heterochromatin (which contains the satellite DNA) and the nucleolus (which contains the ribosomal DNA) have similar chromatin subunit structures.

As observed in Figure 11 there is a gradual decline in the radioactive intensities of both profiles in Figure 14, presumably for similar reasons to those discussed above. There appears to be no significant difference in the decline of radioactive intensity between the two profiles. Initially it might be thought that the similar decline in radioactive intensities for both satellite DNA and ribosomal DNA might argue against there being an actual deficiency of satellite DNA sequences in the lower molecular weight fragments for the reasons discussed in Section 1, namely that heterochromatic regions are more resistant to micrococcal nuclease than euchromatic regions. However, it could simply be that the nucleolus is also packed in a less accessible form than the euchromatic regions.

Interestingly, whereas there is an apparent paucity of satellite DNA sequences at the origin there is not a

Figure 14. Hybridisation of Satellite ^{32}P-cRNA and ^{32}P-rRNA Across an Agarose Gel of DNA Isolated from a Partial Micrococcal Nuclease Digestion of Mouse L Cell Nuclei.

Nuclei were prepared, digested, and the DNA extracted, analysed by gel electrophoresis and transferred to a nitrocellulose filter as described in the legend to Figure 11. This filter was then cut longitudinally in half. To one half was hybridised satellite ^{32}P-cRNA as before; the other half was incubated with ^{32}P-ribosomal RNA (18 S and 28 S species; and checked by electrophoresis on 2.5% polyacrylamide gels) to a Rot value of 2.5 mole nucleotides . sec/litre. Both filters were processed as before, prepared for autoradiography, and scanned in the microdensitometer.


Density of autoradiograph
Ribosomal RNA
Satellite cRNA
origin
Distance migrated

corresponding deficiency of ribosomal DNA sequences, supporting the conclusions of Southern (1975b) that high molecular weight DNA fragments containing satellite DNA sequences form networks and are not transferred from the agarose gels to the nitrocellulose filter.

It could be argued that the ribosomal RNA was not completely pure, since it was not purified by gel electrophoresis but simply analysed by it, leaving the possibility that minor contaminating RNA species were the ones that hybridised. This is unlikely to be the case since the hybridisation was only taken to a Rot value of 2.5 mole nucleotides.sec/litre by which value only repetitive sequences could have hybridised. Furthermore, before the filter was put to expose for autoradiography it was counted in a scintillation spectrometer to obtain a value of the amount of RNA that had hybridised. From this value it was calculated that the expected amount of RNA had hybridised if the RNA was pure ribosomal RNA. Lastly, there does not appear to be other major RNA species on the gel shown in Figure 12.

Section 3. Conclusions.

It has been shown that both satellite DNA sequences and ribosomal DNA sequences are packed in the repeating chromatin subunit structure proposed by Kornberg (1974) and defined, in this instance, by digestion with micrococcal nuclease. From the data presented it cannot be said whether heterochromatin and/or nucleoli are/is less accessible to micrococcal nuclease than euchromatin (which contains the bulk of the DNA) and therefore packed more tightly. If heterochromatin and/or nucleoli ~~are/is~~ is/are packed more tightly

than euchromatin, this presumably arises through some subtle modification to the chromatin structure, since the results of Figures 11 and 14 conclusively demonstrate the subunit structure of both heterochromatin and the nucleolus. This subtle alteration in chromatin structure may be due to a modification of H1 (phosphorylation,methylation,acetylation) or to acquisition (or loss) of non-histone proteins. Nucleolus-specific proteins may have been found (Matsui,1974) but their involvement, if any, in the nucleolus architecture has yet to be demonstrated.

What these experiments say nothing about is whether genes being transcribed are packed in the same repeating chromatin substructure as the bulk of the DNA. The demonstration of ribosomal DNA in this type of structure is not sufficient evidence, since it is not known whether in L cells all the ribosomal genes are switched on or only a small percentage of them. Obviously this question could be adequately answered in another system, namely the amphibian oocyte where, in the zygotene-pachytene stages of early oogenesis, there is amplification of the ribosomal RNA genes resulting in the formation of many hundreds of active extrachromosomal nucleoli per nucleus (Brown and Dawid,1968; Evans and Birnstiel,1969; Macgregor,1968; Gall,1969). Due to unfamiliarity with amphibian oocyte manipulation, another system has been chosen to answer this question and the work is described in the next chapter.

CHAPTER NINE

THE DISTRIBUTION OF TRANSCRIBED GENES WITHIN INTERPHASE CHROMATIN.

CHAPTER NINE

One of the lines of evidence to support the subunit structure for chromatin (Kornberg,1974) was obtained by Noll (1974a) using micrococcal nuclease digestion of isolated chromatin or nuclei. He concluded that at least 80% of the nuclear DNA was packed into chromatin subunits. This value was confirmed in Chapters 6 and 8. The remaining 20% appeared as acid-soluble material. The possible origin of this 20% is the subject of the present chapter.

It has been discussed in Chapter 8 that about 10% of this 20% acid-soluble material could arise through digestion of the DNA not packed into chromatin subunits, but in structures more accessible to nuclease action. A priori, it might be expected that DNA that is undergoing transcription would be less tightly complexed than DNA not being transcribed. This is supported by the packing of satellite DNA in the repeating nucleohistone structure and not necessarily contradicted by the similar localisation of the ribosomal genes, since it is not known what proportion of these genes is active in mouse tissue culture cells. It could be that the ones identified in Chapter 8 were the inactive ones.

It is possibly significant that both saturation hybridisation with total nuclear RNA and nuclear RNA complexity measurements, show that it is usually less than 10% of the genome which is active in transcription in a given cell type (Scherrer et al.,1970; Hahn and Laird,1971; Grouse et al.,1972; McConaughy and McCarthy,1972; Hough et al.,1975).

To answer the question of whether genes being trans-

cribed are packaged into chromatin subunits it is best to use a gene with a readily isolatable mRNA and known protein product; the histone genes from cleavage stage sea urchin embryos were selected as the best system. Following fertilisation there is a period during early embryogenesis of the sea urchin when there is a rapid rate of cell division which continues until late blastula stage, cleavage occurring approximately once every hour. The rapid rate of DNA synthesis necessary for chromosomal replication requires a concomitant rapid rate of protein synthesis, particularly of the histones (Kedes et al.,1969). The existence of 'maternal mRNA' had been inferred for some time (Denny and Tyler,1964; Gross and Cousineau,1964; Gross et al.,1965; Slater and Spiegelman,1966; Whiteley et al.,1966;1970; Glisin et al.,1966; Crippa et al.,1967; Hynes and Gross, 1972; Slater et al.,1972,1973; Wilt,1973); direct evidence for a defined maternal messenger RNA species came from the demonstration that histone mRNA sequences were present in sea urchin eggs (Farquhar and McCarthy,1973; Skoultchi and Gross,1973). However, there is not enough maternal histone mRNA to carry the sea urchin embryo all the way through cleavage since soon after fertilisation there is the appearance of a distinct size class of RNA - a broad peak centering around 9 S - which is found on those polysomes actively synthesising histone proteins (Kedes et al., 1969; Kedes and Gross,1969a; Nemer and Lindsay,1969; Moav and Nemer,1971). The rate of appearance of this RNA species increases to a maximum in mid-blastula stage after which it declines (Kedes and Gross,1969b; Moav and Nemer, 1971). Conclusive proof that this 9 S RNA coded for all

five histone species came from several laboratories where translation of the RNA in cell-free systems showed that the five histones were the major proteins synthesised (Gross et al.,1973; Grunstein et al.,1973; Levy et al.,1975; E.S.Weinberg, unpublished; K.W.Gross, unpublished).

During the blastula stage there is no detectable synthesis of ribosomal RNA (Kedes and Gross,1969b), so that if sea urchin embryos are labelled during the early cleavage stages with ^{3}H-uridine, the polysomes prepared and the RNA extracted, the predominant labelled material will be in a heterogeneous peak sedimenting at about 9 S in a 15-30% sucrose gradient. RNAs of specific radioactivities of several million counts per minute per µg are readily attainable. It is obvious, therefore, that the sea urchin embryo provides a ready source of high specific activity messenger RNA for a specific and characteristic set of proteins, the histones.

If this heterogeneous 9S mRNA fraction is further analysed by 6% polyacrylamide gel electrophoresis, several bands are found (Weinberg et al.,1972; Levy et al.,1975) which can be eluted from the gel, fingerprinted, and translated in cell-free systems (Grunstein et al.,1973; Levy et al.,1975; E.S.Weinberg,unpublished; K.W.Gross,unpublished). To date the mRNA for H1 and H4 has been obtained in pure form.

Another property of the histone genes from the sea urchin which makes them even more useful for the current study is that they are reiterated genes. By DNA excess hybridisation, Kedes and Birnstiel (1971) showed that the DNA sequences complementary to total 9S mRNA were repeated 400

times in the sea urchin gemone; using 9S mRNA which had been fractionated by polyacrylamide gel electrophoresis into three classes, Weinberg, Birnstiel, Purdom and Williamson (1972) raised this figure to 1000. Reiteration makes it easy to investigate them by filter hybridisation methods.

It should be stated that the work described in this chapter was made possible only through the skill and knowledge of Ms P.Mounts concerning sea urchin gamete fertilisation and embryo development. Her continual guidance and assistance is gratefully acknowledged.

Section 1. Materials and methods.

(a) The sea urchin species used.

Two Scottish sea urchins were used in this work: Echinus esculentus (reproductive season April-May) and Psammechinus miliaris (reproductive season June-midAugust). Both were obtained from the University Marine Biological Research Station, Milport, Isle of Cumbrae, Scotland. They were transported in sea water, but on arrival were transferred to artificial sea water (see below) at 4°C.

(b) Fertilisation of sea urchin eggs and development of embryos.

Adaptations of standard techniques were used (see Hinegardner,1967; Kedes and Gross,1969b). Urchins were shed of their gametes by injection of 0.5 M KCl (approximately 5 ml with Echinus, 1 ml with Psammechinus); Echinus sperm was usually collected dry on Petri dishes, whereas Echinus eggs and both eggs and sperm from Psammechinus were collected over artificial sea water. The artificial sea water used throughout this work was that of Horstadius-

Bialascewicz and which comprises the following: NaCl (25.17 g), KCl (0.65 g), $CaCl_2 \cdot 2H_2O$ (1.44 g), $MgCl_2 \cdot 6H_2O$ (5.78 g), $MgSO_4 \cdot 7H_2O$ (4.53 g), $NaHCO_3$ (0.2 g) made to 1 litre; the pH was adjusted to 8.0 with 1 N NaOH. When the urchins had completed shedding, the sea water was removed by aspiration, the eggs screened through two layers of cheesecloth and then washed three times in sea water. With Echinus it was necessary to remove the jellycoat by washing in acid sea water made pH 5.5 with 1 N HCl. The eggs were suspended in the acid sea water, allowed to settle and the liquid removed by aspiration. This process was not found to be necessary with Psammechinus since with this species the jellycoat was removed during the washing in sea water. Between each aspiration the eggs were allowed to sediment under gravity. Each batch of eggs were test fertilised by mixing small aliquots of egg suspension with a one thousandth dilution of 'dry' sperm on a microscope slide. Fertilisation was monitored by phase contrast microscopy; fertilisation membranes usually lifted off within 1 min. Only those eggs and sperm that produced greater than 95% fertilisation were used in any experiment.

When a batch of eggs was found to be suitable a diluted sperm suspension was added, the volume increased to 1 litre and the eggs allowed to settle during which time fertilisation occurred. The sea water was removed by aspiration and the zygotes washed twice with sea water and once with sterile sea water to remove excess sperm so as to avoid polyspermy which could lead to abnormal development. Sterile sea water was prepared by filter-sterilisation and addition of penicil-

lin G to 200 mg/l and streptomycin to 50 mg/l.

Embryos were cultured at 12°C (Echinus), or at room temperature which was never more than 20°C (Psammechinus). Embryos (200 ml cultures) were placed in shallow dishes to allow free exchange of oxygen. For larger volumes (500 ml or more) a slowly rotating top-drive stirrer was employed to prevent the embryos settling and developing oxygen deprivation, which also leads to abnormal development and sometimes death. If the embryos were to be labelled, ^{3}H-uridine was added to 50 μCi/ml. Development was for 8-10 h by which time the embryos had reached mid-blastula.

(c) Isolation of polysomes from sea urchin embryos.

This was carried out essentially as described by Levy et al. (1975). All operations were carried out between 0° and 4°C. All solutions were diethylpyrocarbonate (DEP) treated and, where possible, autoclaved as well. All glassware that was destined to be used for either polysomes or RNA was SDS rinsed, rinsed with DEP-treated water, dried, and heat sterilised (175°C) for at least 3 h before use.

Embryos were harvested by low speed centrifugation (500 g for 5 min) at 2°C using 150 ml Corex tubes in an MSE 6L centrifuge. The supernatants were removed by careful aspiration and the embryos washed twice in either Schapiro's Ca^{++}-free sea water (NaCl 25.88 g; KCl 0.79 g; $MgCl_2 \cdot 6H_2O$ 5.0 g; $MgSO_4 \cdot 7H_2O$ 4.14 g; water to 1 litre; pH to 8.0) or Ca^{++}, Mg^{++}-free sea water (NaCl 29.22 g; KCl 0.671 g; Na_2SO_4 9.022 g; water to 1 litre; pH to 8.0), being transferred to 30 ml Corex tubes after the first wash, washed once in medium M of Kedes and Birnstiel (1971) (0.4 M KCl. 0.01 M $MgCl_2$, 0.05 M

Tris-HCl,pH 7.7) and resuspended in 4 vol of medium M. The washed embryos were homogenised in a Dounce homogeniser (B pestle), about 20 up-and-down strokes generally being required; homogenisation was monitored by phase contrast microscopy. The homogenate was centrifuged at 10,000 g for 10 min in a Sorvall RC2B refrigerated centrifuge to pellet nuclei and mitochondria, and the supernatant decanted into a sterile tube. Aliquots (1.5 ml) of this supernatant were carefully layered onto 5 ml of 50% sucrose in medium M in 10 ml polycarbonate tubes, the tubes topped up with paraffin oil and capped, and the polysomes pelleted by centrifugation at 60,000 rpm for 3 h in a 10 x 10 ml MSE titanium rotor. Firstly the oil was removed by aspiration, followed by the remaining liquid, leaving a small polysomal pellet. After drying the tubes with tissues, they were stored at -70°C.

(d) Extraction of RNA from polysomes.

Polysomal pellets were dissolved in a small volume (1-2 ml) of medium R (0.1 M NaCl, 0.001 M EDTA, 0.5% SDS, 0.01 M Tris-HCl, pH 7.4) at room temperature and combined. An equal volume of phenol-chloroform mixture (redistilled phenol, 500 g; 8-hydroxyquinoline, 0.5 g; an equal volume of choloroform-octan-2-ol (24:1, v/v) was added, mixed and water saturated) was added and the RNA extracted by vigorous agitation on a vortex stirrer for 5 min at 4°C. Centrifugation at 5,000 g for 10 min at 4 C separated the phases; the lower, organic phase was removed and the aqueous phase re-extracted with phenol-chloroform. If an interphase remained then the aqueous phase was extracted a third time. To the clear aqueous phase was added 0.1 vol of 1 M NaCl

followed by 2.5 vol of ethanol, and the RNA precipitated at -20°C overnight. The precipitate was collected by centrifugation at 10,000 g for 30 min at -15°C, the supernatant decanted, the RNA pellet washed with 70% ethanol and drained dry.

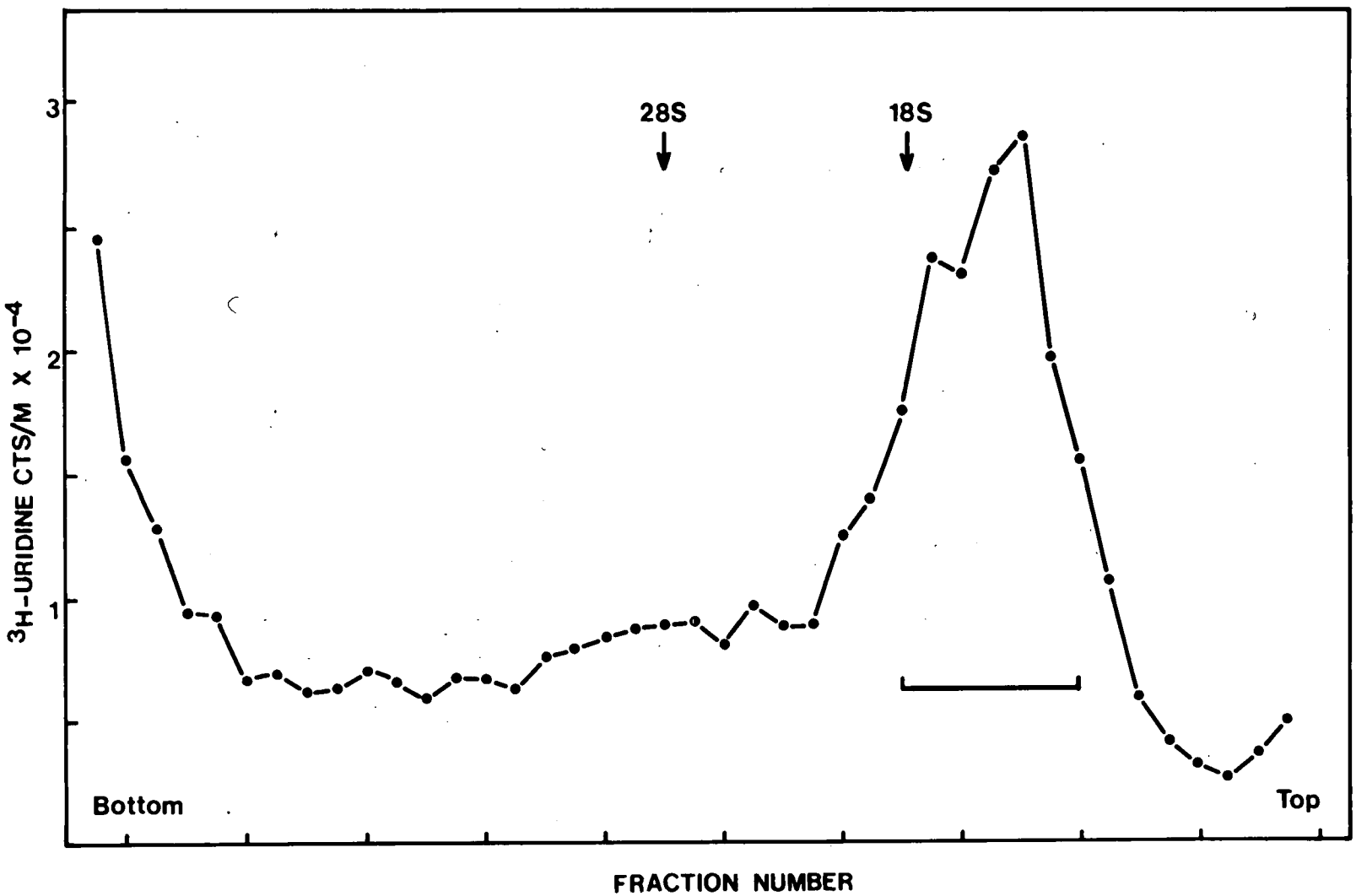
(e) Fractionation of 9 S RNA.

The dried RNA was dissolved in a small volume (0.1-0.2 ml) of medium R and centrifuged on 12.5 ml 15-30% linear sucrose gradients in a 6x14 ml swing out MSE titanium rotor. Details of the preparation of the gradients and their fractionation are given in Chapter 2. Centrifugation was for 16 h at 26,000 rpm at 20°C. Radioactivity was determined in each 0.2 ml fraction by spotting 5 µl samples onto a strip of Whatman GF/A filter, soaking sequentially in 10% TCA, 5% TCA, ethanol-ether (1:1, v/v) and ether, all at 4°C for 10 min each, drying at 100°C for 10 min, and finally counting each spot separately in a liquid scintillation spectrometer. Optical densities at 260 nm were also determined. A typical gradient is shown in Figure 15. The fractions containing the 9 S radioactivity were pooled, E.coli tRNA added to 10 µg/ml as carrier and the RNA precipitated by addition of 0.1 vol of 1 M NaCl and 2.5 vol of ethanol, and leaving at -20°C overnight. Centrifugation at 10,000 g for 30 min at -15°C produced an RNA pellet which was dissolved in 0.5 ml of water or medium R. Specific radioactivities of 5.10^6 cpm/µg RNA were routinely obtained. RNA samples were stored frozen at -70°C.

(f) Analysis of the 9 S RNA by polyacrylamide gel electrophoresis.

Figure 15. Sucrose Gradient Centrifugation of ^{3}H-uridine Labelled RNA Isolated from Mid-blastula Sea Urchin Embryos.

Sedimented polysomes were dissociated with medium R at room temperature, and extracted three times at 4°C with phenol-chloroform mixture. The RNA was precipitated by addition of 1 M NaCl to 0.1 M, 2.5 vol of ethanol, and putting at -20°C overnight. It was collected by centrifugation at 10,000 g for 30 min at -15°C, washed with 70% aqueous ethanol and drained dry. The RNA was dissolved in medium R (0.2 ml) and centrifuged on two 12.5 ml 15-30% linear sucrose gradients in an MSE 6x14 ml Titanium rotor at 26,000 rpm for 16 h at 20°C. Fractions were collected dropwise from the bottom and both absorbance at 260 nm and radioactivity measured.


28S
18S
3
2
1
^{3}H-URIDINE CTS/M X 10^{-4}
Bottom
Top
FRACTION NUMBER

To be sure that the RNA fraction contained discrete RNA species, 3%-6% polyacrylamide gel electrophoresis analysis was carried out according to the method of K.W.Gross (personal communication) and which is described in Chapter 2. The RNA bands were located by fluorography, also described in Chapter 2, and shown in Figure 16. For the reasons outlined above this 9 S fraction was taken to be mainly histone mRNA.

(g) Analysis of the 9 S RNA by oligo(dT)-cellulose chromatography.

A small (0.1 ml) column of oligo(dT)-cellulose was prepared and washed extensively with 0.1 M NaOH to remove any contaminating RNA and RNase. This column was then washed thoroughly with the binding buffer (0.01 M Tris-HCl, pH 7.4, 0.1% SDS, 0.4 M NaCl, 0.001 M EDTA, 10% glycerol; DEP-treated). ^{3}H-uridine-labelled 9 S RNA (60,000 cpm) was loaded onto the column in the binding buffer (0.1 ml) and washed through with a further 0.2 ml. This solution was recycled through the column four times. The column was then washed with the elution buffer (0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.1% SDS; DEP-treated) several times (0.5 ml each) until no further radioactivity eluted.

Both total unbound and bound radioactivities were measured. It was found that greater than 80% of the 9 S RNA fractions did not bind to oligo(dT)-cellulose, indicating that poly A was absent from this RNA. Histone mRNA is reported not to have poly A at its 3' end (Adesnik et al., 1972; Grunstein et al.,1973).

(h) Isolation of nuclei from mid-blastula stage embryos.

Figure 16. 3%-6% Polyacrylamide Gel Electrophoresis of the 9 S RNA Fraction from a Sucrose Gradient. Approximately $3 \cdot 10^4$ cpm of ^{3}H-uridine labelled RNA was loaded onto a 3%-6% polyacrylamide gel, made as described in Chapter 2. RNA samples were heated to 60°C for 2 min prior to loading. Electrophoresis was at 3.5 mM at 37°C for 12 h, after which time the gel was soaked in 2 changes of DMSO, 30 min each, soaked in 20% PPO in DMSO for 3 h, and soaked in water for 4 h. The gel was fluorographed wet, according to the method of Ken Gross which is described in Chapter 2, since the RNA was subsequently recovered from it. Exposure was for 2 days. In this experiment the gel fractured during freezing and this accounts for the darkly staining central band.

A modification of the method described by Hogan and Gross (1972) was finally found to be the most suitable. All operations were carried out at 0°C.

Embryos in mid-blastula stage were harvested by centrifugation at 500 g for 5 min, washed twice in sea water and resuspended in 5 vol of STC buffer (0.5 M sucrose, 0.003 M $CaCl_2$, 0.05 M Tris-HCl, pH 8.0). The embryos were dissociated into individual cells by five up-and-down strokes in a Dounce homogeniser (B pestle); this was monitored by phase contrast microscopy. After adding an equal volume of sea water, the cells were collected by centrifugation at 700 g for 10 min and resuspended in STC buffer containing 0.25% Triton X-100. Cell lysis was accomplished by a further two up-and-down strokes in the Dounce homogeniser (B pestle); this was again checked by phase contrast microscopy. An equal volume of sea water was added immediately and the nuclei collected by centrifugation at 1000 g for 10 min. The clean nuclei were washed twice in 0.01 M Tris-HCl, pH 7.4, 0.002 M $CaCl_2$, 0.01 M NaCl, and resuspended in the same buffer for further analysis.

(i) Isolation of nuclei from sperm.

Several methods of preparation of nuclei from sperm were tried but the simplest one was found to be the following.

Dry sperm, collected by 0.5 M KCl injection, was suspended in sea water and checked for motility. Sperm tails were removed by five up-and-down strokes of a Dounce homogeniser (B pestle). Triton X-100 was added to 0.5%. One more up-and-down stroke of the pestle was enough to lyse the sperm heads which were collected by centrifugation

at 10,000 g for 10 min at 2°C. The supernatant was discarded and the pellet resuspended in sea water by homogenisation and pelleted again, this time at 5,000 g for 7 min. The pellet was resuspended in sea water and digested with micrococcal nuclease in sea water as described in Section 2.

Sperm nuclei produced by such a procedure were far from clean, the cell membrane although lysed, remaining attached. However, for the purpose of isolating chromatin subunit DNA fragments this method was adequate and extremely simple.

Section 2. The subunit structure of sea urchin chromatin.

Nuclei isolated from sperm and mid-blastula stage embryos were digested with micrococcal nuclease at various enzyme concentrations at 37°C for 5 min, and their DNA extracted and analysed as described in Chapter 2. Figure 17 shows the result. It can be seen that a repeating substructure exists for both sea urchin sperm and embryo chromatin similar to all higher eukaryotes studied. The size of the repeating DNA fragment is again about 180 n.p. in length, but, as can be seen from Figure 17, prolonged digestion reduces this size to about 150 n.p. Presumably technical reasons and endogenous nucleases account for the poorer digestion patterns obtained with sea urchins than with other species studied.

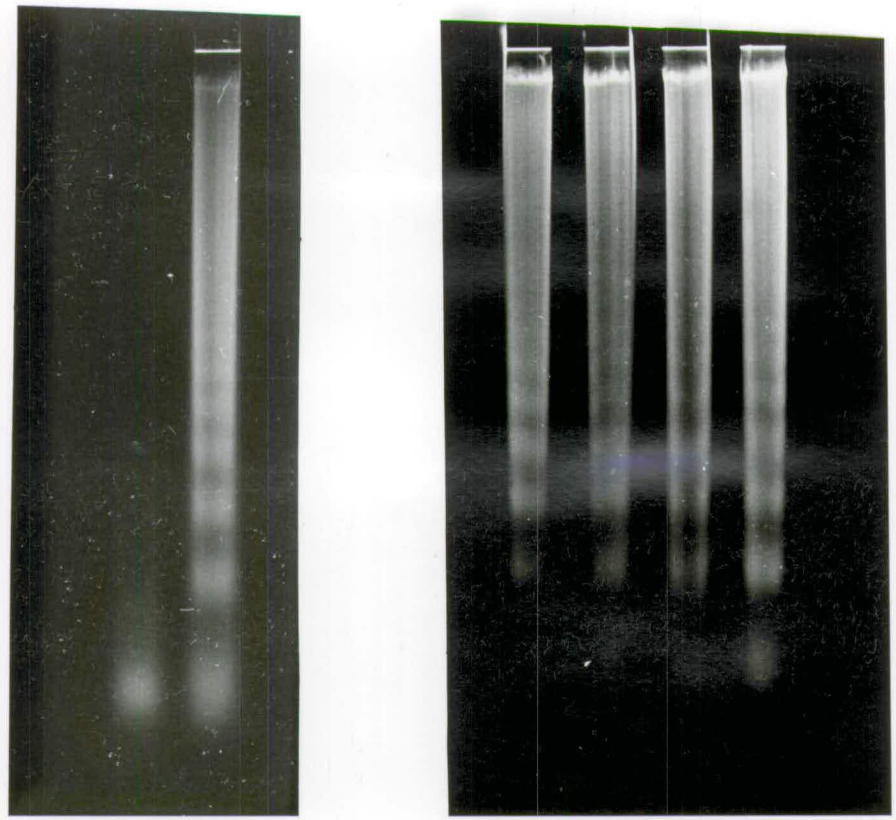
Section 3. Analysis of histone DNA sequences in the chromatin of sperm and blastula embryos.

The experimental approach to this problem was like that used to investigate the distribution of both

Figure 17. Micrococcal Nuclease Digestion of Sea Urchin Nuclei Isolated from Mid-blastula Embryos and Sperm.

Nuclei were prepared and digested as described in Section 1.

(a)	blastula nuclei	50 units/ml
(b)	blastula nuclei	200 units/ml
(c)	sperm nuclei	10 units/ml
(d)	sperm nuclei	50 units/ml
(e)	sperm nuclei	100 units/ml
(f)	sperm nuclei	200 units/ml

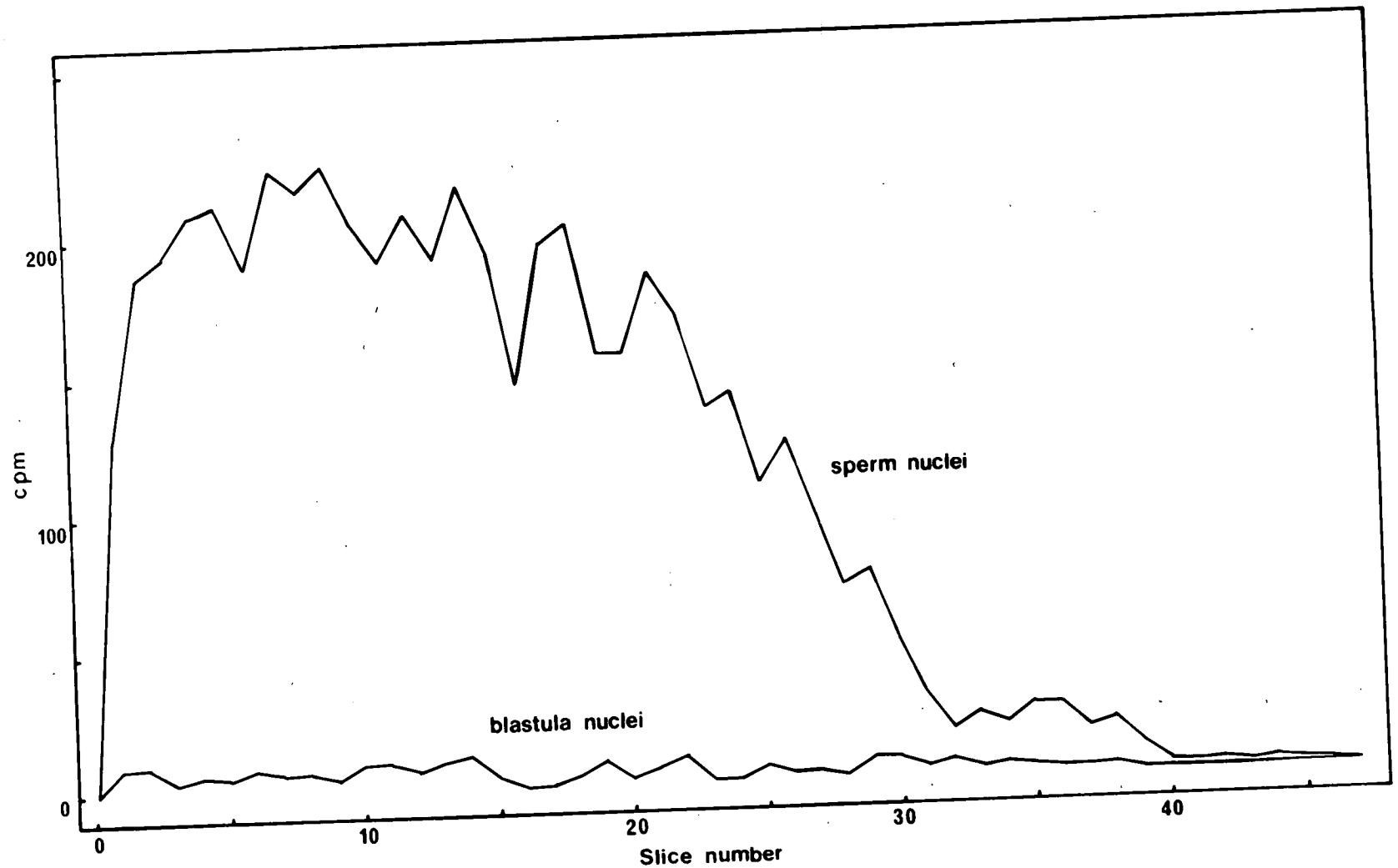


b a c d e f

satellite DNA sequences and ribosomal DNA sequences. Nuclei isolated from both sperm and blastula stage embryos were digested with micrococcal nucleaseto produce partial digests. The purified DNA fragments were separated by tube gel electrophoresis on 1.5% agarose, as described in Chapter 2. After staining the gels in ethidium bromide, the DNA was denatured in situ, and transferred to a nitrocellulose filter as before. Duplicate samples from sperm and blastula nuclei were run in parallel and transferred to the same nitrocellulose sheet, which, after baking, was used in a filter hybridisation assay with ^{3}H-labelled 9S RNA. Hybridisation conditions were $2 \cdot 10^{5}$cpm/ml of RNA at 65°C in 2xSSC, 0.1% SDS for 16 h. To remove the unhybridised RNA, the filter was washed twice in 1 litre of 2xSSC at 65°C for 30 min each, and then dried overnight.

To localise the radioactivity, fluorography, as described in Chapter 2, was initially used. However, although development after 4-5 week exposures produced slight blackening of the X-ray film, this was not enough to distinquish bands or differences between samples. Therefore due to lack of time it was decided to section the individual filters into 1 mm slices and to count these in liquid scintillation spectrometer. This method is rapid but resolution is lost. Figure 18 shows a comparison of the results obtained with nuclei isolated from sperm and mid-blastula stage embryos. It can be seen that there is an approximately ten-fold ~~greater~~ less hybridisation of the histone mRNA with the DNA fragments resulting from the digestion of mid-blastula nuclei. It is felt that the decreased amount of hybridisation reflects a true deficiency of histone DNA sequences in the

Figure 18. Hybridisation of ^{3}H-labelled Sea Urchin Histone mRNA Across an 1.8% Agarose Gel of DNA Isolated from a Partial Nuclease Digest of Nuclei from Sea Urchin Sperm and Mid-Blastula Stage Embryos.
Nuclei were prepared as described in the text and digested with micrococcal nuclease at 50 units/ml at 37°C for 5 min. DNA was extracted, electrophoresed on an agarose gel and transferred to a nitrocellulose filter as described in the legend to Figure 11. ^{3}H-labelled histone mRNA was hybridised to this filter in 2xSSC, 0.2% SDS at 65°C for 18 h, after which time the filter was processed as described for Figure 11, except that it was sectioned into 1 mm strips and counted in a liquid scintillation spectrometer.


200
100
0
cpm
sperm nuclei
blastula nuclei
0
10
20
30
40
Slice number

DNA fragments isolated from digested mid-blastula nuclei for the following reasons: Identical amounts of DNA (50µg) were loaded onto each gel; parallel gelswere transferred to the same nitrocellulose filter; labelled histone mRNA was hybridised to this filter in the same vessel, and thus the conditions of hybridisation must have been identical. The only variable was the DNA sample itself. Since this result has been repeated with different DNA samples, the difference in the amount of hybridisation could not have been due to the quality of the DNA; more likely it was due to the absence of histone DNA sequences in these DNA fragments.

The most obvious reason for this deficiency of histone DNA sequences is that the histone sequences in embryonic chromatin are more accessible to nuclease digestion than those in sperm. However, due to the lack of resolution of these experiments, it cannot be unequivocally stated that the histone genes in sperm are packed in the same repeating subunit structure as is most of the sperm DNA (Figure 17). Nevertheless, it remains the most likely possibility, and this must mean that the packing of the histone genes is altered following fertilisation. Correlated with this altered susceptibiltiy to nuclease digestion is the onset of transcription. Thus these results suggest that transcriptionally active genes are not packed in a nuclease resistant structure, but are in a more open, nuclease accessible form.

Section 4. Discussion.

There are obvious deficiencies in this type of analysis.

Most arise as a consequence of using filter hybridisation methods, some of the limitations of which have been discussed in the preceeding chapter. If time had permitted, and the sea urchin season was longer, several other experiments would have been done to confirm and extend these findings. For example, an obvious experiment that should be done is to use solution hybridisation methods to determine the histone gene number in the DNA fragments isolated from nuclease-digested sea urchin sperm chromatin and from mid-blastula chromatin. If the conclusion about the distribution is correct, then there should be an approximately ten-fold increase in the $Cot_{\frac{1}{2}}$ values of the histone genes in the latter type of DNA fragments (using DNA excess). Another control experiment would be to look for the distribution of the ribosomal genes in both sea urchin sperm and mid-blastula chromatin, for in both these nuclei there is minimal, or no, transcription of these genes; ribosomal RNA synthesis does not occur until about the gastrula stage of embryogenesis (Kedes and Gross,1969b). This experiment would eliminate any methodological artefacts between the use of sperm nuclei and embryo nuclei, as well as testing the transfer and filter hybridisation methods more rigourously.

A major problem with the experiments described in both this chapter and the preceeding one to determine the types of DNA sequences present in the 200 n.p. fragments produced by nuclease digestion of nuclei, is that histone rearrangement could have occurred during both nuclear isolation and subsequent nuclease digestion. Histone rearrangement includes histone exchange and histone migration. If

either had occurred then interpretation of the results becomes impossible. Histone exchange can be assayed for by the addition of labelled DNA to the preparation and looking to see if histones become associated with this DNA; histone migration is less easy to detect, but work is in progress in several laboratories to resolve this point. Earlier experiments by Clark and Felsenfeld (1971) showed that during extensive nuclease digestion of sheared DNP in the presence of divalent cations (greater than 10^{-3}M) or high NaCl concentrations, histones exchanged onto added, free DNA. However, there are obvious differences between this work and the work described in this thesis. Firstly, Clark and Felsenfeld used sheared DNP preparations while whole nuclei were used here. Teleologically it might be expected that histone rearrangement is less likely to occur in nuclei than in isolated chromatin which was later sheared. Also, whole nuclei have been found to mimic such in vivo functions as DNA replication (Friedman,1974; Hallick and Namba,1974) and specific transcription (Shutt and Kedes, 1974) and presumably, therefore, these nuclei have maintained quite substantial structural integrity. Secondly, nuclease digestion times here were much shorter (about 5 min) compared to those used by Clark and Felsenfeld (1.5 h). Thirdly, low ionic conditions were used for digestions (0.01 M Tris-HCl, pH 7.5; 0.001 M $CaCl_2$) although these may be slightly above the value for minimum exchange suggested by Clark and Felsenfeld. However, Varshavsky, Ilyin and Georgiev (1974) claim that it is at low, non-physiological ionic strengths that histone exchange is favoured. Thus this point remains contentious. Fourthly, Sollner-Webb and

Felsenfeld (1975), using similar conditions for nuclear isolation and digestion to those used in this thesis, have shown that histone rearrangement does not occur.

But possibly the most convincing demonstration that histone rearrangement-exchange and migration-has not occurred to any significant extent in the experiments described here is that there is marked difference between sea urchin sperm chromatin and embryo chromatin, with respect to the distribution of histone genes, by the criterion of nuclease accessibility. If redistribution of histones had occurred then little difference between the two should have been found. Thus the evidence suggests that histone rearrangement has not been a problem in the experiments described in Chapters 8 and 9.

These results also indicate that most of the histone genes from both homologues are active during embryogenesis, since if only one chromosomal set was active - a kind of allelic exclusion phenomenon - then presumably 50% of the embryo histone genes would be packed into nucleosomes. Other explanations are, however, also possible.

CHAPTER TEN

GENERAL DISCUSSION

It has been shown in Chapter 4 that a significant and reproducible amount of DNA (about 1% of the nuclear complement) can be digested from isolated chromatin using an endonuclease specific for single-stranded DNA. The endonuclease chosen was that from N.crassa and was shown to be approximately 10,000 times as active with denatured DNA compared to duplex DNA; identical results were obtained using an enzyme prepared in the laboratory and a commercial preparation.

The value of 1% of the chromatin DNA present in the single-stranded form is much greater by two orders of magnitude than the value of 0.01% determined by Levy and Simpson (1973) using an immunochemical and complement fixation assay, and is also greater than the value of less than 0.5% found by Mirzabekov and Melnikova (1974) using dimethyl sulphate. Assuming for the moment that the higher value is more accurate, then one important reason could account for the discrepancy. Erlanger et al. (1972) have pointed out that the antibody molecule (about 180,000 daltons) may be too large to penetrate the chromatin to the possibly small unpaired regions of DNA. Levy and Simpson argue that reaction can occur between single-stranded DNA in chromatin and the antibody, since by heating the DNP to 95°C for 1 min followed by rapid cooling - a process that would be expected to generate unpaired DNA regions - they found an enhanced binding of the antibody. However, it could be that the heating produced much longer regions of unpaired DNA, and it was these which could then be recognised by the antibody, whereas the 'native' short unpaired regions could not. This arguement could obviously not

apply to the dimethyl sulphate method.

If the lower value (0.01%) is more accurate, then the reason for the higher estimate could be an obvious one, namely that the endonuclease is not specific for single-stranded DNA. This, however, is unlikely in view of the lack of reactivity of the endonuclease on duplex λ DNA. An alternative explanation could be that once small single-stranded regions had been digested, exonuclease activity, whether endogenous or as a minor activity of the added endonuclease, could continue the digestion by nibbling in from the ends. This would have the effect of increasing the apparent amount of single-stranded DNA. Consideration of the kinetics of digestion, though (figure 2), suggests that this may be the explanation for the gradual and continual release of TCA-soluble material after about 5 h incubation of DNP with the endonuclease. Thus the 1% figure may be a more correct estimate for the amount of single-stranded DNA in interphase chromatin than the 0.01%, but is probably in excess of the real value, which may be about 0.5%, derived from the dimethyl sulphate method; this excess is due to accompaning exonucleolytic action after the initial endonucleolytic digestion.

The function of these regions is not known. As the cells were not synchronised, some cells would have been isolated in S phase in which single-stranded DNA regions are generated during the process of DNA replication. Thus at least some TCA-soluble material would be expected to be derived from this source, although since the DNA was uniformly labelled, this would only be a minor contribution. (Similar

contributions by replicating DNA would be made to the estimates of unpaired regions in the studies of both Levy and Simpson and Mirzabekov and Melnikova).

One alternative source of unpaired DNA regions in interphase chromatin could be regions for initiation of transcription. There is some preliminary evidence that RNA polymerase initiates on umpaired DNA regions (Groner et al.,1975). Thus it could be that these regions are sensitive to the endonuclease. As derived in Chapter 1, Section 10, if control regions (operators, promotors etc.) are unpaired regions of about 30 nucleotides in length, then 1% of the mammalian genome would represent about $1 \cdot 10^6$ such sequences, far more than the number of genes estimated for mammalian cells by Ohta+Kimura (1971) and Ohno (1971). Thus there are certainly enough single-stranded regions for there to be at least one per gene. Although the control of gene expression in eukaryotes is far from understood, and there is certainly a possibility that the sequences of DNA recognised by 'control proteins' are in the unpaired form, it seems likely that all genes whether active or not have an associated single-stranded region; it would seem more likely that on being switched on, the 'initiator region' becomes unpaired. If this is true, namely that the 'initiator region' is activated by becoming unpaired, then what could bring about this effect? From the work of Engle and vonHippel (1974) it is known that the introduction of a methyl group on the exocyclic nitrogen of adenine reduces the stability of the duplex by 1-2 kcalories per mole of methyl group relative to the same nucleotide sequence in unmethylated DNA. This lowered stability, the authors claim, may favour the looping

out of the duplex molecule into clover leaf type structures where the methylated adenines are located at the ends of the hairpins. Methylation of adenine might be expected to lower the propensity of this molecule to form hydrogen bonds with thymine and this presumable is the reason for the destabilisation. But in eukaryotes the methylated base is predominantly that of cytosine not adenine. Methylation of cytosine does not directly interfere with its ability to form hydrogen bonds with guanine. Whether it does so indirectly, perhaps through altering the electron density over the heterocyclic ring, has not yet been studied. If it turns out that methylation of cytosine residues also destabilises duplex DNA, this would provide a mechanism of changing the structure of DNA at specific, sequence-determined regions.

Assuming all the above were true, then it might also be expected that, once destabilised by methylation, these regions could be digested from the chromatin with an enzyme specific for single-stranded DNA. The products released by such a digestion would thus be expected to be enriched for methylated residues. When this experiment was done (Chapter 5) such an enrichment was not found. It seems, therefore, that methylation is not the cause of the apparent unpairing of the DNA duplex, and may not have anything whatsoever to do with it. The results of Chapter 5 certainly show there is very little, if any, correlation between the occurrence of single-stranded DNA regions and DNA methylation.

Thus the involvement of unpaired DNA regions in the control of gene expression remains a tantilising possibility

although the experiments described here do not point to any particular function for them.

Unfortunately the same is true for DNA methylation in eukaryotes - it certainly occurs but its function remains unknown. One interesting observation comes from the results of Chapter 6 which show a slight enrichment for 5-methylcytosine residues in the DNA of the subunits of interphase chromatin. If genes involved in transcription are not packed into these subunits (see below), then the enrichment for 5-methylcytosine in the non-transcribed sequences may be more than coincidental. The higher degree of methylation that is found in satellite DNA is consistent with this observation and contributes to it. Moreover, it has been suggested that methylation of DNA may be correlated with gene inactivity such as X chromosome inactivation (see Riggs,1975; Holiday and Pugh,1975).

Chapter 7 demonstrates the widespread occurrence of a repeating subunit structure for interphase chromatin. In light of recent work by Gorovsky and Keevert (1975b), however, the tentative suggestions made concerning Tetrahymena chromatin structure needs to be revised. It was suggested in Chapter 7 that since discrete fragments were not in the main visible in the DNA isolated from micrococcal nuclease digested nuclei, then the macronucleus of Tetrahymena (the transcriptionally active one) may not have the same structure as do all higher eukaryotes. In addition, since faint DNA bands were visible on the negatives, it was suggested that the micronucleus may have the more usual subunit structure. Recently, though, Gorovsky and Keevert (1975b), using a different method for nuclear

isolation, observed a regular repeating structure for both macronuclei and micronuclei. This is interesting in view of the reported absence of both H1 and H3 from macronuclear chromatin (Gorovsky and Keevert,1975a,b). However, at the recent British Society for Cell Biology Symposium on Chromatin held in Glasgow (September 28-30,1975) it was pointed out by several participants that earlier reports of the absence of certain histones from lower eukaryotes such as yeast, Neurospora and Aspergillus were incorrect, and can be explained either by incomplete extraction or proteolysis during extraction, particularly acid extraction. In view of the fact that Gorovsky and Keevert (1975a) were able to extract histones H1 and H3 from the micronucleus, this might argue against degradation or incomplete extraction occurring in this case, but a more thorough search is still warranted.

The Glasgow Symposium resolved the earlier discrepancy between different laboratories concerning the actual size of the DNA fragment within the subunit (see Table 2). Estimates varied from a little over 100 n.p. to just over 200 n.p. As was suspected this variation was due to the digestion conditions used. Mild digestion at 37°C yields DNA fragments in multiples of about 180 n.p. (as reported in this thesis). Long digestions (over 30 min at anything above 10 units/ml) reduces this size to a stable 140 n.p. These latter fragments are quite resistant even after prolonged incubations but eventually smaller digestion products become evident. Thomas reported that if digestion is carried out at 2°C instead of 37°C, then the repeat fragment size is 200 n.p. The explanation resides in the finding that micrococcal endonuclease has some exonuclease activity which

is much reduced at low temperatures. Thus it now seems that there are two stages in the nuclease digestion of nuclei: the first, a rapid, endonucleolytic cleavage between the subunits generating 200 n.p. fragments; and a slower, exonucleolytic digestion of the exposed ends to stable 140 n.p. fragments. These results suggest that there are two classes of DNA within the subunit: a tightly complexed 140 n.p. fragment, and a more loosely bound 60 n.p. piece. Removal of H1 from isolated chromatin appears to increase the rate of appearance of the 140 n.p. fragments from the 180 n.p. fragments.

Further evidence in support of two classes of DNA within the subunit has emerged from the denaturation studies of Woodcock and Frado (1975) on isolated chromatin subunits. These workers compared the melting profiles of the DNA within the isolated monomer, dimer, trimer chromatin subunits, total DNP and purified DNA. Woodcock and Frado suggest that their results support the conclusion that the DNA within chromatin subunits is complexed in at least two different ways.

Thus in conclusion it now seems certain that the fundamental repeating DNA unit in higher eukaryotes is 200 n.p., the lower values arising through both exonuclease activity and prolonged digestions. Lower eukaryotes appear to have a slightly smaller fundamental repeating unit, the reason for which is not immediately apparent.

The experiments described in Chapters 8 and 9 are attempts to determine the kind of DNA sequences which are contained within the chromatin subunits. In Chapter 8 it was shown by hybridisation that both the simple sequence

or satellite DNA, and the ribosomal DNA is present in the nuclease-resistant DNA fragments produced by micrococcal nuclease digestion of mouse L cell nuclei. In Chapter 9 it was suggested, although not unambiguously proven, that the corresponding fragments isolated from sea urchin sperm chromatin contain the sequences for the histone genes. Echinoderm sperm DNA is complexed with histones (Easton and Chalkley,1972) unlike the sperm of mammals which contain protamines.

To answer the question of whether genes undergoing transcription are packed in the same repeating nucleohistone subunit structure as is the majority of the DNA, the following conditions should be fulfilled: the provision of a defined gene product (the mRNA) that can be isolated in pure form, a nucleus in which this mRNA is not being transcribed (the control), and a situation in which a nucleus synthesises this mRNA (the test).

There are many obvious candidates. The massive extrachromosomal rRNA synthesis that occurs during amphibian oogenesis is one. Any differentiated cell that produces large quantities of a single protein and its mRNA would also be suitable; for example, the avian erythropoietic system, conveniently approximated in tissue culture when Friend cells are stimulated with dimethylsulphoxide (globin genes), the chick oviduct system stimulated with oestrogen (ovalbumin genes), the chick embryo feather system (keratin genes), to mention a few. However, if only one gene (or set of genes) from one chromosome is active while the other gene on its homologue is inactive any interpretation will be difficult since half of these genes will presumably be present in the

inactive structure while the other half will be present in the active type of chromatin; this problem will exist whether the genes are 'unique' or repetitive. Ideally what is required is either a haploid cell line or a system in which the majority of genes from both chromosome sets are active. Such a system is approached by the amphibian rRNA genes mentioned above where most of the extrachromosomal copies appear, in the electron microscope, active in transcription. Another system might be the histone genes during sea urchin embryogenesis where large quantities of histones are required for the high rate of chromosome replication that occurs during the very rapid cell divisions of cleavage. Some of this histone synthesis is from the stored maternal histone mRNA, but this supply cannot sustain the embryo throughout cleavage and the newly synthesised histone mRNA is required. Since so much histone is necessary and so much of the polysomal RNA is histone mRNA, it seems likely that most of the sea urchin histone genes are active at mid-blastula stage.

In this system the inactive nucleus is provided by the sperm in which there has not been any convincing demonstration of RNA synthesis. Obviously the mid-blastula nucleus represents the transcriptionally active nucleus for the histone genes. And the histone mRNA is readily isolated from this later stage of embryogenesis. All of the above reasons, plus the information already gathered on the sea urchin system by others (see Chpater 9), made it an attractive one to determine whether, on becoming transcriptionally active, there is any major structural alterations in the nucleohistone complex.

From the results presented in Chapter 9 it can be said that, firstly, in sperm the histone genes are packed in a nuclease-resistant structure which is probably, but not unambiguously proven, the same type of repeating chromatin subunit structure described for both satellite DNA and the ribosomal genes in mouse L cells (Chapter 8), and secondly, by mid-blastula stage most of these histone genes have become more accessible to nuclease digestion; those genes remaining in the nuclease-resistant structure amount to about 10% of the original genes, assuming all of the histone genes in sperm are ~~identified~~ present in chromatin subunits.

This conclusion, if true, is very significant. As mentioned in Chapter 9 time did not permit more control experiments and further stufies to be done. Nevertheless, preliminary results from other groups using different methods add support to this conclusion. At the British Society for Cell Biology Symposium on Chromatin referred to above, Werner Francke showed electron micrographs of the putative ribosomal RNA cistrons from several different species, using a method similar to the one developed by Oscar Miller. Most of the extrachromosomal nucleoli were being transcribed as judged by their 'Christmas tree' like appearance due to the RNA chains. In this case it was immpossible to say whether chromatin subunits or RNA polymerase molecules accounted for the densely staining granules along the DNA axis; it is more likely they are polymerase molecules as there seemed to be one per RNA chain. In several places, however, there were regions along the DNA axis not being transcribed although these regions should contain rRNA cistrons if strict periodicity is maintained throughout. In such regions no

nucleosomes were apparent, although nucleosomes were abundant on surrounding chromatin from the same nucleus. If it can be shown that those non-transcribed regions do in fact contain sequences for the ribosomal genes, then this result suggests that genes destined for transcription may not be ~~un~~packed in ~~regard to~~ chromatin subunits. Francke also showed electron micrographs of dispersed liver cell nuclei in which amongst the mass of nucleosomes, there were a few regions of DNA which gave the appearance of 'Christmas trees' depleted of most of their branches; in other words only a few suspected RNA chains were present. If these are genes being transcribed in the liver cell nucleus at a low rate then this rate of transcription is probably what is expected for a 'normal' liver function gene. Strikingly, no nucleosomes were present on these DNA regions either. Thus Francke's results point to the conclusion that any transcription of chromatin involves the removal, temporarily or otherwise, of histones from the DNA.

Corroborative evidence came from the work of John Sommerville, reported at the same Symposium, using fluorescein-conjugated antibodies raised against histones. When these anti-histone antibodies were applied to the lampbrush chromosomes of the amphibian oocyte and visualised by fluorescence microscopy, fluorescence was not found on the loops - the putative regions where RNA synthesis is occurring- but was found on the chromosome axis, again suggesting that transcription and the presence of nucleosomes is incompatible.

Thus it seems likely that high rates of transcription, and perhaps all transcription, may involve dissembling nu-

cleosomes along the chromosome axis, although whether the dissociation of histones from DNA in localised regions is enough to initiate transcription is not known at present. If it is, then modification of histones is of obvious interest. Furthermore, these results have important consequences for the credibility of numerous published experiments purporting to have fractionated 'active' from 'inactive' chromatin. In the vast majority of these DNP was sheared prior to fractionation. Recent experiments on the detrimental effects of shearing isolated chromatin have been referred to in the Introduction and thus throw additional doubts upon the meaningfulness of the results obtained. One method, developed by Marushige and Bonner (1971) does not involve shearing DNP to reduce its molecular weight but rather farsightedly employs brief nuclease treatment, in this case with DNase II. Since this enzyme has a pH optimum of about 4.5, if the incubation is carried out between 7 and 8, the activity of the nuclease is greatly reduced with the result that only a limited number of double-stranded breaks are introduced into the DNA. Using this procedure and separation of the digestion products on sucrose gradients, Gottesfeld et al. (1975) have reported the isolation of a component of chromatin of about 14 S which, they claim, has the properties expected of DNA undergoing transcription. This method is possibly the most hopeful one so far to study the structure of transcribing chromatin.

From all the results presented in this thesis and others it can be concluded that:

1. Most of the nuclear DNA (greater than 80%) is present as chromatin subunits.

2. Whenever specific sequences are sought in the DNA of the subunit they are found. For example, the simple sequence or satellite DNA, the ribosomal genes, the histone genes (all from this thesis), and the globin genes (Axel,1975; Sollner-Webb and Felsenfeld,1975; Lacy and Axel,1975).

3. When the repeated histone genes from sea urchin embryos are switched on after fertilisation there is an increase in nuclease susceptibility of these genes, suggesting that rapidly transcribed genes may not be so tightly complexed with subunits during this time.

Recently, however, Lacy and Axel (1975) have reported convincing solution hybridisation studies demonstrating that globin gene sequences are present in the 180 n.p. fragments produced by micrococcal nuclease digestion of duck reticulocyte nuclei. These nuclei are actively synthesising globin mRNA. This conflicts with the results reported here. It could be that genes being transcribed at low rates (possibly globin genes) can be packed into subunits during transcription, but this is not possible for genes being transcribed at high rates (the amphibian ribosomal genes and possibly the sea urchin histone genes). More work will be necessary to resolve this point.

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A Rapid and Simple Method for the Detection of Mycoplasma and Other Intracellular Contaminants

There are many published methods for the detection of mycoplasmas in cultured eukaryotic cells (for reviews see[1,2] and references within), most of which are complex, time consuming and often inaccurate giving false-negative results. Recently a method has been published[3] that involves detection of cytoplasmic DNA-containing cell contaminants by staining their DNA with either Hoechst 33258 or 4'-6-diamidino-2-phenylindole and viewing by fluorescence microscopy. This method, as are several others, is based on the fact that mycoplasmas and some DNA animal viruses replicate in the cytoplasm. However, one drawback of this procedure is that cells must be grown on coverslips. In the method I describe, a small culture vessel (e.g. a 6 oz medical flat) routinely used for sub-culture is adequate for the assay. An answer can be obtained within 2 h, thus making it feasible to check for the presence of mycoplasma in sister cultures before each experiment, as suggested by LEVINE[2].

The method I describe is a simpler and more sensitive double-label modification of the one described by SCHNEIDER et al.[4] and makes use of the fact that mycoplasmas synthesize the enzyme pyrimidine phosphorylase[5] which both hydrolyses uridine (and thymidine) to uracil (and thymine), and can 'salvage' pyrimidines to nucleosides by the reverse reaction; this means that exogenous uracil can be incorporated into RNA in mycoplasma-infected cells but not in uninfected cells[5,6] where this enzyme is in the main absent (but see ref.[2]).

Methods. A 50 times concentrated mixture of ^{3}H-uridine and ^{14}C-uracil was added to a rapidly growing, subconfluent bottle of cells to final levels of 1 μCi/ml and 0.1 μCi/ml respectively, without changing the medium. This bottle was allowed to incorporate label for 1 h, after which time the medium was poured off and the attached cells washed twice with a balanced salt solution (e.g. Earle's, Hank's, phosphate buffered saline). 5 ml of 0.1% sodium dodecyl sulphate (SDS; w/v) was then added and, after leaving for 5 min to allow complete lysis, the viscous solution was poured into 10 ml of 20% trichloroacetic acid (TCA; w/v) at 0°C. A further 5 ml of 0.1% SDS was used to rinse the culture vessel, and this was also added to the 20% TCA. The mixture was left for 20 min at 0°C before filtration onto a Whatman GF/A or GF/C filter; this filter was washed sequentially with 20 ml of ice-cold 10% TCA, 10 ml of ethanol-ether (1:1 by vol.) and 10 ml of ether. Finally the filter was dried in an oven at 100°C for 5 min, added to a vial containing PPO-POPOP toluene scintillant and counted in a Packard liquid scintillation spectrometer; the settings were such that ^{3}H and ^{14}C could be differentiated e.g. ^{3}H: window 50–400, gain 90%; ^{14}C: window 350–1000, gain 10%. With these settings ^{3}H is counted at 47% relative efficiency of counts, and ^{14}C at 35%; spillover from ^{14}C channel into ^{3}H channel is 15% of the corrected ^{14}C cpm, while there is no spillover of ^{3}H cpm into the ^{14}C channel. Background values were obtained by stopping the incorporation at time zero (i.e. immediately after addition of label), and processing exactly as above.

Total cellular DNA was isolated essentially as described by MARMUR[7].

Results and discussion. The importance of using radioactively-labelled nucleic acid precursor compounds in a wide variety of types of experiments with tissue culture cells over the last 20 years or so is obvious. If cell lines are contaminated with mycoplasma, then the host's metabolism is radically altered [1–6]. For example, mycoplasmas synthesize pyrimidine phosphorylase, an enzyme that hydrolyses pyrimidine nucleosides to ribose and the free base. An important consequence of this is that the use of thymidine or uridine either to label cellular nucleic acids, or in the case of thymidine, to synchronize cells, becomes impractical, since these coupounds are degraded by the mycoplasma contaminants. The presence of pyrimidine phosphorylase, however, can be exploited in a convenient assay for the presence of mycoplasma.

SCHNEIDER et al.[4] labelled separate cultures with either ^{3}H-uridine or ^{3}H-uracil, purified total cellular RNA from both, and determined their specific radioactivities. Thus the ratio of the specific radioactivities of the RNA labelled with ^{3}H-uridine to the RNA labelled with ^{3}H-uracil gave an indication of elevated levels of uridine phosphorylase and hence of mycoplasma contamination. This method has now both been simplified and made more sensitive by using uridine and uracil labelled with different radioisotopes in the same culture vessel, and also speeded up by labelling for much shorter periods. Using 2 radioisotopes obviates the necessity for determining the precise amounts of RNA present.

A comparison of ^{3}H-uridine and ^{14}C-uracil incorporation in healthy, mycoplasma-infected, and kanamycin-treated infected mouse L cells

Condition of cells	Corrected TCA-insoluble radioactivity (cpm)	
	^{3}H-uridine	^{14}C-uracil
Healthy cells	81,540	7
Infected cells	3,050	5,285
Kanamycin (200 μg/ml) treatment for 7 days	7,488	1,522

If cells are labelled for 1 h with a mixture of ^{3}H-uridine and ^{14}C-uracil to final concentrations of 1 μCi/ml and 0.1 μCi/ml respectively, the incorporation into RNA can be measured as described in Methods. The Table shows results from a typical experiment using mouse L cells. Isolation of total cellular DNA followed by analytical isopycnic CsCl gradient centrifugation (data not shown) confirmed the presence of mycoplasma DNA (density approx. 1.68 g/ml). Furthermore, treatment of mycoplasma-infected cells with kanamycin (200 μg/ml) for 7 days suppressed the infection but did not eliminate it; this suggests that incorporation of ^{14}C-uracil indicates the presence of some cell culture contamination.

Since the medium used (modified 199) contains uracil (2.68 μ*M*) but nor uridine, its was necessary to check if the addition of cold uridine (to 3 μ*M* and 30 μ*M*) had any effect on either ^{3}H-uridine or ^{14}C-uracil incorporation. As expected, ^{3}H-uridine incorporation was decreased by addition of cold uridine, but ^{14}C-uracil incorporation was unaffected. Also using a meduim (e.g. F10) lacking uracil slightly increased ^{14}C-uracil incorporation in mycoplasma-infected cells.

So far this method has been successfully applied to the following cell types: to human lymphocyte suspension cultures; to monolayer cultures – human HEP, D98/AH2 and FLA cells, mouse L and 3T3 cells, kangaroo rat (*Dipodomys ordii*) cells, marsupial (*Sminthopsis crassicaudata*) cells and *Xenopus* kidney cells; and to several human-mouse somatic hybrid cells. Obviously this method will also detect other cell culture contaminants (e.g. viruses) if they produce enzymes that utilize uracil. Mycoplasmas, however, are the most common and troublesome cell culture contaminants.

Summary. A simple, rapid and sensitive double radioisotopic method is described for the detection of mycoplasma infection in tissue culture cell lines.

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