CHROMATIN RNA COMPONENTS

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SCOPE AND CONTENTS: Almost all the genetic information of the eucaryotic cell is contained in the nuclear chromosomes. How regulation of the expression of this information takes place still remains as one of the great mysteries of the Biological Sciences. In this thesis an attempt is made to look at a few of the chemical components that may be involved in this process.

Chromatin (the interphase form of the eucaryotic cell genome) of a number of tissue culture cell lines has been fractionated using some novel techniques into its four major components: DNA, RNA, histones and non-histone proteins.

Of these four fractions the RNA fraction has remained the least well characterized. This thesis attempts to demonstrate that the RNA fraction in chromatin, is contrary to the opinion of a number of people at present, a complex fraction, quite distinct from other cellular RNA components. A model is presented whereby gene regulation in eucaryotic cells takes place via the interaction of chromosomal protein and RNA components.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	=	adenylate
C	=	cytidylate
CHMW-RNA	=	chromatin high molecular wt. RNA
CLMW-RNA	=	chromatin low molecular wt. RNA
Cot	=	conc. of DNA (moles nucleotides) X sec/litre
cpm	=	counts per minute
cyto.	=	cytoplasm
cyt-RNA	=	cytoplasmic ribonucleic acid
cyt-rRNA	=	cytoplasmic ribosomal ribonucleic acid
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
D-RNA	=	DNA-like nuclear RNA
DOC	=	deoxycholate
EDTA	=	ethylene diamine tetra acetate
G	=	guanylate
g	=	gram
HEPES	=	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
mRNA	=	messenger ribonucleic acid
NHP	=	non-histone protein
μ	=	micron = 1×10^{-6} meter
nm	=	nano meter = 1×10^{-9} meter

RNA	=	ribonucleic acid
RNase	=	ribonuclease
rRNA	=	ribosomal ribonucleic acid
SDS	=	sodium dodecyl sulphate
Tris	=	tris (hydroxymethyl) amino methane
tRNA	=	transfer ribonucleic acid
U	=	uridylate
U.V.	=	ultra violet

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I INTRODUCTION

The principles involved in regulation of gene expression was born in 1866, with the pioneering work of George Mendel, it lay unnoticed by his contemporaries until the beginning of the 20th century. In the years following the rediscovery of Mendel's laws, by De Vries, Correns, and Tschermak, the new science of genetics was formed. Morgan in 1919, working with the fruit fly Drosophila, concluded that genes are unique entities carried in the chromosomes of the cell nucleus. Even in those pioneering years the gene had emerged as the elementary unit of the blueprint of the organism. It became the biologists atom. Today in the case of such simple living things as viruses and bacteria, it immerges as a structure of never ceasing wonder, the epitomy of simplicity and complexity molded together in harmony to present the mysterious feature of life — the power of self-duplication.

Since 1950 there has been a spectacular growth in our understanding of the nature of the gene. This growth is due in fact to, firstly, our successful application of biochemical and biophysical techniques to dismantle the gene and examine its numerous components, and secondly, the exploitation of microorganisms such as viruses and bacteria to study how the gene functions in vivo.

The genetic studies of the first half of the century had

indicated that the cell's blueprint was stored in a defined manner in the chromosome. How then were the plans of the blueprint transformed into a living organism?

It was eventually realized that the primary functional property of the gene was to code for the enzymes within the cell. This functional property was decisively demonstrated by Beadle and Tatum when they showed that different genes in Neurospora crassa controlled the presence of specific enzymes involved in complex metabolic pathways in the cell. Mutants which had lost certain genes, could not survive under certain environmental conditions because they no longer had the required enzyme to metabolize one or more metabolic components the new environment had presented to the cell.

Our present understanding of the chemical nature of the gene comes principally from the elucidation of the structure of DNA (Watson and Crick, 1953), the chemical substance of which genes are made. This quickly led to an appreciation of how the gene reproduces (Meselson and Stahl, 1958) and the role genes play in the synthesis of the vast array of proteins in the cell (Hoagland, Stephenson, Scott, Hecht, and Zamecnik, 1957; Nirenberg and Matthaei, 1961; Warner, Kropf and Rich, 1962; Smith, Salas, Stanley, Wahba and Ochoa, 1965). Since the early sixties, the study of how the genetic information stored on the DNA is processed into individual cellular proteins has grown into a very complex branch of science. Particularly for some procaryotic cells, details about the mechanisms of protein synthesis are known, which a few years before would have been thought of as unattainable. It was through a massive trust in public interest and

support, and the judicial use of a limited number of biochemical and biophysical techniques, that the basic aspects of the mechanism of protein synthesis emerged.

It soon became clear that while all cells carried out protein synthesis in essentially the same manner, the cells from one species to another differed in two important respects. Firstly, cells of different species contained different base sequences in their DNA - a result that is not too surprising when we consider that the cells' genetic information is stored in the form of a triplet nucleotide sequence along one strand of the DNA. The second way one cell differs from the other is the basis of this thesis, namely different regions of the DNA are transcribed into proteins in different cells. In this way, one cell type may have a very different set of protein components from another depending upon its environment, its passage through evolution, and its passage through the differentiation process in the organism. The degree to which any one gene will be transcribed can vary from almost no detectable transcription at all, up to almost continuous transcription throughout the lifetime of the cell. It is an understanding of this gene regulation that the forces of nature still retain from us, and thus prevent us from understanding and possibly further modulating the process of life.

Considerable progress has been made in understanding the mechanism of regulation of bacterial gene expression (Jacob and Monod, 1961; Gilbert and Mueller-Hill, 1966; Gilbert and Meuller-Hill, 1967; Epstein and Beckwith, 1968). Although the study of gene regulation in procaryotes has

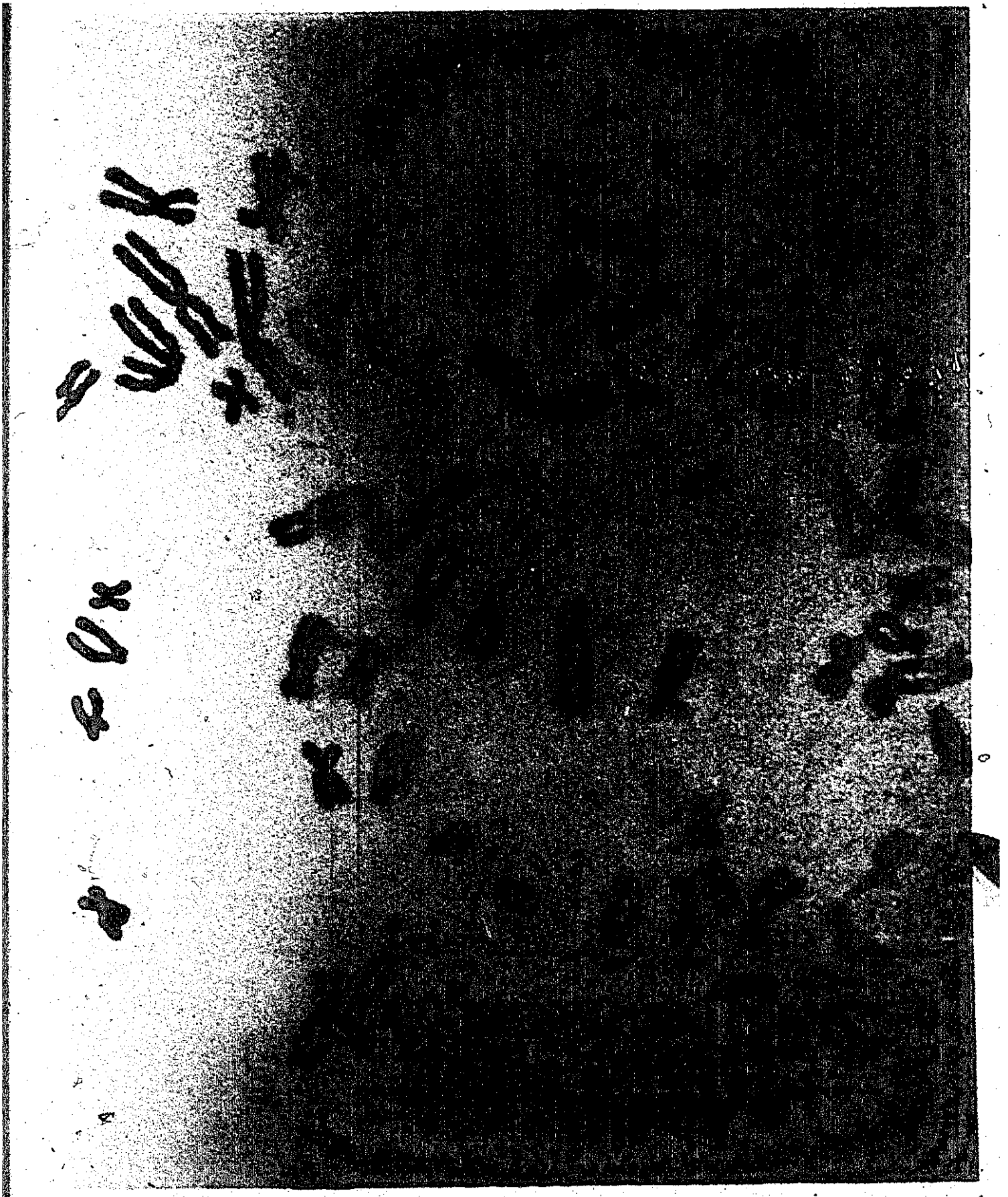


Figure 1. The 73 metaphase chromosomes of a HeLa cell.
(From Watson 1970)

been both interesting and important, it has nevertheless some limitations. Many feel that a direct extrapolation of processes in bacterial cells to higher organisms is far too simple-minded. The eucaryotic cell contains many times more DNA with greater complexity than the bacterial cell and carries vast amounts of information. So many complex processes are going on simultaneously with thousands of proteins being synthesized at a time, that it seemed virtually impossible to segregate individual processes for detailed analysis. However, attempts to unravel the eucaryotic system have improved considerably during the past few years due to technical progress in isolation and examination of various sub-cellular components and the ability to reproduce, in vitro, conditions and processes similar to those that occur in the eucaryotic cell in vivo.

There are many clear morphological differences between procaryotic and eucaryotic cells. For example, eucaryotic cells possess nuclei, nucleoli, endoplasmic reticulum and a golgi apparatus. These structures alone would indicate that the eucaryotic cell has a very much more complex structure than that of the procaryotic cell.

When we look at the nature of the genetic material in the eucaryotic cell we see that not only is there much more DNA present in terms of absolute amount per cell, but this DNA is only part of a very complex structure - the eucaryotic chromosome - which also contains considerable amounts of protein and RNA.

The chromosome can exist in two forms in the eucaryotic cell. It can exist as a compact, highly ordered structure during cell mitosis. Fig. 1 shows a preparation of some typical metaphase

chromosomes obtained from a human cell line (HeLa cells). An enormous body of work has gone into studying the metaphase chromosome, particularly its morphological characteristics (see Du Praw, 1970 for a review). The metaphase chromosome is, however, quite inactive as far as gene transcription is concerned (Kasten and Strasser, 1966). In this thesis we shall be concerned with the other form the eucaryotic cell genome takes up. During cell interphase, the total genome becomes disorganized into an apparently random fiberous structure which we call chromatin. It is as chromatin that the genome is genetically active in the cell. Fig. 2 shows an electron micrograph of a typical chromatin preparation.

It is now believed that the decision of which regions of the eucaryotic cell DNA will be transcribed into RNA and translated into protein rests upon the highly complex interaction of the cellular protein and RNA components with the DNA. There is a vast amount of experimental data for this conclusion, for example: Stone (1950), McCarthy and Hayer (1964), Davidson (1968). The experiments of Gurdon (1962) have shown that nuclei from a sizable proportion of the differentiated intestinal cells of a feeding Xenopus tadpole possess the capability of developing into a normal adult when implanted into enucleated eggs from the same species. This demonstrated that differentiation, and thus highly complex changes in gene regulation, need not involve irreversible changes to any significant part of the total cellular genome. Thus any irreversible gene inactivation such as loss of unused regions of cellular DNA cannot be regarded as a

Figure 2. Calf thymus chromatin. X 42,800 (From Bram and Ris, 1970).

fundamental mode of control. It has become clear that only a small portion of the total cellular genome is active in any one differentiated cell. The fraction which is active will depend on the type of differentiated cell. Though this was briefly proposed by Morgan in 1934 (Morgan, 1934), the serious proposal that variable gene activity could underlie differentiation can be considered to date from the early 1950's, and the writings of Mirsky (1953 and 1951), Stedman and Stedman (1950), and Sonneborn (1950). In the last decade the theory has been tested directly in many ways. Strong evidence now exists supporting the existence of an inactive chromatin (heterochromatin) fraction in differentiated cell nuclei which includes the major portion of the genomic DNA. Allfrey and Mirsky (1958, 1962, 1963), for example, have demonstrated that more than 75% of the DNA in isolated calf thymus nuclei can be removed with DNase without impairing RNA synthesis, provided that the histones thus released are inactivated and an adequate ATP supply is ensured. This RNA synthesis, however, is dependent on the presence of the remaining minority fraction of the nuclear DNA. In the same laboratory the inactive chromatin of the thymus nucleus has been visualized in the electron microscope and has even been partially isolated.

The fraction of the genome actually functional in differentiated cells (euchromatin) has also been studied. Measurements based on RNA-DNA hybridization procedures and on chromatin template activity now exist for a number of differentiated cell types. In the hybridization experiments radioactively labeled, newly synthesized RNA extracted from

various differentiated cell types is annealed with homologous DNA under conditions favoring hybrid formation between the RNA and complementary stretches of DNA. Comparison between the amount of DNA hybridized and the amount which could have engaged in hybridization under the conditions used have shown that in differentiated cells only about 10% or less of the genomic fraction assayed is actually active in RNA synthesis (Davidson, Crippa, Kramer and Mirsky, 1966; Shearer and McCarthy, 1967; Paul and Gilmour, 1966; Georgiev, Ananieva and Kozlov, 1966). Similarly, in a variety of tissues, only about 10% of the template activity displayed by pure DNA appears to be available in differentiated cell chromatin preparations. It has been shown in several such studies; furthermore, that the chromatin preparations employed function in a "normal" manner in that the spectrum of gene products which they produce in vitro correspond to those synthesized in that cell type in vivo (Paul and Gilmour, 1968).

Other RNA-DNA hybridization experiments demonstrate that the spectrum of genes active in each tissue is indeed distinct, exactly as the theory of variable gene activity necessitates, so that the RNA gene products of each cell type hybridize with partly overlapping but partly nonoverlapping regions of the genomic DNA.

Furthermore, both template activity and hybridization studies demonstrate that the spectrum of gene activity changes as the state of cell differentiation changes, for instance in liver regeneration (Thaler and Villee, 1967) and in hormone response (e.g., O'Malley, McGuire, Middleton, 1968). The presence of partially diverse RNA populations specific to given differentiated cell types and to given

states of differentiation clearly represents a direct verification of the variable gene activity theory of cell differentiation. It is not yet established how the template activity of chromatin is regulated, nor is it known what are the components in the cell that are involved in deciding which fraction of the genome should exist as euchromatin, and thus be genetically active, and which fractions should exist as heterochromatin.

Chromatin as it is isolated from the cell contains three major components, DNA, protein and RNA. Let us quickly summarize what we know about each of these fractions.

DNA

In 1874 the Swiss biochemist Miescher reported the purification of a substance from salmon sperm that he called nucleic acid; later it was found that there are two types of nucleic acid in all cells, and that Miescher's compound was the substance of the genes, deoxyribonucleic acid (DNA). After Feulgen introduced a specific stain for DNA in 1924, this material was shown to be largely localized in the nuclei and chromosomes of both plant and animal cells; by contrast, the other major class of nucleic acid, ribonucleic acid (RNA), occurs primarily outside the nucleus in the cytoplasm. In 1949 Mirsky and Ris quantified Feulgen's stain and succeeded in measuring the DNA content of individual cell nuclei; they concluded that the amount of DNA per chromosome set is approximately constant in a given species, a characteristic that had been predicted for the hereditary material.

The chromosomes of mammals have about 5×10^9 base pairs in the DNA of each haploid set, and it would not have been surprising if the sequences comprising this vast number of bases had properties which differed so little from the mean, that further analysis would have been impractible. The last five years have shown, however, that the genomes of higher organisms have many groups of sequences with properties so different from the mean that they can be purified and even their basic sequence determined. Some of these special groups of sequences are peculiar to higher organisms and are not found in bacteria or phage. Some of them have known functions; others have not. One working hypothesis is that these unknown special sequences are somehow concerned with processes peculiar to higher organisms, the organization of their chromosomes or differentiation, for example.

Two discoveries in the molecular biology of DNA have made this work possible; the demonstrations by Meselson, Stahl and Vinograd (1957) that DNA molecules of different composition can be separated by banding in dense salt gradients and by Marmur and Doty (1961) that denatured DNA will in suitable conditions reform a duplex structure very like its original native form. It now appears that the DNA contains in addition to its base sequences for specific gene proteins special base sequences which are redundent, in that they repeat over and over again throughout the cell genome. Their presence can be demonstrated by looking at the renaturation kinetics of denatured DNA (Britten and Kohne, 1968).

If low molecular weight DNA from a higher organism is heat

denatured and then returned to a temperature some 25° below its Tm, it will reassociate (or renature) and the time course of this reassociation can be followed either optically or by periodical column fractionations which distinguish reassociated from denatured molecules. The time course of such a reassociation is usually discontinuous as shown in Fig. 3 for the calf. This figure taken from Britten and Smith (1970) shows clearly the difference between the "intermediate fraction" reassociating between Cot 0.0001 and Cot 1 and the "slow fraction" reassociating at Cot values above 100. Different organisms have different shapes for this kind of reassociation curve.

Britten and Smith (1970) have calculated that for some rodents a few sequences present in the "intermediate DNA fraction" occur as often as 66,000 times and contain sequences up to 17,000 nucleotides long. Repeated DNA has now been identified in all higher species that have been examined (Britten and Davidson, 1971). It constitutes as little as 20% of all the DNA in the cell nuclei of some species to as much as 80% of the DNA of the species Bufo (Britten and Davidson, 1971).

One important feature of intermediate DNA is its ability to bind in vivo synthesized RNA. It now appears that in earlier experiments in which RNA was hybridized to DNA the measurement gave only the ability of the RNA to bind to intermediate or fast DNA (Bishop, 1968; Walker, 1968; Melli and Bishop, 1969; Britten, 1970). The reasons for this are as follows; let us take as a model the situation in which RNA is transcribed equally from one strand of a DNA, which contains a 10% fraction in which each sequence is repeated a hundred times.

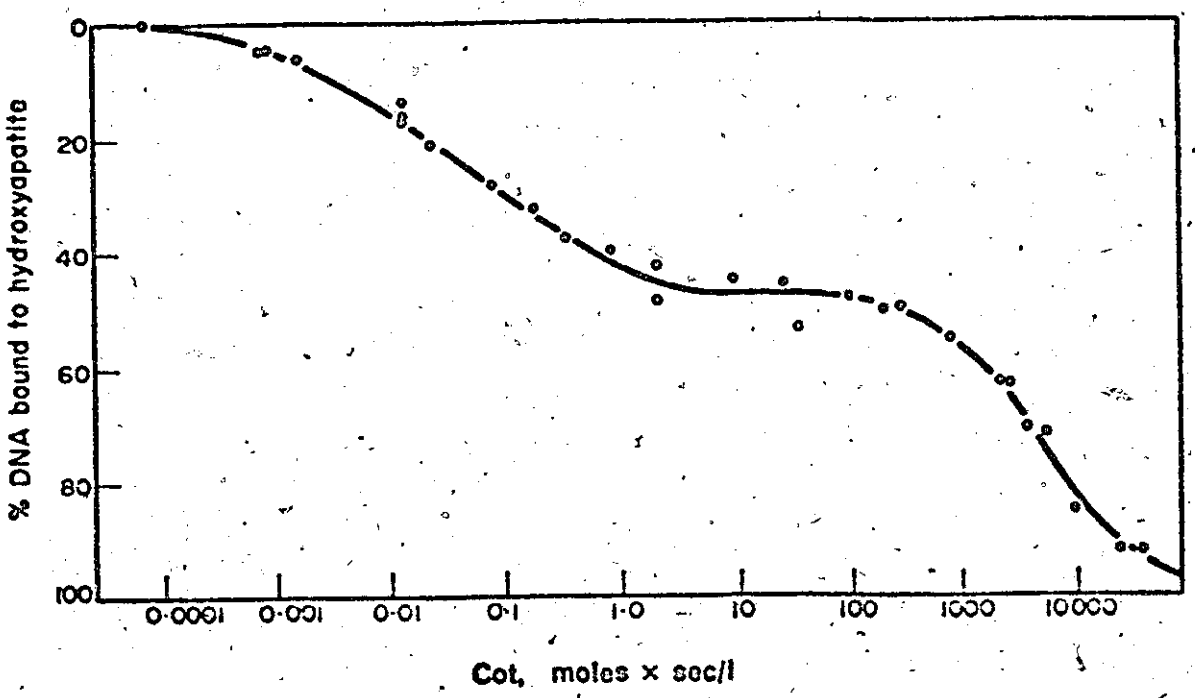


Fig. 3. The reassociation of calf DNA. This DNA was sheared into pieces approximately 400 nucleotides long, denatured and annealed at 60°C in 0·12 M phosphate buffer, and the proportion of reassociated DNA was assayed by hydroxyapatite fractionation. (From Britten and Smith, 1970.)

Then it is easy to see that in a hybridization experiment each repetitive DNA has a 100-fold better chance of reacting with a complementary RNA molecule than any fragment of unrepeated DNA. Therefore, under conditions of DNA excess it is the RNA products of repeated DNA sites which first react and when RNA is in excess it is the repeated DNA sites which are saturated earliest. Since under most experimental conditions only the early part of the reaction is recorded, these experiments give information primarily about repetitive DNA and its products. This simple picture is much complicated in vivo because there will be unknown proportions of the different RNA classes and their origin will also be undetermined. However, besides ribosomal, transfer and 5S RNA which are known to be transcribed from multiple genes, it can be concluded from the conditions of the experiments that part at least of the giant RNA from nuclei and of the messenger RNA of polysomes binds to intermediate or fast DNA (Scherrer and Marcaud, 1965; Attardi et al., 1966; Soeiro, Birnboim and Darnell, 1966).

What then is the function of the RNA transcribed from the "intermediate DNA fraction"?

Callan (1967) has suggested that structural genes in the eucaryotic cell are present in multiple copies. Since, however, the genetic evidence is only consistent with the presence of a single copy of each gene he had to propose that all copies had to follow exactly the mutations which occurred in a "master gene". The theory is based primarily on cytological evidence. For example, the genome is

organized into chromomeres, which contain much more DNA than is required for either a cistron or an operon in the bacterial sense. There is a close correlation between genetic markers and bands (equivalent to chromomeres) in Diptera. In amphibia, in particular, there are widely differing amounts of DNA per genome which cannot be explained by widely different complexities in these organisms, and are best explained by DNA multiplication. A difficulty is that no molecular mechanism for the rectification of "slaves" is known, although models have been suggested (Whitehouse, 1967). The difficulty is not overwhelming, however, since, as we have seen, the cistron coding for 5S RNA appears to remain identical as if such a mechanism existed. The theory has been recently considered in considerable detail by Thomas (1970), and has received experimental support from Thomas et al. (1970), who have observed a high proportion of ring structures in the electron microscope after native sheared DNA has been treated with an exonuclease or after denatured DNA has been renatured.

A function for intermediate DNA in regulating differentiation has been suggested by Britten and Davidson (1969) and Georgiev (1969). These two hypotheses differ in important respects since Britten and Davidson suppose that RNA, possibly the high molecular weight RNA found in nuclei, is the main controlling element. They therefore require a fraction of DNA which is only transcribed into this kind of RNA. The latter is then able to bind to sites adjacent to structural genes which are both transcribed and translated. Many structural genes might therefore have common controlling sites responding to the same RNA sequence and hence there is a need for repetitive DNA.

Georgiev's model, on the other hand, gives the controlling role to proteins, like the bacterial repressors, which react with acceptor sites on the DNA. There may be many such acceptor sites close together each reacting with different controlling proteins. In the absence of repression a very large RNA molecule is transcribed, which includes both the multiple acceptor sites and the structural genes adjacent to them. Before the structural genes pass to the cytoplasm for translation, the acceptor sites are removed enzymatically, thus accounting for the turnover within the nucleus of a large proportion of this high-molecular weight RNA.

The main difference between these hypotheses is the role they ascribe to the high-molecular weight nuclear RNA. Britten and Davidson consider this or perhaps a low molecular weight RNA to be a positive controlling element or activator, while Georgiev considers the former to be the precursor in part of messenger RNA. There are difficulties with both ideas; it is not clear why these activator molecules should be of such high molecular weight (100s) on the one hand, and there is no evidence that any of this RNA reaches the cytoplasm on the other.

Amongst the repetitive DNA sequences is a rather special fraction known as "satellite DNA". When cellular DNA is centrifuged in a isopicnic CsCl gradient in many cases the DNA forms not only one main band around its mean density, but also one or more smaller side bands appear at densities quite different from the main band. In the domestic horse, for example, this "satellite DNA" can represent as much as 28% of the total cellular DNA (Arrighi, Mandel, Bergendahl and Hsu, 1970).

Schildkraut and Maio (1968) prepared nucleoli by light sonication and differential centrifugation and found that they were considerably enriched in satellite DNA. This unexpected result has been confirmed by the recent *in situ* hybridization results of Jones (1970), which shows interphase nuclei with radioactivity concentrated round the nucleoli and by Smith's (1970) results which demonstrate that when satellite is being preferentially synthesized after polyoma infection, the nucleolus is preferentially labelled. If mouse metaphase chromosomes are separated into eight size classes, which somewhat overlap their neighbours, each class contains satellite DNA (Maio and Schildkraut, 1969). The five larger classes ranging in average length from 2.1 to 3.8 μ contain between 7 and 8% satellite. The three smaller classes of 0.8-1.4 μ length have between 11.4 and 12.8% satellite. They also reported another significant observation; when metaphase chromosomes are washed with 2 M NaCl, about 70% of the DNA is extracted into the supernatant, but the remaining 30% of resistant DNA contains all the satellite fraction.

These observations were extended by the investigations of Yasmineh and Yunis (1969, 1970) who have isolated chromatin fractions from nuclei by mild ultrasonic treatment and subsequent differential centrifugation. They distinguish three fractions which they have called heterochromatin, intermediate and euchromatin DNA. They find satellite DNA to be greatly enriched in the heterochromatin fraction for the mouse (Yasmineh and Yunis, 1969, 1970a), the guinea pig (Yunis and Yasmineh, 1970b) and the calf (Yasmineh and Yunis, 1971).

Most recently the newly developed technique of *in situ* molecular

hybridization has been used by Jones (1970) and Pardue and Gall (1970) to indicate that a large fraction of mouse satellite DNA is located near the centromere in the metaphase chromosomes and near the nucleolus and chromatin granules in the interphase nucleus. The technique consists of partially deproteinizing and then denaturing the cells by heat or alkali while they still adhere to the slide. During cooling or after neutralizing, highly radioactive RNA enzymatically prepared from satellite DNA or DNA from the single strands themselves is added at high concentration to the slide when some of the label is taken up by the different parts of the nucleus.

These results might suggest that "satellite DNA" has a structural function in the chromosome.

Clearly we have a lot yet to learn about the arrangement of base sequences in the eucaryotic cell DNA until we can get some insight into how it regulates gene expression.

The histones

There are two major protein fractions in chromatin, the histones and the non-histone proteins (NHP). Let us first consider the histones. Like DNA the histones had an early historical introduction. Kossel (1884), almost one hundred years ago, isolated histones from red cells of goose blood. The name remains a mystery, he merely says "I recommend the term histone" for his fraction. His reasons for studying histones are not clear. He had been impressed by Miescher's work on the transformation occurring in the tissues of the salmon as it swam up the Rhine to Basel - the diminution of the muscles and the increase

in mass of the gonads. Kossel supposed that the explanation of this change in the tissues was that a developmental transformation of the original tissue proteins occurred. Protamine was the most far-reaching state reached in this transformation, histone an intermediary state. Kossel said that he was particularly interested in this developmental change of the tissue proteins.

It was not until about 1942 that the investigation of histones were taken up again after Kossel's work. Mirsky and Pollister (1952) clearly established them as a unique group of cellular protein. Around this time Stedman and Stedman (1950) suggested a possible role of histones in gene regulation. They suggested that gene activity is suppressed in differentiation and that this is done by histones. While this role of genes in differentiation is quite the norm today, it was not a point of view frquently expressed in those days, particularly not by geneticists or embryologists.

Ever since their discovery by Kossel it was known that histones are heterogeneous proteins. The polycationic character and the similar amino acid composition of histones frustrated many attempts to fractionate these unique proteins. The chemical differences between two groups of histones (i.e. histones rich in lysine and histones rich in arginine) proved sufficient to permit their chemical separation (Stedman and Stedman, 1950, 1951; Johns et al., 1960). The peculiar amino acid composition of the very lysine-rich histone (approximately 30% lysine and only about 3% arginine) allowed its early separation and characterization (Daly and Mirsky, 1955; Davison and Butler, 1954;

Crampton, Moore and Stein, 1955; Crampton, Stein and Moore, 1957; Crampton, 1957). However, only the advent of more sophisticated tools for protein fractionation (such as ion exchange chromatography, gel filtration, and counter-current distribution) frequently combined with selective extraction of chromatin by a variety of solvents permitted further investigations of histone heterogeneity.

It now appears that there are five major histone fractions (Hnilica, 1967; Stellwagen and Cole, 1969; Johns, 1971; Phillips, 1971). There are three major histones in the lysine-rich groups. These are commonly known as F_1, F_{2a1} and F_{2a2}. There are only two major histones in the arginine-rich group known as F_{2b} and F_3. Several other histones occur in specialized tissues such as nucleated erythrocytes and spermatozoa (Hnilica, 1967; Nielin, Callahan, Lamb and Murry, 1964; Paoletti and Huang, 1969). Unfortunately another terminology also exists in the literature for the same histone fractions. The F_1, F_{2a1}, F_{2a2}, F_{2b} and F_3 are also referred to as: I, IV, IIa, IIb and III. We shall use the former notation in this thesis.

It also appears that histones, especially the lysine-rich fractions, can be phosphorylated (Ord and Stocken, 1966; Allfrey and Mirsky, 1966), methylated (Shepherd, Hardin and Noland, 1971) and acetylated (Shepherd, Noland and Hardin, 1971). These histone modifications introduce multiplicity in electrophoretic and chromatographic patterns. However, the reasons for these modifications remain unclear.

There is now little doubt that as suggested by Stedman and Stedman (1951) histones are one of the key components involved in the control of gene expression in the eucaryotic cell. Numerous experiments

have shown that they inhibit the action of DNA dependent RNA polymerase by restricting the amount of DNA template available to the enzyme (Huang and Bonner, 1962; Allfrey, Littau and Mirsky, 1963; Barr and Butler, 1963; Sonnenberg and Zubay, 1965). However, it is believed that they are not the primary factors involved in specific gene regulation. Other component(s) within the cell determine which genes will be repressed and this repression is mediated via the histones. This suggestion is based on a number of observations. With a few exceptions (Bustin and Cole, 1968; Fambrough, Fujimura and Bonner, 1968) the ratio of histone to DNA, the composition, and the properties of histones are similar in tissues which differ strongly with respect to their synthetic activity (Dingman and Sporn, 1964; Hnilica and Kappler, 1965; Beeson and Triplett, 1967; Mohberg and Rusch, 1970). There is a strong similarity between the histone compositions of hetero- and euchromatin (Frenster, 1965; Gorwski and Woodard, 1966; Comings, 1967). It appears that the histones do not possess the ability to recognize selectively a polydeoxyribonucleotide sequence to a degree required for specific gene regulation although some do display a preference for certain bases or certain regions of the DNA (Johns and Butler, 1964; Paul and Gilmour, 1968; DeLange and Smith, 1971).

It thus appears that the primary factors involved in specific gene regulation in the eucaryotic cell must be contained in some other component(s) of the chromosome. There are two other major components present in the chromosomes of eucaryotic cells that may be possible candidates for this function, the non-histone proteins (NHP) and RNA.

The non-histone proteins

Unlike the histones and DNA which were discovered almost a century ago the NHP have a relatively recent history.

Stedman and Stedman (1943, 1944) first isolated non-histone chromosomal proteins and suggested that they were involved in chromosome structure. However, research on non-histone nuclear proteins evolved very slowly, mainly because of the absence of unique chemical properties by which the individual non-histone nuclear proteins could be identified or readily extracted and fractionated. Only during the last few years has the improvement of extraction procedures together with the electrophoresis in polyacrylamide gel permitted better identification of the non-histone proteins in chromatin together with some initial studies on their biochemical and biological properties.

The NHP are classified as the protein material remaining in chromatin after extraction of the histones with acid. They have an acidic amino acid composition and show an excess of glutamic and aspartic acids. For this reason they are often called the "nuclear acidic proteins". They have one other distinguishing feature, they have a very high thiol content. They represent, for example, over 80% of the thiol groups in rat liver nuclei (Granow, 1969). These proteins also contain significant amounts of phosphorus (Longan, 1967; Kleinsmith and Allfrey, 1968; Takeda, Yamamura and Ogo, 1971). Subfractionation of the NHP has been extremely difficult in the past, due to the harsh treatment with dilute alkali and to the tendency of these proteins to aggregate once removed from the DNA. Results of the

NH_2-terminal amino acid analyses of nuclear non-histone proteins (Steele and Busch, 1963; Busch and Steele, 1964; Busch, 1965) indicated a considerable degree of heterogeneity. The scope of the heterogeneity of these proteins was not fully realized however, until the advent of polyacrylamide gel electrophoresis. Using gels containing SDS and/or urea or special buffer solutions, numerous bands were observed by several investigators for the non-histone nuclear proteins in various tissues (Benjamin and Gellhorn, 1968; Elgin and Bonner, 1970; Shaw and Huang, 1970; Shelton and Allfrey, 1970; Dastugue et al., 1970b; Loeb and Creuzet, 1970; Platz et al., 1970; MacGillivray, Carrol and Paul, 1971). As determined from their electrophoretic migration in SDS-containing polyacrylamide gels, the molecular weights of these proteins range from 5,000 to more than 100,000 daltons.

The NHP have a number of properties that we might expect a component involved in specific gene regulation to have. The content of NHP is different in parts of the chromosome that have different RNA synthesizing activities (Georgiev, Ermolaeva and Zbarskii, 1960; Frenster, Allfrey and Mirsky, 1963; Swift, 1964; Commings, 1967). There also appear to be marked tissue variations in the number and content of NHP components between different tissues (Loeb and Creuzet, 1969; Teng, Teng and Allfrey, 1970; Platz, Kish and Kleinsmith, 1970; Kostraba and Wang, 1970; MacGillivray, Carroll and Paul, 1971). There are indications of selective synthesis of some NHP fractions after cell stimulation by hormones (Tata, 1966; Deisseroth, 1969; Teng and Hamilton, 1970; Buck, Schauder and Weser, 1970; Stein and Baserga, 1970a;

Shelton and Allfrey, 1970; Barker, 1971; LeStourgeon and Rusch, 1971; Helmsing and Berendes, 1971) as well as specific binding of a NHP fraction to DNA (Kleinsmith, Heidema and Carroll, 1970; Salas and Green, 1971).

Thus, unlike the histone which appear to be relatively non-specific in their binding to DNA, the NHP appear as good candidates for components that may be involved in control of specific gene expression.

While the reported tissue specific distribution of NHP is a great improvement to the monotony of similar histone patterns in various tissues, it should be interpreted with caution. In the early days of histone fractionation and characterization there were reports of tissue and species specific histones. Most of these reports were later found to be due to aggregation, limited proteolysis or contamination of the histones with other proteins.

Recently Elgin and Bonner (1970) investigated in detail the tissue and species specificity of non-histone proteins in purified chromatin. Using SDS-acrylamide gel electrophoresis they were able to resolve rat liver chromatin non-histone proteins into thirteen major bands with molecular weights between approximately 5,000-100,000 daltons. A homologous pattern was found for similar proteins from chicken liver. We should remember that many of the NHP bands found in chromatin may only represent enzymes essential to nucleic acid metabolism or are just structural proteins, and have little to do with controlling specific gene expression. Perhaps the true proteins involved in gene control are too heterogenous and present in too low an amount

to be seen on the polyacrylamide gels used to fractionate the NHP fractions.

Chromatin RNA's

The RNA components of the chromosome are an even less well defined group of components. There seems to be a considerable degree of variation in the total RNA content of chromatin (interphase chromosomes) in different tissues (Dingman and Sporn, 1964; Bonner et al., 1968; Shaw and Huang, 1970; Bekhor and Bavetta, 1971). A wide spectrum of RNA components of differing molecular weights has been isolated from chromatin (Huang and Bonner, 1965; Benjamin, Levander, Gellhorn and DeBellis, 1966; Prestayko and Busch, 1968; Pelling, 1970; Dahmus and Bonner, 1970; Gasaryan et al., 1971; Kanehisa, Fujitani, Sano and Tanaka, 1971; Monahan and Hall, 1972).

Perhaps the best studied chromatin RNA component is a chromatin low molecular wt RNA component called "chromosomal RNA" first described by Huang and Bonner (1965). This RNA was characterized by its small size, chain length of 40 nucleotides, and its high content of dihydrouridylic acid. This RNA is complementary to about 5% of the nuclear DNA and is organ-specific (Bonner and Widholm, 1967). The unusual properties of chromosomal RNA led Huang and Bonner to suggest that it may be involved in gene regulation; that is, in conferring specificity on the histone-DNA interaction. Chromosomal RNA has since been isolated from rat liver, rat ascites cells (Dahmus and McConnell, 1969), calf thymus (Shih and Bonner, 1969), and chick embryos (Huang, 1967). The role of

chromosomal RNA in the histone-DNA interaction has been investigated by studying the conditions required for the sequence-specific reconstitution of chromatin. When chromatin is dissociated in the presence of 2 M NaCl, the histones, a portion of the non-histone protein, and the chromosomal RNA, which under these conditions is bound to the chromosomal proteins, are completely dissociated from the DNA. The dissociated chromatin can be reconstituted by slowly dialyzing away the NaCl. The template activity (the percentage of DNA in chromatin available for E. coli RNA polymerase to transcribe RNA) of such reconstituted chromatin is nearly identical to that of native chromatin. The RNA transcribed from this chromatin was then compared by RNA-DNA hybridization competition experiments with the RNA transcribed from native chromatin. If the histone returns to the same sites, the RNA generated should contain the same sequences contained in the RNA transcribed from native chromatin, and should thus compete on an equal basis in DNA-RNA hybridization competition experiments. If, however, the histones return to random sites on the DNA, the RNA generated will not be identical to the RNA transcribed from native chromatin and will not be an effective competitor in the hybridization reaction.

It was found that RNA transcribed from chromatin did not compete with RNA transcribed from reconstructed chromatin. Thus under these conditions the chromosomal proteins did not return to their original sites.

When chromatin is reconstituted by gradient dialysis from 2 M NaCl, the ionic bonds between histones and DNA are reformed. If,

however, chromosomal RNA directs the binding of histones, by base pairing with a complementary segment of the DNA, then conditions must be employed which allow the specific reconstitution of chromosomal RNA. Conditions were therefore sought which would allow specific chromosomal RNA-DNA interactions. The 5 M urea in the presence of NaCl was found to be a suitable condition. Chromatin, reconstituted from 2 M NaCl containing 5 M urea by first removing the NaCl and then the urea, results in chromatin with a template activity identical to that of native chromatin. RNA transcribed from such reconstituted chromatin competes on an equal basis with the RNA transcribed from native chromatin. The histone has therefore returned to the same or to equivalent portions of DNA.

If the specificity of the histone-DNA interaction lies in the ability of chromosomal RNA to recognize a complementary base sequence in the DNA, destruction of the chromosomal RNA should result in conspecific reconstitution. Chromosomal RNA was therefore degraded by RNase treatment followed by reconstitution of the chromatin and removal of the RNase. Control experiments have shown that under these conditions the chromosomal RNA is totally degraded. The RNA transcribed from such chromatin does not compete with the RNA transcribed from native chromatin. Treatment of native chromatin with bentonite, under conditions identical to those used for the removal of RNase from reconstituted chromatin, did not change the spectrum of RNA sequences synthesized.

An additional method to selectively remove the chromosomal RNA is $Zn(NO_3)_2$ treatment. The Zn^{2+} catalyzes the hydrolytic scission

of RNA, Zn^{2+} treatment of the dissociated chromatin again results in nonspecific reconstitution. From these experiments it was concluded that chromosomal RNA is required for the sequence-specific reconstitution of chromatin (Bekhor, Kung and Bonner, 1968).

Sevolap and Bonner (1971) have recently shown that this chromosomal RNA interacts with the repetitive sequences of the DNA. They further demonstrated that almost immediately after partial rat liver hepatectomy there is an increase above normal in the amounts of liver chromosomal RNA. This is followed by an increase in the template activity of chromatin, DNA synthesis and mitosis (Mayfield and Bonner, 1971a). Distinct tissue difference in rat chromosomal RNA species have been demonstrated (Mayfield and Bonner, 1971b). The above work would appear to demonstrate that there is a unique RNA fraction present in chromatin.

However, not everybody agrees with interpretations of Bonner's work. Heyden and Zachau (1970) feel that chromosomal RNA is nothing more than degraded tRNA. Starting from calf thymus, and adhering closely to the procedure described by Shih and Bonner (1969), Heyden and Zachau found that commercial pronase (a proteolytic enzyme extract used to remove protein associated with chromosomal RNA) contained some nuclease activity. Using the method described by Shih and Bonner they found that electrophoresis of chromosomal RNA on polyacrylamide gels gave only a broad smear of low-molecular weight material. If the pronase treatment of the pellicle was omitted, however, the RNA showed instead a major component migrating in the position associated with

tRNA. The identification was confirmed by the demonstration in the extract of high amino-acid acceptor activity, which was substantially destroyed by the pronase treatment.

Artman and Roth (1971), Commerford and Delihas (1966), Loeb (1967), using different techniques could not isolate any protein bound RNA components. Szeszak and Pihl (1971), treating chromatin with RN'ase H, an enzyme specific for the RNA moiety of DNA-RNA hybrids, found that only about 0.1% of the DNA of rat liver chromatin is hybridized to RNA. (Bonner's group indicated that chromosomal RNA hybridizes to a large fraction of the genome (Bonner and Widholm, 1967; Dahmus and McConnell, 1969; Shih and Bonner, 1968)). Szeszak and Pihl (1972) recently indicated that the RNA moiety of chromosomal RNA from rat liver is not a well defined entity but consists of different types of RNA, the major part of which is not chemically bound to protein. Arnold and Younge (1971) have isolated a low molecular wt. RNA fraction from rat liver chromatin using the method of Bonner et al. (1968), they isolate an RNA fraction in many ways similar to that of chromosomal RNA. However, their RNA differed in one important respect, it contained no dihydropyrimidine bases, a distinguishing feature of chromosomal RNA (Bonner, Dahmus, Fambrough, Huang, Marushige and Tuan, 1968; Jacobson and Bonner, 1971). De Fillippes (1970) attempting to purify chromosomal RNA from Hela cells failed, due to the contamination of native small RNA species with fragments of degraded larger RNA's.

Thus we see that there is a considerable air of cloudiness surrounding the true nature and function of chromosomal RNA. This has at times given rise to some heated editorial comments and replies in

the literature (Nature molecular biology correspondent, 1971a; Bonner, 1971b; Nature molecular biology correspondent, 1971b; Holmes, Mayfield, Sander and Bonner, 1972).

Chromatin contains other RNA components which appear to be chemically unbound to protein. Even less is known about these RNA components.

Prestayko and Busch (1968) described a number of low molecular wt RNA's (4-7S RNA) from the "chromatin fraction" of rat liver and rat Novikoff hepatoma nuclei. However, it was not clear if this "deoxyribonucleo protein RNA" came solely from the chromatin fraction. Using a more traditional method to isolate chromatin from chicken liver nuclei Kanehisa, Fujitani, Sono and Tanaka (1971), Kanehisa, Tanaka and Kano (1972), Tanaka and Kanehisa (1972) have obtained five low molecular wt RNA fractions (7-10S) from chicken chromatin. They have demonstrated that at least two of these fractions stimulate the template activity of chromatin by in some way interacting with the histones (Tanaka and Kanehisa, 1972). Sato, Arinke, Gaito and Sugimura (1972) have recently demonstrated that a rapidly labeled RNA fraction is bound to DNA in Ehrlich tumor cells - a result in marked contrast to that obtained by Szeszak and Phil (1971b), discussed earlier.

Thus, we see that unlike the other components present in the eucaryotic cell chromosome the RNA fraction has remained confused. This thesis sets out to illustrate that there is indeed a very distinct RNA fraction present in chromatin. This fraction is considerably more complex than is presently appreciated. In studying the RNA

a new procedure to isolate and fractionate chromatin was developed. A theoretical model has also been drawn up to suggest how gene regulation takes place in eucaryotic cells.

II METHOD AND MATERIALS

1. Growth of Tissue Culture Cells

a. Mouse L cells were derived from Earle's original fibroblast line (Sanford, Earle & Likely, 1948). The established line was obtained from Dr. K.B. Freeman, McMaster University. The cells were grown in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 5% (v/v) foetal calf serum, amphotericin B 2.5 µg/ml and tylocine 60 µg/ml. The composition of minimum essential medium is given on table 1.

b. Human KB cells were originally derived from an epithelial cheek carcinoma (Eagle, 1965). The established line was obtained by Dr. S. Mak from Dr. M. Green, St. Louis, Missouri. Cells were grown in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 5% (v/v) foetal calf serum, amphotericin B 2.5 µg/ml and tylocine 60 µg/ml.

c. Rat hepatoma cells, derived from a solid hepatoma (Thompson, Tomkins & Curran, 1966) were originally grown in monolayer culture in Swim's medium but were adapted to grow in suspension in Joklik's modification of minimum essential medium (Eagle, 1959) with 3% (v/v) foetal calf serum, 3% calf serum, amphotericin B 2.5 µg/ml and tylocine 60 µg/ml. The established line was obtained from Dr. K.B. Freeman, McMaster University.

d. DON-C3 Chinese Hamster Cells were obtained from a single cell clone of DON chinese hamster cells. These are a euploid cell line and were obtained from Grand Island Biological Company. These cells were

grown in monolayer culture in minimum essential medium (see table 1) supplemented with 10% foetal calf serum, amphotericin B 2.5 µg/ml and tylocine 60 µg/ml.

Cells were maintained in exponential growth by diluting the suspension to a concentration of 2×10^5 cells/ml each day with fresh prewarmed medium. Cell concentrations were determined with a Hemocytometer (American Optical). All cell lines were free of mycoplasma as assayed with "a mycoplasma test kit" (North American Biologicals Inc.).

To study incorporation of labeled precursors into chromatin, the cells were grown in the presence of the precursor at a concentration of 2×10^6 cells/ml. In a typical experiment cells were collected from a one litre suspension culture by centrifugation for 10 min at 650 x g, the pellet was resuspended in 100 ml of fresh medium containing the labeled precursor. When ^{14}C leucine, or ^{32}P H_3PO_4 were labeled precursors the cells were first washed, then grown in minimal essential medium free of unlabeled leucine, or H_3PO_4. In all incorporation experiments involving labeled precursors the medium was supplemented with 5% dialyzed foetal calf serum and 20 mM HEPES buffer, pH 7.3 - 7.4. ^3H Uridine incorporation into chromatin of DON-C3 cells was obtained by replacing the medium with medium containing labeled uridine and 10% dialyzed foetal calf serum. All tissue culture reagents were obtained from Grand Island Biological Company.

Preparation of chromatin and its fractionation into its major components

Numerous methods to isolate chromatin and isolate some or all of its individual components exist. The methods generally involve procedures which denature or discard some of the chromatin components in yielding

TABLE 1

CHEMICAL COMPOSITION OF MEM, MINIMUM ESSENTIAL MEDIUM (JOKLIK-MODIFIED) FOR SUSPENSION CULTURE

Component	mg/L	Component	mg/L
NaCl	6500.00	L-Tryptophan	10.00
KCl	400.00	L-Tyrosine (Disodium)	47.00
$MgCl_2 \cdot 6H_2O$	200.00	L-Valine	46.00
$NaH_2PO_4 \cdot H_2O$	1327.00	Choline Cl	1.00
Dextrose	2000.00	Folic acid	1.00
L-Arginine HCl	105.00	i-Inositol	2.00
L-Cystine 2HCl	32.40	Nicotinamide	1.00
L-Glutamine	294.00	D-Ca pantothenate	1.00
L-Histidine	31.00	Pyridoxal HCl	1.00
L-Isoleucine	52.00	Riboflavin	0.10
L-Leucine	52.00	Thiamine HCl	1.00
L-Lysine	58.00	Phenol red	10.00
L-Methionine	15.00	$NaHCO_3$	2000.00
L-Phenylalanine	32.00	Potassium penicillin G	75 units/ml
L-Threonine	48.00	Streptomycin sulfate	50 mcg/ml

MEM, MINIMUM ESSENTIAL MEDIUM (EAGLE WITH EARLE'S BALANCED SALT SOLUTION FOR SUSPENSION (SPINNER) CULTURES

Identical to formula above, except WITHOUT $CaCl_2$ and 10 times the phosphates.

one purified component. Examples of such procedures are acid extraction to isolate the histones (Johns, 1964; Murray, 1969), strongly alkaline conditions, or ionic detergents to isolate the NHP (Benjamin and Gellhorn, 1968; Shirey and Huang, 1969). Phenol has been used to isolate the nucleic acid or protein components (Prestoyko and Busch, 1968; Shelton and Allfrey, 1970). Some milder extraction methods recover only a portion of the NHP fraction (Longon, 1967) or do not separate the RNA components from the NHP fraction (Shoshana, Simpson and Sober, 1972).

In the present work a procedure based on the use of high urea and salt concentrations is described. This method is capable of fractionating small amounts of chromatin into its four major components, DNA, RNA, histones and non-histone proteins. As little as one monolayer of 14×10^6 cells can be handled conveniently. Although only the results for L cell chromatin will be described in detail, the method has been used successfully with chromatin from KB, DON, and rat hepatoma cell lines. The procedure used does not involve use of phenol, ionic detergents or other substances that may irreversibly denature one or more of the above fractions. In fact the high urea and salt conditions used are those experimental conditions initially required to bring about renaturation of chromatin from the individual chromatin fractions (Dahmus and Bonner, 1970).

Isolation of nuclei

It was found that the preparation of purified chromatin isolated directly from whole cells grown in tissue culture by a presently used procedure (Marushige and Bonner, 1966) when stained with Pyronine-Methyl Green stain (Stern, 1968), contained a considerable amount of cytoplasmic

contamination. Further, nucleolar contamination was suspected and was later confirmed by solubilization of chromatin in I M NaCl. An insoluble nucleolar fraction was obtained using the method of Wang (1967).

To eliminate possible complications due to contamination from these sources chromatin was isolated from pure morphologically intact nuclei rather than from whole cells in which both the cell membrane and nuclear membranes were broken together to liberate the chromatin.

Cell nuclei were prepared as follows. The cells (4×10^7 to 4×10^0 cells/ml) were harvested by sedimentation at 650 x g for 10 min at 4° C. The cells were washed once in ice-cold Earle's Balanced Salt solution and collected by recentrifugation. Nuclei were isolated essentially as described by Penman (1966) except that the cells were allowed to swell in hypotonic buffer (0.01 M NaCl, 0.01 M tris, 0.0015 M $MgCl_26H_2O$, pH 7.4) for five min. Triton X-100 was added to a final concentration of 0.2% and the cells were ruptured in a Dounce glass homogenizer (Kontes Glass Co., Vineland, N.J.) using 5-10 strokes. The amount of cell breakage was determined by staining a drop of the homogenate with methyl green/pyronine B stain (1968). Microscopic examination reveals blue nuclei free of red cellular membranes when the cell breakage is complete. The outer nuclear membrane was removed from the washed nuclei by treatment with the non-ionic detergent Tween 80, and the ionic detergent sodium deoxycholate as described by Penman.

It is essential to add the sodium deoxycholate dropwise with vigorous mixing on a vortex type mixer to prevent loss of chromosomal protein components, as well as partial lysis of the nuclei. The rapid mixing probably prevents a local buildup in concentration of the ionic

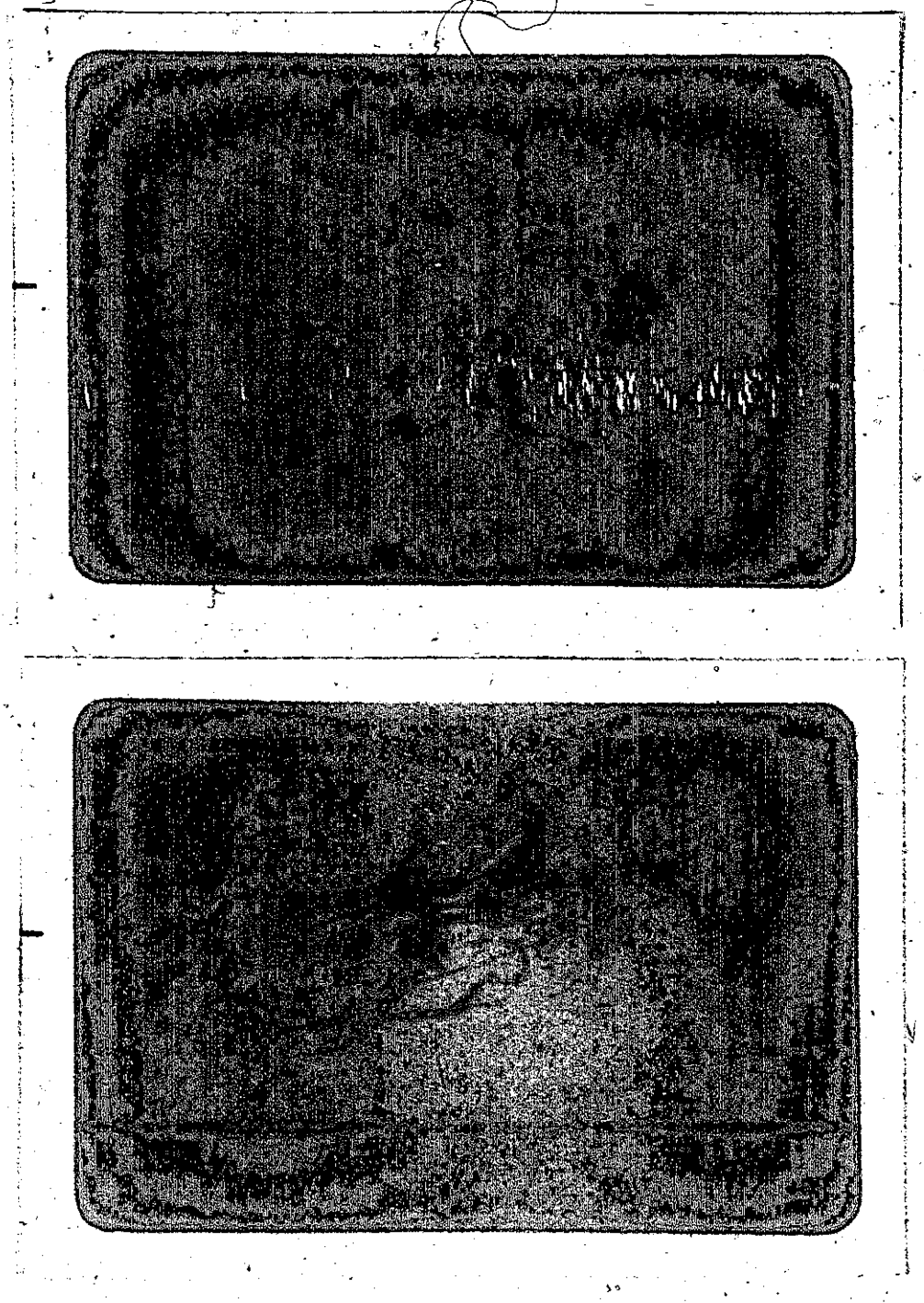


Figure 4. a. A preparation of purified L-cell nuclei stained with methyl green/pyronine B stain.

b. A preparation of purified L-cell chromatin stained with methyl green/pyronine B stain.

detergent to such an extent that it causes loss of some histone or non-histone protein components (Smart and Bonner, 1971). The detergent treated nuclei are collected by centrifugation, 650 x g for 7 min. Figure 4a shows a preparation of L cell nuclei isolated as described above.

Isolation of chromatin

A general flow diagram of the isolation procedure is given in Fig. 5.

When small amounts of chromatin are being isolated it is essential to siliconize all glassware with Silicled (Clay Adams, Parsippany, N.J.) in order to prevent loss due to binding of chromatin to glass.

The pellet obtained after centrifugation of the detergent-treated nuclei was suspended in saline-EDTA (0.075 M NaCl, 0.025 M EDTA, pH 8.0), 2×10^7 nuclei/ml. Purified bentonite (Brownhill, Jones, Stacey, 1959) was added to a final concentration of 100 µg/ml. The bentonite appears to serve three functions. It acts as an RNA'ase inhibitor (Brownhill, Jones, Stacey, 1959; Littauer and Sila, 1962), its abrasive action during homogenization facilitates the breaking of nuclei and nucleoli, and probably it aids in shearing the chromatin. Also for small amounts of chromatin (for example chromatin from a single monolayer of 14×10^6 cells) it serves as an excellent carrier in the numerous homogenizing and centrifugation steps. The nuclei in the saline-EDTA buffer containing bentonite are resuspended using a teflon glass tissue grinder (A.H. Thomas Co., Philadelphia). The nuclear membrane was ruptured by three two-sec sonication treatments (the lowest setting on a Branson model B125 sonifier). The lysed nuclei were examined under the microscope after staining with methyl green-pyronine B stain. The bentonite stains red and appears to aggregate to the blue filaments of chromosomal material. No round intact nuclei could be seen.

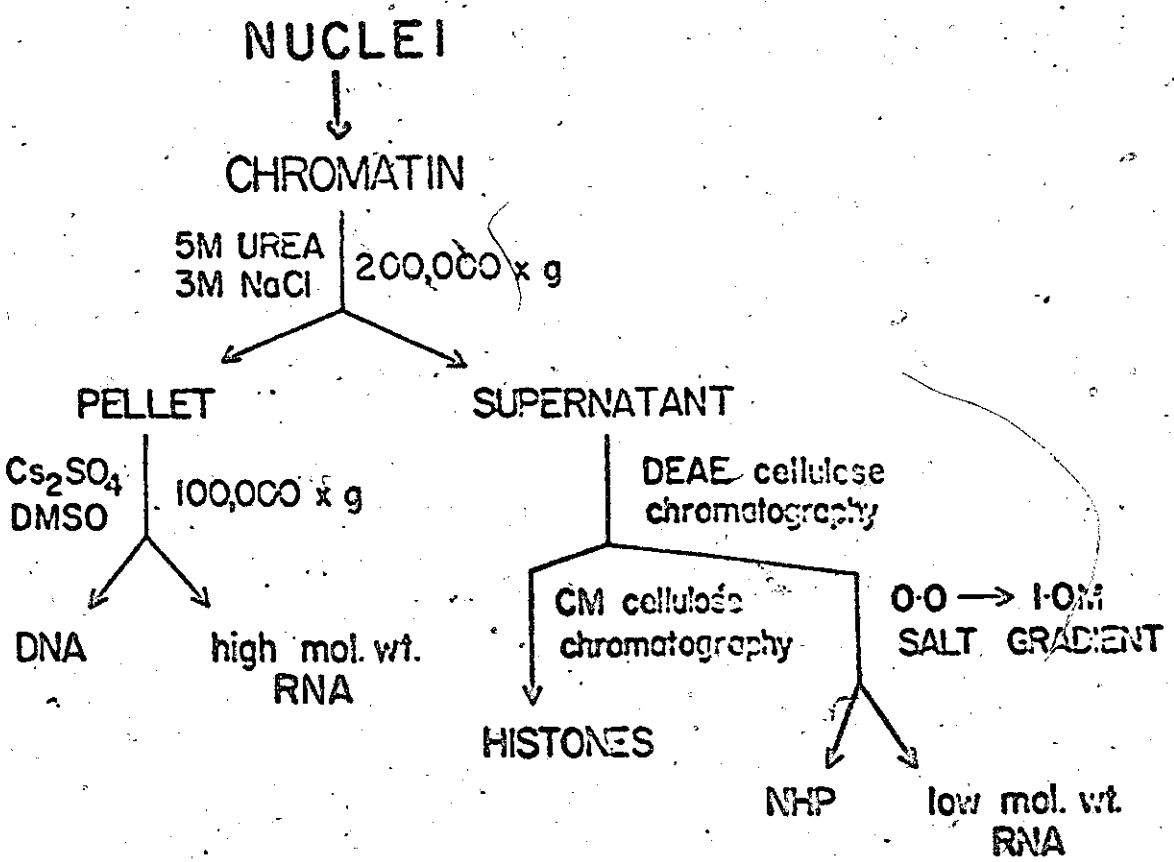


Figure 5. Flow sheet for fractionation of chromatin.

The crude chromatin and bentonite were collected by centrifugation for 10 min at 10,000 x g. The pellet was suspended in the saline-EDTA buffer by homogenization and recollected by centrifugation. The pellet was washed three times with 0.01 M tris, pH 8.0, by repeating the above homogenization and centrifugation steps. Figure 4b shows a preparation of L cell chromatin isolated as described above.

To remove the bentonite and some nucleolar material the chromatin was dissolved in 1 M NaCl, 0.01 M tris, 0.002 M EDTA, 0.002 M 2-mercaptoethanol, pH 8.0 by stirring slowly for 30 min. The viscous solution was centrifuged for one hr at 30,000 x g. The supernatant was carefully removed leaving behind the "nucleolar residual fraction" (Wang, 1968) and bentonite as a pellet. We refer to the material soluble in the 1 M NaCl buffer supernatant as chromatin. This method of obtaining chromatin has been used before (Prestayko and Busch, 1968; Wang, 1968) and yields at least 96% of the total nuclear DNA in our hands. The material insoluble in the 1 M NaCl buffer appears to contain bentonite, nucleolar material and some nuclear membrane-like material (Wang, 1968). The chemical composition of chromatin isolated from KB, L, DON and Rat Hepatoma cells is shown on Table 2.

Fractionation of chromatin components

1. DNA and high molecular wt RNA

Crystalline urea (Schwartz/Mann ultra pure) and solid NaCl was then added to the above chromatin solution such that their final concentration were 5 M and 3 M respectively. The chromatin solution was centrifuged for 24 hr at 200,000 x g, yielding a clear pellet of DNA containing 0.5% RNA. The relative effectiveness in obtaining maximal dissociation of chromatin components using urea, NaCl, guanidine

TABLE 2

CHEMICAL COMPOSITION OF CHROMATIN

CELL TYPE	CONTENT RELATIVE TO DNA		
	DNA°	RNA°°°	PROTEIN°°
KB	1	0·08	1·71
L	1	0·05	1·73
HTC	1	0·07	1·85
Don	1	0·08	1·70

° Determination by the method of Burton, J. Biochem. 62 (1956) 315.

°° Determination by the method of Lowry et al, J. Biol. Chem. 193 (1951) 265.

°°° Determination by the method of Webb, J. Biol. Chem. 221 (1956) 635.

hydrochloride, and SDS have already been published (Martines and Fuks, 1971). I have favoured the use of a system combining two of the above components: 5 M urea, 3 M NaCl. Under these conditions in the presence of 2-mercaptoethanol and EDTA at pH 8.0 the DNA pellet formed during the high speed centrifugation contains only 0.1% of the total chromatin protein. This protein fraction has not been further characterized. From previous work it seems possible that it contains protein components most of which were already dissociated by the 3 M NaCl 5 M urea buffer (Shoshana, Simpson and Gober, 1972).

Throughout the procedure care was taken to keep shearing of the DNA to a minimum. However, brief sonication treatment of the purified nuclei was found to be necessary to ensure rupture of the nuclei. The minimal effect of this treatment on the DNA finally obtained in the high speed centrifugation pellet is seen in Fig. 6. Chromatin was obtained from $H_3{}^{32}PO_4$ labeled cells. The cells were grown in 1.0 mC $H_3{}^{32}PO_4$ in 100 ml of cells (4×10^6 cells/ml) for 6 hr. The DNA pellet from the high speed centrifugation step was dissolved in 0.01 M sodium acetate, 0.001 M 2-mercaptoethanol, 0.2% SDS, 15% sucrose pH 5.4. Unlabeled calf thymus DNA, L-cell r-RNA, t-RNA and bromophenol blue dye were added to act as markers. Chromatography on sepharose 2B shows that almost all of the counts are in the high molecular weight region. However, a small fraction of the counts appeared to reside in the r-RNA region. Treatment with purified RN'ase and DN'ase (Worthington) showed that both DNA and RNA labeled components were present in the r-RNA region. The RNA was separated from the large excess of DNA by equilibrium density centrifugation in a cesium sulfate solution containing

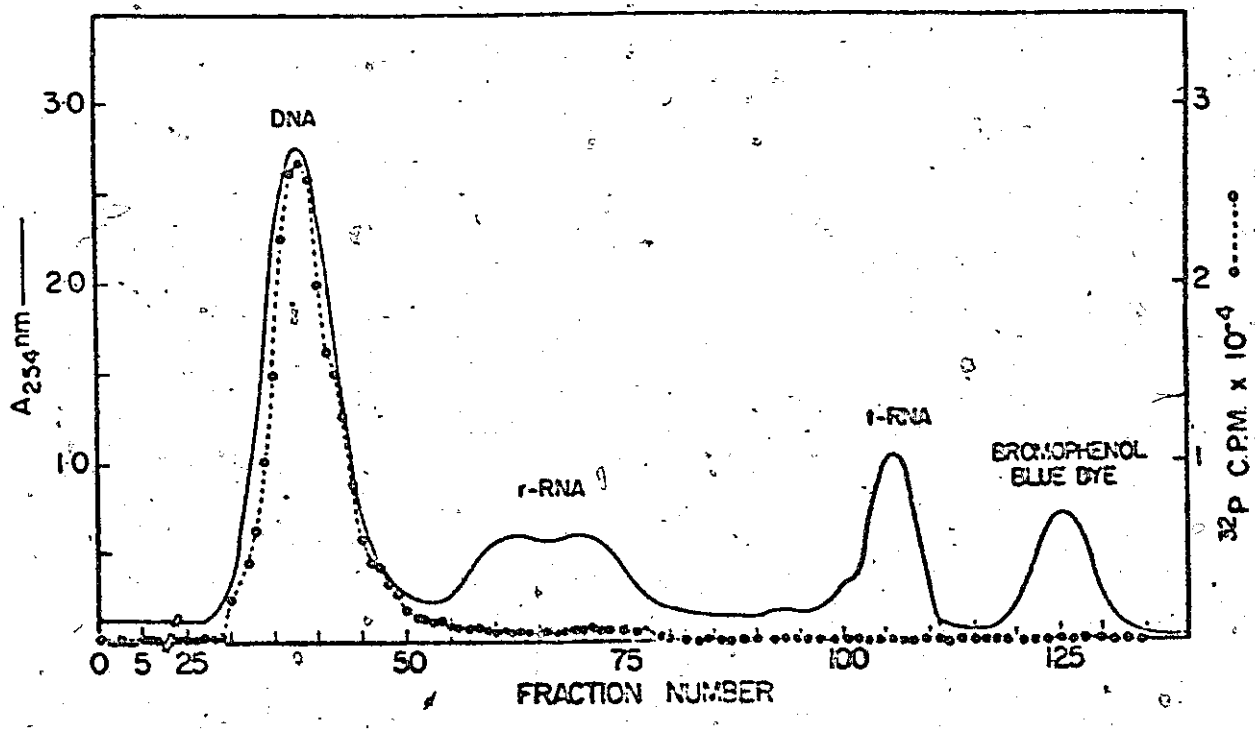


Figure 6. Chromatography on a column 2.5 cm diameter x 100 cm of sepharose 2B of material present in the high speed centrifugation pellet. A_{254} profile of unlabeled calf thymus DNA, L-cell r-RNA, t-RNA and bromophenol blue dye markers. o——o ^{32}P CPM profile of labeled DNA and RNA present in pellet.

dimethylsulfoxide (Williams and Vinograd, 1971). In a typical experiment the chromatin DNA pellet formed from 10^7 L-cells P^{32} labeled was dissolved in 4.0 ml of a solution containing 0.01 M tris; 0.002 M EDTA; 0.002 M 2-mercaptoethanol; 1.491 M Cs_2SO_4; 10% DMSO pH 8.0.

The presence of 10% DMSO was found to be essential to prevent precipitation of the RNA. The solution was filtered through a glass fiber filter (Reeve Angel 934AH) into a centrifuge tube. The remainder of the tube was covered with liquid paraffin and centrifuged for 58 hr at 32,000 rpm in a Spinco SW 50 rotor at 25° C. Forty fractions were collected from each gradient. Fig. 7 shows the complete separation of DNA from the high molecular weight RNA. The RNA fraction was pooled and dialyzed against 0.01 M sodium acetate pH 4.5 after adding cold carrier KB cell r-RNA and t-RNA. The RNA was then collected by precipitation with ethanol and stored at -20° C.

2. Histones

The supernatant of the solution from the high speed centrifugation step containing histones, non-histone proteins and RNA was poured off and dialyzed for six hours against two changes of 100 volumes of 5 M urea, 0.01 M tris, 0.002 M EDTA, 0.002 M 2-mercaptoethanol pH 8.0. The dialyzed solution was passed through a DEAE-cellulose column (for a sample derived from 4×10^9 cells, a column 0.5 cm diameter x 12 cm is sufficient). Whatman microgranular pre-swollen DE-52 DEAE-cellulose was used. The histone fraction passes through the column in the runoff peak. For small quantities of cells it is not possible to quantitatively concentrate the protein in this fraction by precipitation with organic solvents (Johns, 1964). The runoff peak fraction was brought to pH 4.5

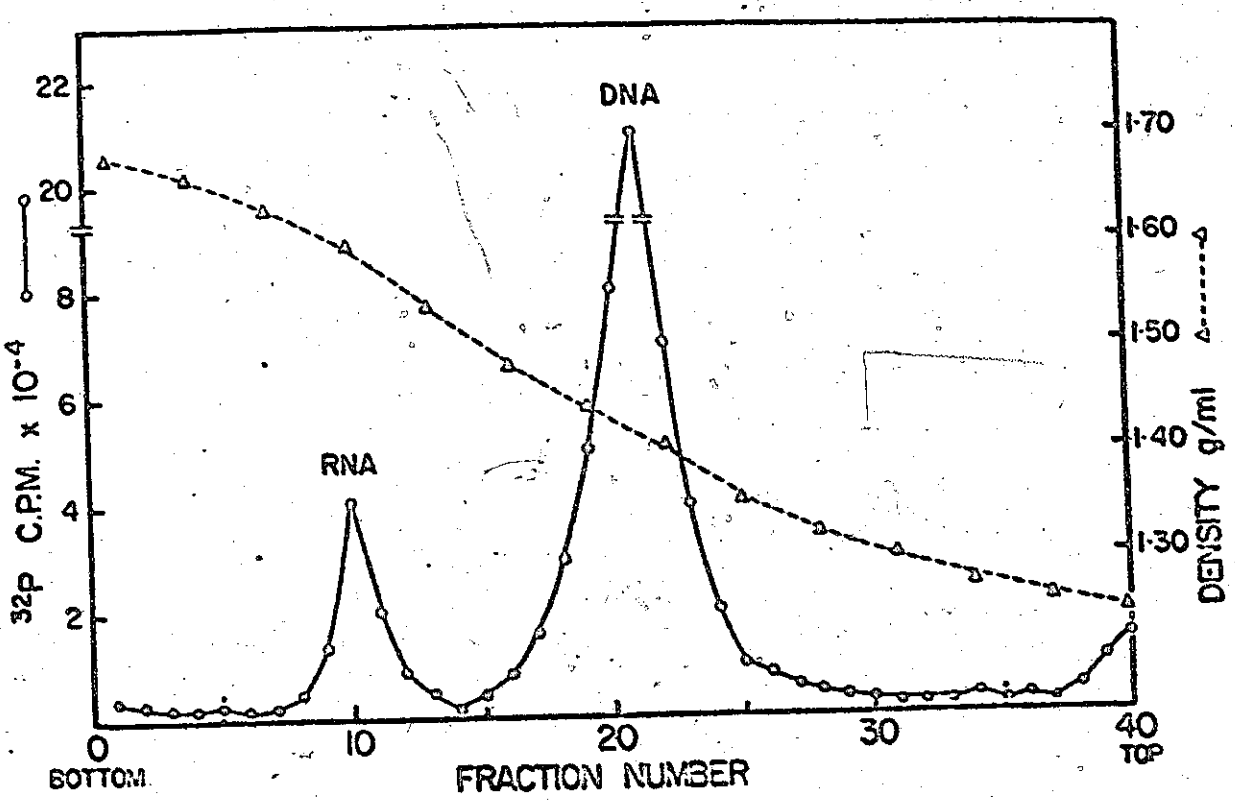


Figure 7. Equilibrium density centrifugation in Cs_2SO_4 of ^{32}P labeled DNA and RNA present in high speed centrifugation pellet of L-cells.

by addition of glacial acetic acid and the solution passed through a CM-cellulose column (0.6 cm diameter x 8 cm). The column was washed with 0.5 M acetate buffer, pH 4.5 containing 0.005 M 2-mercaptoethanol. The total histone fraction was collected in a few drops by elution with 0.02 M HCl. (Subfractionation of the total histone fraction can be obtained using different elution buffers instead of 0.02 M HCl which elutes all the histones from the column together (Johns, Phillips, Simpson and Butler, 1969); this was not attempted here). The histone fraction was lyophilyzed and stored at -20° C.

3. NHP and low molecular weight RNA fractions

The NHP and low mol wt RNA components remained bound to the DEAE-cellulose column. They were eluted with a salt gradient of 0.0 M NaCl to 1.0 M NaCl in a buffer containing 5 M urea, 0.02 M tris, 0.002 M EDTA and 0.002 M 2-mercaptoethanol. A high protein or RNA concentration in a small volume of elution buffer facilitates the further analysis of these components with polyacrylamide gel electrophoresis. For this reason we use an accurate steep salt gradient. The gradient was made with a sucrose gradient maker (Buchler Instruments, North Lee, N.J.) using 40 ml of buffer in each reservoir. We have found that although the RNA components are relatively insensitive to the presence of urea in the eluting buffer the NHP fraction is sensitive. Complete absence of urea appears to allow the NHP to remain bound to the column at concentrations close to that required to elute the chromatin RNA components (Patel and Wang, 1964). Five M urea in the elution buffer allows almost complete separation of the NHP fraction and the RNA fraction.

Fig.8 illustrates the A_{254} elution profile from the DEAE-cellulose

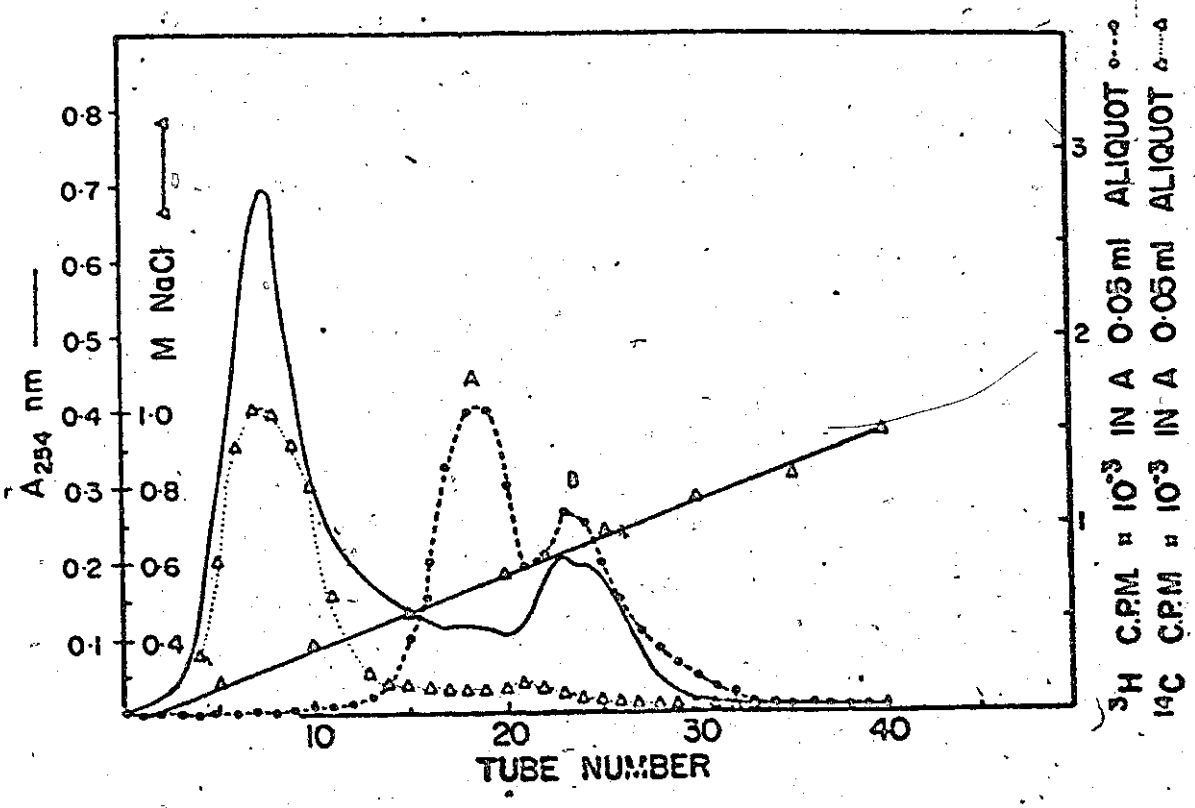


Figure 8. A_{254} elution profile of L-cell NHP and chromatin low molecular wt. RNA from a 0.5 cm diameter x 12 cm DEAE cellulose column. Δ.....Δ ^{14}C CPM profile of material labeled with ^{14}C leucine. o——o ^{3}H CPM profile of material labeled with ^{3}H uridine.

column. Two ml fractions were collected. This is a profile that was obtained from chromatin starting with 2 x 10^8 L-cells grown in the presence of ^{3}H-uridine 1 mC/100 ml and ^{14}C-leucine 50 µC/100 ml of medium for 6 hr. Fractions 0 to 12 contain most of the ^{14}C leucine counts and will be termed the "NHP fraction". The ^{3}H uridine counts elute as two radioactive peaks; the first, fractions 15-20 we call peak A, the second fractions 22-27 we call peak B. We shall consider each of the pooled fractions in turn.

The pooled fractions of NHP or RNA were dialyzed against 0.1% SDS, 0.1% 2-mercaptoethanol and analyzed by gel electrophoresis. When less than 4 x 10^8 cells was used as starting material, 0.2 vols of 1 M sodium acetate, pH 4.5, was added to the pooled fractions followed by unlabeled yeast soluble-RNA (B.D.H., England) and 2 vols of ethanol. The total pooled fraction was then collected as a precipitate and stored at -20° C.

Polyacrylamide gel electrophoresis

1. Analytical acidic gels

For analysis of the histone fraction the method of Panyim and Chalkley (1969) was used. Acrylamide, N,N'-bis-acrylamide and TEMED were purchased from Eastman Chemicals.

Acrylamide gel formation requires the mixing of acrylamide with polymerization accelerator and catalyst, all of which are stored in separate containers. These solutions can be prepared in the calculated buffer and urea concentrations to give the desired experimental conditions in the final gel. The following solutions were employed to give a 15%

polyacrylamide gel in 2.5 M urea at a final pH = 3.2 (after pre-electrophoresis): Solution A: 60% acrylamide (w/v) and 0.4% N,N'-bisacrylamide (w/v) in H_2O; Solution B: 43.2% of glacial acetic acid (v/v) and 4% of TEMED (w/v) in H_2O; Solution C: 0.2% $(NH_4)_2S_2O_8$ (w/v) in 4 M urea, freshly prepared. Solutions A and B were stored at 0° and warmed to room temperature before mixing in the ratios: 1 part of B, 2 parts of A, and 5 parts of C. Polmerization takes about 2 hrs to go to completion. The final dimensions of each gel was 0.75 cms internal diameter x 15 cms long.

All gels were pre-run to remove all charged species other than protons and acetate anions. This electrophoresis was routinely monitored by observing benzene-azo-α-naphthylamine through one gel. The dye moves about 1.65 cm/hr at 2 mA/tube. The dye is red in the pH range used, carries a single positive charge and has a molecular weight greater than that of the other ions; pre-electrophoresis is, therefore, judged complete when the dye is eluted.

Electrophoresis of histones. Histones (1 mg/ml) were dissolved in 0.9 N acetic acid, 15% sucrose. The amount of solution applied to the gel was 20 µl. A spacer gel is neither required nor used in this system. Ohmic heat should be minimized to the extent that it does not cause curved bands. This is achieved at the ionic strength used if the current is maintained at 2 mA/gel. Both the upper (anode) and lower (cathode) buffer was 0.9 M acetic acid.

After electrophoresis each gel was stained for at least 5 hrs with 0.1% amido black in 20% ethanol, 7% acetic acid, and water. The gels were destained with 7% acetic acid. The positions of the stained

histone fractions was then determined by scanning the gel in a Gilford gel scanner (Model no. 2410) using a visible light source, 600 nm.

After staining each gel was cut into 2 mm slices. The slices were individually combusted in a Packard Tri-Carb sample oxidizer and the amount of ^{3}H arginine and ^{3}H lysine was determined in a Nuclear Chicago Mark 1 Scintillation Counter.

2. Analytical basic gels

For analysis of the NHP and low molecular wt RNA fractions a two-phase gel system was used. A 0.75 cm internal diameter x 15 cm "separation gel" was prepared by polymerizing 10 ml of a solution containing: 12.5% acrylamide, 0.375% N,N'-methylene-bisacrylamide, 0.499 M tris and 0.1% SDS, with 10 µl of TEMED and 100 µl of a 10% solution of ammonium persulfate. A 0.5 cm high "stacking gel" was polymerized on top of the separation gel with a solution containing: 3% acrylamide, 0.3% N,N'-methylene-bisacrylamide, 0.042 M tris and 0.1% SDS, 10 ml of this solution was polymerized in the same way as the separation gel. The upper buffer (gel cathode) and the lower electrode buffer (the gel anode) was 0.025 M tris, 0.192 M glycine and 0.1% SDS, pH 8.1.

In many instances there were sufficient counts (> 7200,000 CPM/ml) in the CLMM-RNA or NHP fractions to analyse them directly unconcentrated with this gel system.

The fractions were dialyzed against 0.1% SDS and 0.1% 2-mercapto-ethanol. 100 µl aliquots were mixed with a few crystals of urea, 2 µl of 0.05% bromophenol blue and 0.04% trypan blue, and carefully layered on top of the stacking gel. In instances where small amounts of chromatin

were used as the starting material there were usually not sufficient counts in 100 µl aliquots from the DEAE-cellulose fractions to give good resolution of individual RNA or protein components after acrylamide gel electrophoresis. In such cases it was first necessary to precipitate the total CLMW-RNA or NHP fractions with two volumes of ethanol after addition of 0.2 vol of 1 M sodium acetate pH 4.5 and unlabeled yeast soluble RNA (B.D.H. England) 0.2 mg/ml. The precipitate was collected after 4 hrs at -20° C by centrifugation and dissolved in 100 µl of 1% SDS and 1% 2-mercaptoethanol. Before placing the sample on the gel, urea, bromophenol blue and trypan blue were added as described above. Electrophoresis was for about eight hours at 4 mA/gel. After electrophoresis each gel was cut into 2 or 1 mm slices. For ^{32}P labeled material the slices were counted directly in 10 ml of Aquasol (New England Nuclear Inc.). For ^{3}H and ^{14}C labeled material the slices were individually combusted in a Packard Tri-Carb sample oxidizer. The amount of ^{3}H or ^{14}C labeled material in each slice was determined in a Nuclear Chicago Mark 1 Scintillation Counter.

In experiments where an accurate estimation of the S value of the individual CLMW-RNA fractions was obtained the "stacking gel" was omitted. The RNA fractions were separated by electrophoresis directly using the 12.5% acrylamide separation gel. Under these conditions the volume of sample applied to the gel should be less than 20 µl.

For analysis of the chromatin high molecular wt RNA fraction obtained from the DNA pellet was analyzed on a different acrylamide gel system since due to its large size it is unable to penetrate the 12.5% acrylamide separation gel. This component was analyzed on a 2.09%

acrylamide, 0.11% N,N',-methylene-bisacrylamide, 0.2% agarose gel system. The procedure described by Bartoov (1971) was followed. The positions of the unlabeled marker rat liver r-RNA and t-RNA was determined by scanning the gel in a Gilford gel scanner (Model no. 2410).

3. Preparative basic slab gels

This technique was found to be particularly useful for fractionation of ^{32}P-labeled RNA with high specific activity, since the slabs can be easily subjected to autoradiography, a method of choice for detection of ^{32}P-labeled compounds. Visualization of the resolved polyribonucleotide bands by other means, e.g., by staining, is equally possible. Of the many apparatus for gel slab electrophoresis proposed in the literature, the cell described by De Wachter and Fiers (1971) was preferred for its simplicity and because it can be easily dismantled. A few modifications were introduced, in particular the size of the cell and the method of sample application.

The gel slab is enclosed between two glass plates, 4 mm thick, 25 cm high, and 25 cm wide. The space between the plates is 2 or 4 mm and is determined by the thickness of two Perspex (Lucite, Plexiglas) strips, 1.5 x 2.5 cm, which close the cell at the sides. Grease provides a leak-proof joint between the glass and the Perspex, and steel clips keep the plates firmly together. The lower side of the cell is temporarily closed with plasticine while the acrylamide solution is poured into it and allowed to polymerize. Slots for sample application are formed in the gel by inserting a Perspex slot former, 15 x 10 cm, of the same thickness as the strips, in the liquid before polymerization occurs. The number and width of the slots depend on the number of

samples to be applied, 1 cm distance being left between the slots.

For electrophoresis the cell is put in a 30 x 30 x 5 cm wide tank provided with a platinum wire electrode, which serves as lower buffer reservoir. The gel is covered with a layer of buffer and electrical contact with a similar upper buffer reservoir is established by a wick of Whatman 3 MM paper soaked with buffer. The wick is 16 cm wide, and one layer of paper is used per millimeter gel thickness. The distance between the cell and the buffer reservoir is kept as small as possible to minimize the electrical resistance of the wick. The wick is lined with flexible polyethylene sheet to prevent crystallization.

The same type of separation and stacking gel is used as for the analytical basic gel system, namely a 12.5% acrylamide separation gel 15 cm high, on top of which is placed a 0.5 cm high 3% acrylamide stacking gel.

Before the samples are loaded on the gel, the density is increased by adding a few crystals of urea. Also added are bromophenol blue and trypan blue dye markers.

For loading, the paper wick is taken away and samples are applied from a micropipette or pointed capillary. The tip is held under the surface of the buffer a few millimeters above the gel surface while the pipette is slowly blown out. The dense sample falls through the buffer and forms a neat layer on the gel.

The sample volume is related to the size of the slot so that the layer formed does not exceed 5 mm. The presence of a dye makes it easy to observe the loading operation while the migration of the dye band gives a measure of the progress of the electrophoresis. As soon as all

the samples are loaded on the gel, the wick is carefully replaced. Electrophoresis was maintained at 10 volts/cm of gel vertical height until the bromophenol blue dye marker was within two cm from the bottom of the gel.

After completion of the run the wick is taken away and the buffer layer on top of the gel is sucked off with a Pasteur pipette. The gel cell is taken out of the buffer compartment and placed horizontally, the clips are taken off, and the top glass plate is carefully loosened from the gel slab by inserting a knife between the plate and the Perspex strip. The strips are removed as well and most of the grease is carefully rubbed from the bottom plate with a cotton pad wetted with acetone. The gel slab is covered with a sheet of thin plastic film (Saran wrap) which is folded back over the edges of the glass plate. This prevents the gel from drying out while virtually no radiation is absorbed. The gel slab is taken to the darkroom and covered with a 30 x 25 cm sheet of Kodak royal blue medical X-ray film. Good contact with the gel surface is obtained by putting a glass plate on top. The exposure time depends on the activity in the sample, the number of bands over which it is distributed, and the thickness of the slab. Times usually ranged from 1-3 days. The X-ray film was developed in a Kodak RP developer.

The RNA bands of interest are cut out of the slab. The excised gel band is ground in a small mortar and the resulting granular paste is taken up in 10 ml ice-cold 0.2 M NaCl. Carrier yeast soluble RNA was added to this suspension. The crushed acrylamide was removed by centrifugation and the RNA in the supernatant precipitated with a final concentration of 2.5% perchloric acid.

4. Preparative two-dimensional polyacrylamide gel electrophoresis of ^{32}P-labeled RNA

Fractionation of RNA at pH 8 results in a pattern of bands, the mobility of which decreases with the logarithm of the chain length of the fragments they contain (De Wachter et al., 1971). If the vertical height of gel containing these bands is cut out of the slab placed horizontally above another gel at pH 3.5 then when the RNA bands pass down into this large slab the separation of the RNA components will be a function of not only the chain length but also the base composition. This is because at pH 3.5 the net charge is a function both of chain length and of base composition. Moreover, protonation of the cytosine and adenine residues and the presence of SDS should result in a dissociation of chains kept together by base-pairing between complementary segments leading to conformational changes in the RNA. This type of two-dimensional polyacrylamide gel electrophoresis was originally proposed by Raymond and Aurell (1962). The procedure reported here is a modification of the method described by De Wachter and Fiers (1972). It is suitable for fractionation of complex RNA mixtures. The apparatus was the same as that used for the preparative basic slab gels.

Since the net charge of an RNA molecule is appreciably lower at pH 3.5 than at pH 8, the acrylamide concentration of the acid gel must be lower than that of the neutral gel if the molecule is to have approximately the same mobility in both directions. It is then advantageous to choose the acid gel as the first dimension and the neutral gel as the second. At the start of the second separation the pH difference between the two gels rapidly disappears. The RNA moves from a more diluted

to a more concentrated gel, which results in a sudden drop in mobility and a concomitant zone sharpening. Apart from these considerations it is desirable to separate first under the most denaturing conditions, i.e., at acid pH and in the presence of SDS.

The acrylamide concentrations chosen for the first dimension acidic gel was 6.25% acrylamide, 0.187% N,N'-methylene-bisacrylamide. The buffer in the reservoirs and in the gel has the same composition, 0.025 M citric acid, 0.1% SDS, the pH was adjusted to 3.5 with NaOH. Conditions of assembly of the gel and electrophoresis is the same as that described for the preparative slab basic gels. The RNA bands from this gel are located by autoradiography. The strip of gel is transferred to a second glass plate which will form the back wall of the electrophoresis cell for the second dimension.

The cell is then assembled, put upright into a trough with Plasticine, and filled with the pH 8.0 gel and buffer system. This is the same gel as used for the preparative slab basic gel. The procedure to separate and locate the RNA on this second dimension is essentially the same as that described for the preparative slab basic gel, the thin pH 3.5 6.25% acrylamide gel along the top of the 12.5% acrylamide replacing the 3% acrylamide separation gel.

Column chromatography

1. Sephadex

This is a bead-formed, dextran gel. It is prepared by cross-linking selected dextran fractions with epichlorohydrin. It is a chromatographic material capable of separating substances according to

molecular size. The separation method is most commonly known as gel filtration or gel chromatography. Sephadex is strongly hydrophilic and thus swells in water and electrolyte solutions. Various types of Sephadex are available, differing in their swelling properties. The degree of swelling is an important characteristic of the gel. Gels in which the matrix is a minor component are used for fractionation of high molecular weight substances, whereas the denser gels are used for separation of low molecular weight compounds.

The CLMW-RNA was subfractionated by gel filtration through sephadex G-100 using a column 1 cm diameter x 250 cm. Correct packing of the sephadex in this column is of the utmost importance for good performance of a gel filtration column. Irregularities in the packing give rise to uneven flow through the column which often results in zone broadening. This will inevitably give rise to extensive dilution which can lead to mixing of otherwise well separated zones. The flow rates obtainable are affected by the packing technique. If too high an operating pressure is used during packing the gel bed becomes compressed thus causing high resistance to flow.

The gel is previously swollen in the elution buffer of 0.01 M sodium acetate, 0.001 M 2-mercaptoethanol, 0.2% SDS pH 5.4. An overnight excess of eluant is removed until a fairly thick slurry is formed. This slurry should not be so thick that it retains air-bubbles. The slurry should be poured carefully into the column either down the wall of the column or down a glass rod. If the column is not filled with slurry add eluant until the column is completely full. Connect an eluant reservoir to the column and remove the last traces of air through the

air vent in the column top piece. An air pocket in the top of the column will cause the eluant to drop into the gel suspension and will thus disturb the even settling of the gel particles. It may not always be possible to fill the column completely with gel at one time. In such cases an extension tube should be mounted on top of the column. A Gel and Eluant Reservoir has been specially designed for this purpose by Pharmacia Fine Chemicals. Pour all the gel required for the experiment into the column and the gel reservoir and start the flow immediately. Two to three column volumes of eluant should be passed through the column in order to stabilize and equilibrate the gel bed.

To apply the CLMW-RNA fraction most of the eluant above the gel surface is removed by suction. The column outlet is opened and the remaining eluant drained away. After closing the outlet the sample is carefully layered on top of the bed. The column outlet is then opened. After the sample has drained into the bed, the gel surface and the column wall in contact with the sample are washed with a small amount of eluant. Under no circumstances should the bed run dry during this procedure. The column is then filled with eluant and connected to a reservoir of elution buffer. Four ml fractions were collected and the amount of radioactivity in each fraction was determined by removing 0.1 ml aliquots and adding it to 10 ml of Aquasol (New England Nuclear) and counting in a Beckman LS-2008 scintillation counter. Marker cytoplasmic 4S, 5S and 5.5S RNA's were similarly purified on this column.

2. Sepharose.

This is a bead-form agarose gel. It has proved to be an excellent matrix for gel filtration of high molecular wt. molecules totally excluded

by sephadex G-200. The column is made up and run exactly as described for the sephadex columns.

L-cell DNA was characterized by gel filtration through sepharose 2B (Pharmacia, Sweden) using a column 2.5 cms diameter x 100 cms. The elution buffer was 0.01 M sodium acetate, 0.001 M 2-mercaptoethanol, 0.2% SDS pH 5.4. Four ml fractions were collected and the amount of radioactivity in each fraction was determined by removing 0.1 ml aliquots and adding it to 10 ml of aquasol and counting as described above.

3. DEAE-cellulose

This is a microgranular form of cellulose which has a number of sugar hydroxyl groups substituted with diethylaminoethyl groups. This material has the ability to absorb acids or ampholytes above their isoelectric point. This absorption process is inhibited to a certain degree - depending upon the species absorbed, by increasing the ionic strength of the buffer in which the DEAE-cellulose is surrounded. The differential release of protein or RNA components from a column of DEAE cellulose with an increasing salt gradient in the elution buffer is the basis of this chromatographic procedure.

Whatman "New Fiberous DE-52" cellulose was used for all DEAE cellulose columns. This material is supplied in a wet, fully swollen form and thus does not require pre-cycling. All columns were made up and used exactly as recommended by the manufacturer. The columns were always washed with ten times their own volume of buffer before placing the sample on the column.

4. CM-cellulose

This is a microgranular form of cellulose which has a number of

its sugar hydroxyl groups substituted with carboxymethyl groups. This material has the ability to absorb bases and ampholytes below their isoelectric point. As for the DEAE-cellulose columns we can take advantage of the differential release of protein components from the CM cellulose by changing the ionic strength (or pH) of the buffer passing through the column.

Whatman "New Advanced Fiberous CM-23" carboxymethyl cellulose was used for all CM-cellulose columns. All columns were made up and used exactly as recommended by the manufacturer.

5. Methylated albumin kieselguhr

The total content of RNA present in KB cell chromatin was analyzed on a MAK column. Lerman (1955) first introduced the use of a column of methylated serum albumin-coated kieselguhr for the fractionation of pneumoccocal DNA. This method was later improved and extended by Mandell and Hershey (1960) to the fractionation of DNA and RNA. The modified technique has been successfully used by many workers for the separation of RNA from DNA, fractionation of various classes of RNA or DNA, and fractionation of sRNA. The total chromatin RNA was analyzed on a MAK column according to Osawa and Sibatani (1967). Hyflo supercel was washed with 0.1 N HCl, H_2O until neutral, 0.1 N NaOH, and again with H_2O until neutral and dried. Methylated albumin was prepared by dissolving 5 g of bovine serum albumin fraction V in 500 ml of absolute methanol to which 4.2 ml of 12 N HCl was added. The solution was kept in the dark for 3 days at 25° C and was shaken from time to time. The methylated albumin sediment was washed twice with absolute methanol and twice with peroxide-free ether (distilled over ferrosulphate), dried and

stored at -20° C.

Thirty gms of washed Hyflo supercel was suspended in 150 ml of 0.1 M buffered saline (0.1 M NaCl, 0.05 M sodium phosphate, pH 6.7) boiled for 1 minute then cooled to 35° C. Ten ml of 1% (w/v) methylated albumin was added, stirred and then poured quickly to make a 160 x 22 mm column which was jacketed and heated to 35° C. After the MAK settled down a layer of 0.5 cm of Hyflo supercel suspended in 0.1 M buffered saline was added, to serve as a mechanical barrier to the working portion of the column. The bed volume of the column was about 60 ml and the liquid displacement volume was about 54 ml. The column was washed with 100 ml of 0.1 M buffered saline then loaded with about 4 mg of RNA dissolved in 40 ml of 0.1 M buffered saline. Reservoirs of 400 ml of 1.2 M NaCl 0.05 M sodium phosphate, pH 6.7 and 800 ml of 0.1 M NaCl, 0.05 M sodium phosphate, pH 6.7 were incubated at 35° C and connected to a Phoenix varipump Model 4000 which pumped a linear salt gradient through the column at a rate of 1 ml/minute. Three ml fractions were collected, the optical density at 254 nm was recorded continuously by an ISCO UV analyzer Model 222 and an ISCO chart recorder Model 170. Fractions were checked for NaCl concentration with a conductivity bridge, Model 31 (Yellow Springs Instrument Co.) and counted for radioactivity.

Sucrose gradients

The chromatin high molecular weight RNA fraction was analyzed using an isokinetic 15% to 30% sucrose gradient in 0.1 M sodium acetate and 0.5% SDS, pH 6.0 (Noll, 1967). Ten ml gradients were centrifuged for 17 hr in an IEC SB-283 rotor at 25,000 rpm at 25° C. Optical density profiles were monitored with a Pharmacia UV optical unit during

withdrawal of the gradient from the bottom of the tube. The amount of radioactivity in each fraction (0.2 ml) was determined by removing 0.1 ml aliquots and adding it to 10 ml of aquasol (New England Nuclear) and counting in a Beckman LS-2008 scintillation counter.

Base composition of RNA

The ^{32}P-RNA fractions were hydrolyzed with 10% aqueous piperidine as described previously (Sedat and Hall, 1965). Two chromatographic systems were used to separate the nucleotide components. One system capable of separating many of the modified nucleotide components involves two-dimensional chromatography on cellulose plates (Eastman 6065 cellulose, 20 cms x 20 cms) (Seno, Kobayashi and Nishimura, 1968). The solvent systems were isobutyric acid - 0.5 M NH_3; 5:3, by vol, and isopropanol-conc HCl-water; 70:15:15 by vol.) The one dimension chromatographic system of Lane (1963) was used when it was established that there were no modified nucleotide components present in an RNA fraction. The location of each nucleotide was determined by exposing the chromatogram to a Kodak RP/M X-OMAT rapid processing X-ray film. Exposure time ranged from one to four days. The autoradiography correlated exactly with the position of the ultra-violet absorbing spots of the unlabeled carrier nucleotides. The amount of radioactivity in each spot was determined by cutting out the spot and counting it in toluene scintillator fluid.

Preparation of t-RNA and r-RNA

Total KB and L-cell cytoplasmic RNA was isolated from the cytoplasmic fraction using the method of Attardi, Parnas, Huang and Attardi (1966). Partial separation of the t-RNA and r-RNA fractions was

obtained with m-cresol precipitation (Kirby, 1968). Each fraction was further purified by chromatography on a Sephadex G-100 column (Gilbert, Larsen, Lelong and Boiron, 1965). The t-RNA or r-RNA fraction was concentrated by lyophilyzation, resuspension in a small volume of water and precipitation with ethanol.

Preparation of ^{14}C labeled 4S, 5S and 5.5S RNA's

About 4 x 10^8 cells were grown in the presence of 10 µC of ^{14}C uridine, 60 mC/mM in 200 ml of minimum essential medium for 17 hr at 37° C. The 4S, 5S and "7S" RNA components were isolated exactly as described by Pene, Knight, and Darnell (1968). More recent data suggests that the 7S RNA has an S value of 5.5S (Sy and McCarthy, 1970).

Preparation of histones

To identify the histone fraction isolated in this new procedure to fractionate chromatin, unlabeled histones were isolated from L cells using the classical method of extraction with 0.4 M H_2SO_4, 0.05 M $NaHSO_3$ (Panyim and Chalkley, 1969). Five distinct bands were obtained in the 15% acrylamide acidic gel electrophoresis system. The proteins were stained by suspending for two hours the gels in a solution of 0.2% amido black in 7% acetic acid. The gels were destained electrophoretically in 5% acetic acid and immediately scanned at 600 nm on a Gilford 2000 spectrophotometer equipped with a Gilford gel scanner (Model no. 2410).

Chemical analysis

DNA was assayed by the method of Burton (1956) using calf thymus DNA (Sigma) as a standard. RNA was assayed by the method of Webb (1956) using yeast soluble RNA (D.D.H. England) as a standard. Protein was assayed by the method of Lowry, Rosenbough, Farr and Randall (1966)

using bovine serum albumin (Sigma) as a standard. The density of the cesium sulphate gradient was determined as described by Williams and Vinograd (1971).

Reagents

Only the purest available chemicals were used. Urea solutions were purified by passing an 8 M urea solution (Baker, analar grade) through an AG 501-X8 (Bio-Rad Laboratories) column 4 cm diameter x 4 cm. Other reagents were added diluting the urea concentration to 5 M. The buffers were always stored at 4° C and used within 36 hr of preparation. Leucine-^{14}C (uniform labeled) 270 mCi/mmole, lysine-4,5-^{3}H 5000 mCi/mmole, arginine-5-^{3}H 5000 mCi/mmole, uridine-5-^{3}H 20,000 mCi/mmole, uridine-2-^{14}C 60 mCi/mmole were obtained from Amersham/Searle, Toronto. ^{32}P-H_3PO_4 carrier free was obtained from New England Nuclear, Montreal. 5,6-Dihydro uridine (2' or 3') monophosphates (mixed isomers) was made by hydrogenation of UMP with hydrogen over a rhodium catalyst (Schein and Schein, 1968).

III RESULTS

To understand how the eucaryotic cell regulates the expression of specific genes in its chromosomes, it is first necessary to know exactly what are the molecules present in this very complex material. We might then ask are all of these molecules present in chromosomes in the cell, or do they arise by, in some way, binding to the chromosomal complex during its isolation? Are the chromosomal components isolated degradation or otherwise modified products of larger molecules that exist in the cell? What part do these molecules play in the transcriptional process? How is the whole chromosomal apparatus built, duplicated and regulated? The answers to these questions will occupy the minds of men for many years to come. The reward will be the possibility of explaining and possibly modulating the troubles of man those of which genetic basis for their existance.

Initial work with chromosomal RNA

We have seen that the chromosomal RNA component isolated by Bonner et al. (1968) is unique in that it appears to have some protein covalently bound to it. It is thus often known as "Protein bound RNA".

They have isolated this RNA by utilizing the difference in density one would expect between free RNA and a protein/RNA complex. High speed centrifugation of pure chromatin in 4 M CsCl yields a pellet of DNA (also some high molecular wt. RNA). The protein present floats as a "skin" on the surface of the denser CsCl. Present in the skin also

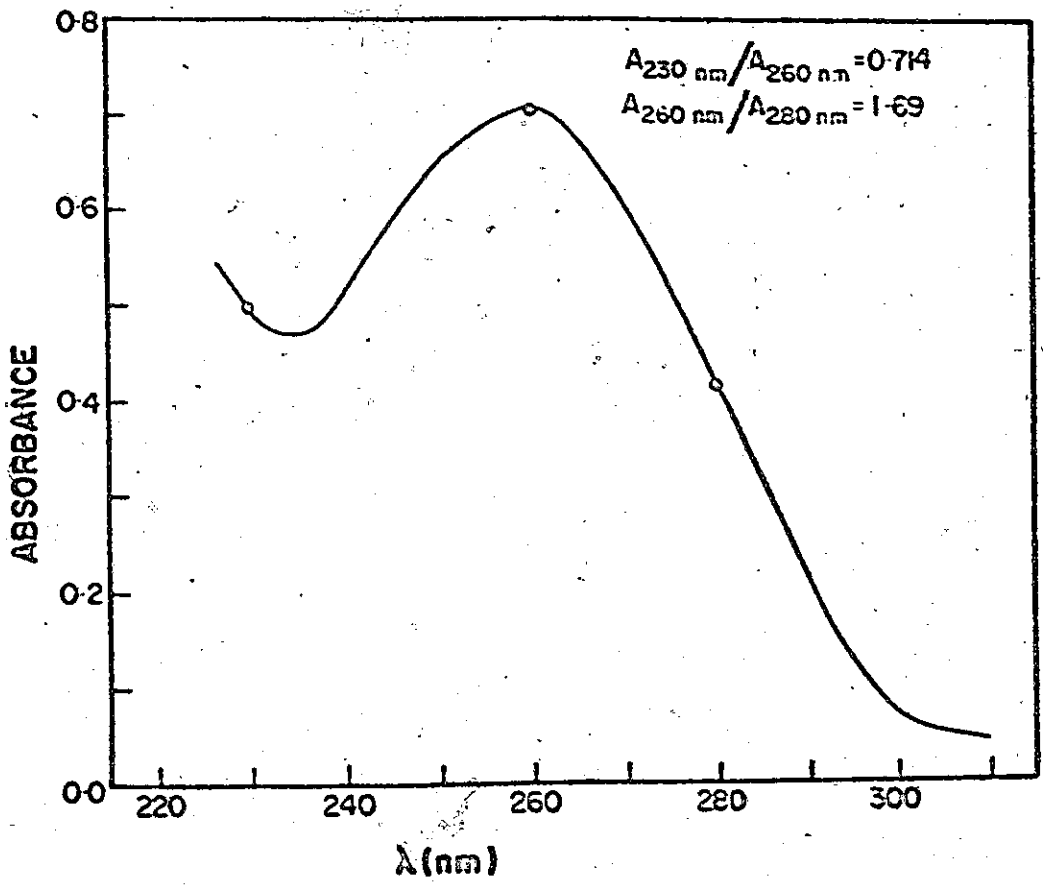


Figure 9 . U.V. spectrum of calf thymus isolated as described by Shih and Bonner (1969).

TABLE 3

CHEMICAL COMPOSITION OF PURIFIED CALF THYMUS CHROMATIN

COMPONENT	MASS RATIO
DNA	1·00°
PROTEIN	1·79°°
RNA	0·01°°°

° Determination by the method of Burton, J. Biochem. 62 (1956) 315.

°° Determination by the method of Lowry et al, J. Biol. Chem. 193 (1951) 265.

°°° Determination by the method of Webb, J. Biol. Chem. 221 (1956) 635.

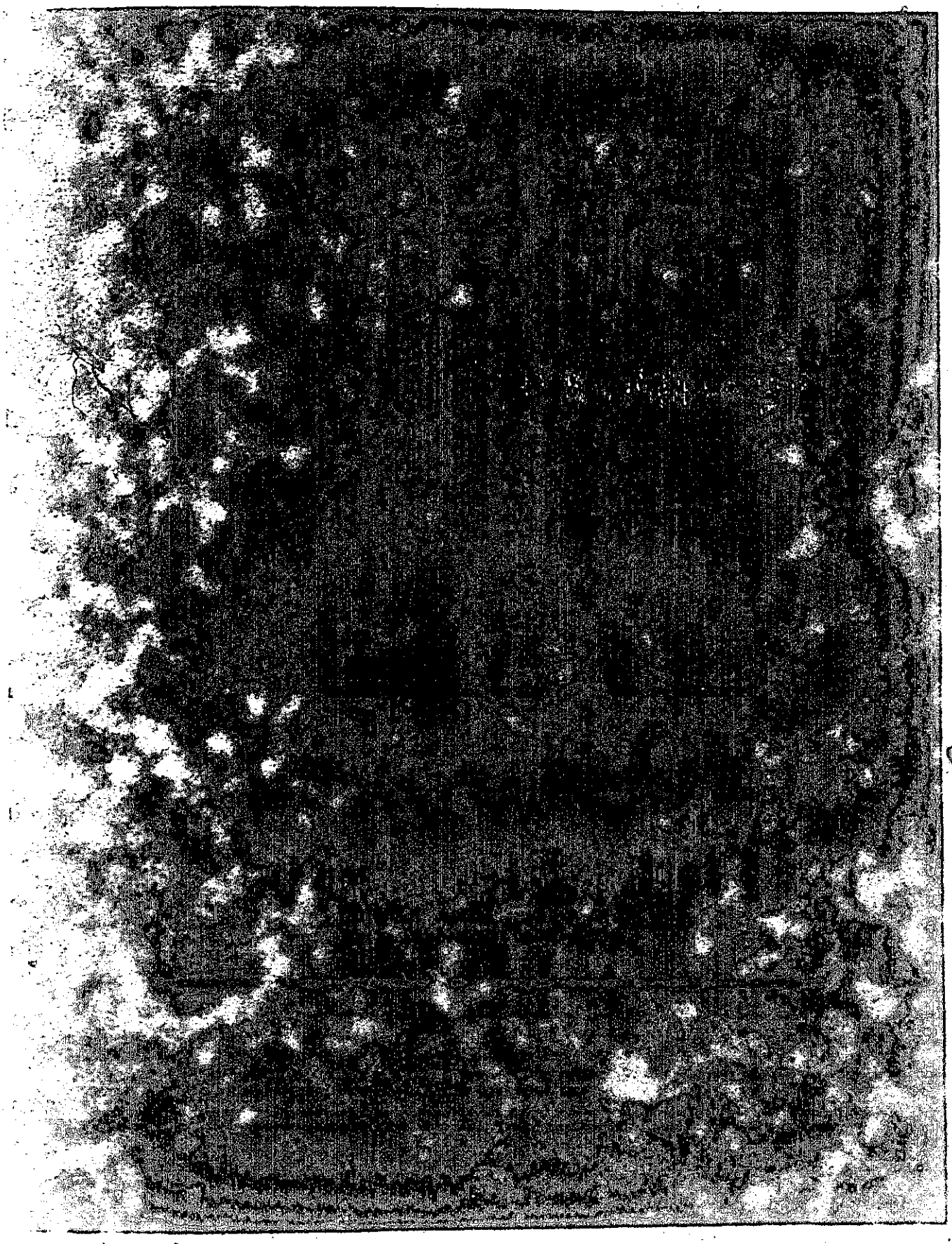
is the protein bound RNA fraction. If the protein is digested with pronase (a group of proteolytic enzymes) the RNA component of the protein bound RNA can be isolated on a DEAE cellulose column.

About the time this work was initiated a number of people reported in the literature that they could not reproduce the work with chromosomal RNA (Commerford and Delihas, 1966; De Fillippes, 1970; Heydén and Zachau, 1971). This has at times given rise to some heated editorial comments and replies (Nature, 1971a; Nature, 1971b; Nature, 1971c).

In the light of these reports it was decided to re-examine the RNA fraction of chromatin; to ascertain the number, type and structure of RNA components present in the eucaryotic cell chromosome.

First experiments involved isolation of chromatin from calf thymus tissue to get familiar with the isolation techniques using methods then already described (Shih and Bonner, 1969; Dahmus and McConnell, 1969; Huang and Bonner, 1965; Bonner et al., 1968). A sample of pure chromatin was obtained having the correct UV spectra (fig. 9), and DNA, RNA and protein composition (table 3) to that of previously published values (Shih and Bonner, 1969).

Electron micrographs of such chromatin preparations stained as described by Parsons (1967) resembled chromatin isolated and similarly examined by Frenster, Allfrey and Mirsky(1963), fig. 2. It was possible also to see some dense regions, fig.10, suspected to be nucleoli. In this chromatin isolation procedure the "nucleoli are largely disintegrated by the blending process" (Bonner, 1968) and this should not appear in the final chromatin preparation. The presence



ure 10. Electronmicrograph of calf thymus chromatin showing some nucleolar contamination. X 60,000.

of RN'ase activity at various stages in the chromatin isolation procedure was also suspected. This has since been confirmed by other workers (De Fillippes, 1970). A protein bound RNA fraction could be obtained through a 10 fold lower amount to that obtained by other workers (Shih and Bonner, 1969).

It was felt, however, that this method of looking at the RNA components in chromatin was unsatisfactory in that it was not possible to isolate the protein components (histones and nonhistone proteins) and any protein bound RNA components from each other, since despite many attempts, the CsCl "protein skin" was completely insoluble, in common buffer media even with the addition of ionic or nonionic detergents, reducing agents or urea. The free RNA was obtained only after digesting all of the protein with pronase.

It had been my early experience that this procedure leads to hydrolysis of RNA present in the chromatin during digestion of the protein due to contamination of the pronase with RN'ase activity and bacterial growth. The bacterial growth could be overcome by the addition of ethanol to a final concentration of 5%. This does not appear to affect the proteolytic activity of the enzymes (Nomoto et al., 1960). No satisfactory method could be obtained to overcome the nuclease problem. These observations have since been confirmed by other workers (De Fillippes, 1970: Hayden and Zachau, 1971).

The procedure described above to isolate chromosomal RNA takes advantage of the unique density an RNA-protein complex would have in a CsCl density gradient. Such a procedure would not yield RNA

components present in chromatin but not bound to protein as an RNA-protein complex. Nor would RNA components bound to a small peptide (Huang and Huang, 1969) be obtained with this method. We might ask how many RNA components are present in purified chromatin? Is there just one low molecular wt. protein bound RNA fraction, or is there a whole spectrum of components not seen by the presently used purification procedures simply because they are discriminated against during the chromatin fractionation?

To answer this question advantage was taken of the unique properties of a MAK-column. Such a column can separate t-RNA, 5S RNA, DNA, 16S and 23S RNA's. An important feature of this column is that removal of DNA from any chromatin associated RNA is not necessary. This eliminates the possibility of treating the final purified RNA preparation with DN'ase that is contaminated with RN'ase activity. This is a very important point since the DNA is in chromatin present to a 100 fold excess to that of the RNA. Many of the other techniques used to fractionate RNA are impractical with such an excess of DNA.

It was first necessary to spend some time "perfecting the art" of fractionation of nucleic acids on a MAK column, to insure that any peaks obtained from the column were in fact real RNA species. To do this E. coli nucleic acids were characterized on the column. Their profiles from the MAK column have been well characterized (Osawa and Sabatani, 1967). ^{32}P labeled E. coli B DNA and RNA was prepared and eluted from the column as described in the methods section and by Osawa and Sabatani (1967).

Chromatin was prepared from 3 rats (= 100 gms each) which were given I.P. 5 mC of 5-^{3}H-uridine. The procedure used to prepare and purify the chromatin was essentially that as described by Dahmus and McConnell (1969). The total nucleic acids content of the rat liver chromatin was obtained free from the protein components by repeated extraction with a chloroform/isoamyl alcohol mixture (Marmur, 1967). Fig. 11 illustrates the profile obtained when rat liver (^{3}H-uridine labeled) chromatin nucleic acid components are coeluted from a MAK column along with E. coli (^{32}P-H_3PO_4 labeled) nucleic acids. From the profile of the ^{3}H labeled material it is clear that there is a wide spectrum of RNA components present in rat liver chromatin. These range in size and structure from low molecular wt. RNA components like t-RNA up to high molecular wt. RNA structures like r-RNA. However, since the mechanism for the differential elution of RNA species from a MAK column is incompletely understood we can say little with this type of column about the structure of the RNA's eluted.

What was apparent from this work was that chromatin did not contain just one low molecular wt. RNA component but, in fact, had a very heterogenous population of RNA molecules.

The major portion of the rest of this thesis will be concerned with the isolation, purification, characterization and possible functional role of these RNA molecules.

A new method to isolate and subfractionate chromatin

The wide spectrum of RNA components present in chromatin necessitated the development of new procedures to isolate and subfractionate

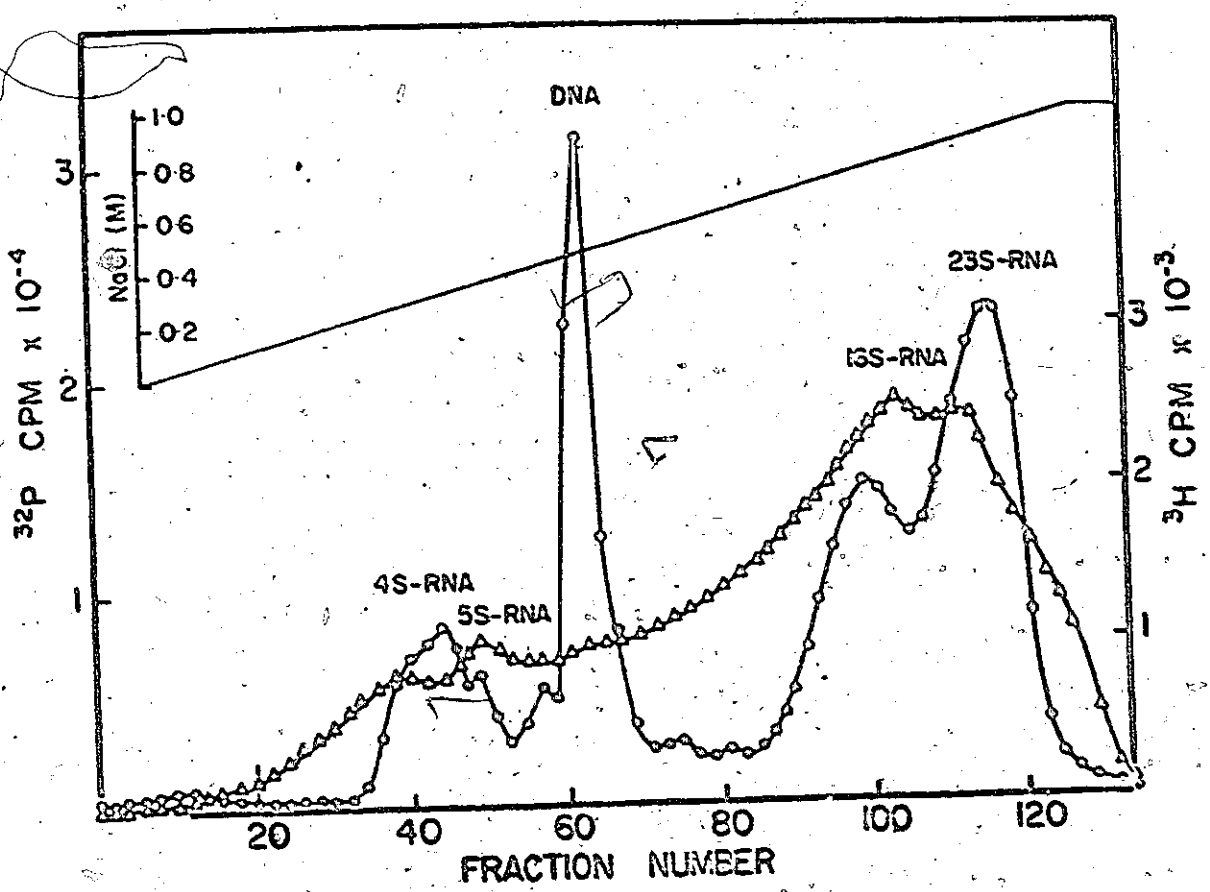


Figure 11. Elution profile of E. Coli ^{32}P labeled nucleic acids, from a MAK column. o——o, Elution profile of total ^{3}H-uridine labeled material from rat liver chromatin Δ——Δ.

chromatin. It was particularly important to obtain the RNA fractions free of protein or DNA components since these fractions could easily cloud over metabolic or regulatory roles of the RNA fraction. DNA in particular, readily interferes with the resolution of RNA components during polyacrylamide gel electrophoresis. Eventually a procedure for the fractionation of chromatin into its four major components DNA, RNA, histones, and nonhistone proteins was established. In summary: the chromatin components are dissociated in a 3 M NaCl, 5 M urea solution containing 2-mercaptoethanol and EDTA. The DNA and high molecular weight RNA are collected by high speed centrifugation as a pellet. The DNA is separated from the RNA in this pellet by Cs_2SO_4 equilibrium density centrifugation. In the supernatant of the high speed centrifugation step are the histones, the nonhistone protein and CLMW-RNA fractions. These fractions can be separated from each other by DEAE cellulose column chromatography.

Let us look at some of the components present in these major complex chromatin fractions.

The pellet from the high speed centrifugation step

Chemical analysis indicated that the DNA pellet formed during the high speed centrifugation contains only 0.1% of the total chromatin protein. This fraction has not been further characterized. From previous work it seems possible that it contains protein components most of which were already dissociated by the 3 M NaCl 5 M urea buffer (Shoshana, Simpson and Sober, 1972).

Throughout the chromatin isolation procedure care was taken to

keep shearing of the DNA to a minimum. However, brief sonication treatment of the purified nuclei was found to be necessary to ensure rupture of the nuclei. The minimal effect of this treatment on the DNA finally obtained in the high speed centrifugation pellet is seen in Fig. 6. Chromatin was obtained from ^{32}P-H_3PO_4 labeled L cells. The cells were grown in 1.0 mC ^{32}P-H_3PO_4 in 100 ml of cells (4×10^6 cells/ml) for 6 hr. The DNA pellet from the high speed centrifugation step was dissolved in 0.01 M sodium acetate, 0.001 M 2-mercaptoethanol, 0.2% SDS, 15% sucrose pH 5.4. Unlabeled calf thymus DNA, L cell r-RNA, t-RNA and bromophenol blue dye were added to act as markers. Chromatography on sepharose 2B shows that almost all of the counts are in the high molecular weight region. However, a small fraction of the counts appeared to reside in the r-RNA region. Treatment with purified RN'ase and DN'ase (Worthington) showed that both DNA and RNA labeled components were present in the r-RNA region. The RNA was separated from the large excess of DNA by equilibrium density centrifugation in a cesium sulfate solution containing dimethylsulfoxide (Williams and Vinograd, 1971). In a typical experiment the chromatin DNA pellet formed from 10^7 L cells P^{32} labeled was dissolved in 4.0 ml of a solution containing 0.01 M tris; 0.002 M EDTA; 0.002 M 2-mercaptoethanol; 1.326 M Cs_2SO_4; 10% DMSO pH 8.0.

The presence of 10% DMSO was found to be essential to prevent precipitation of the RNA. The solution was filtered through a glass fiber filter (Reeve Angel 934AH) into a centrifuge tube. The remainder of the tube was covered with liquid paraffin and centrifuged for 58 hr

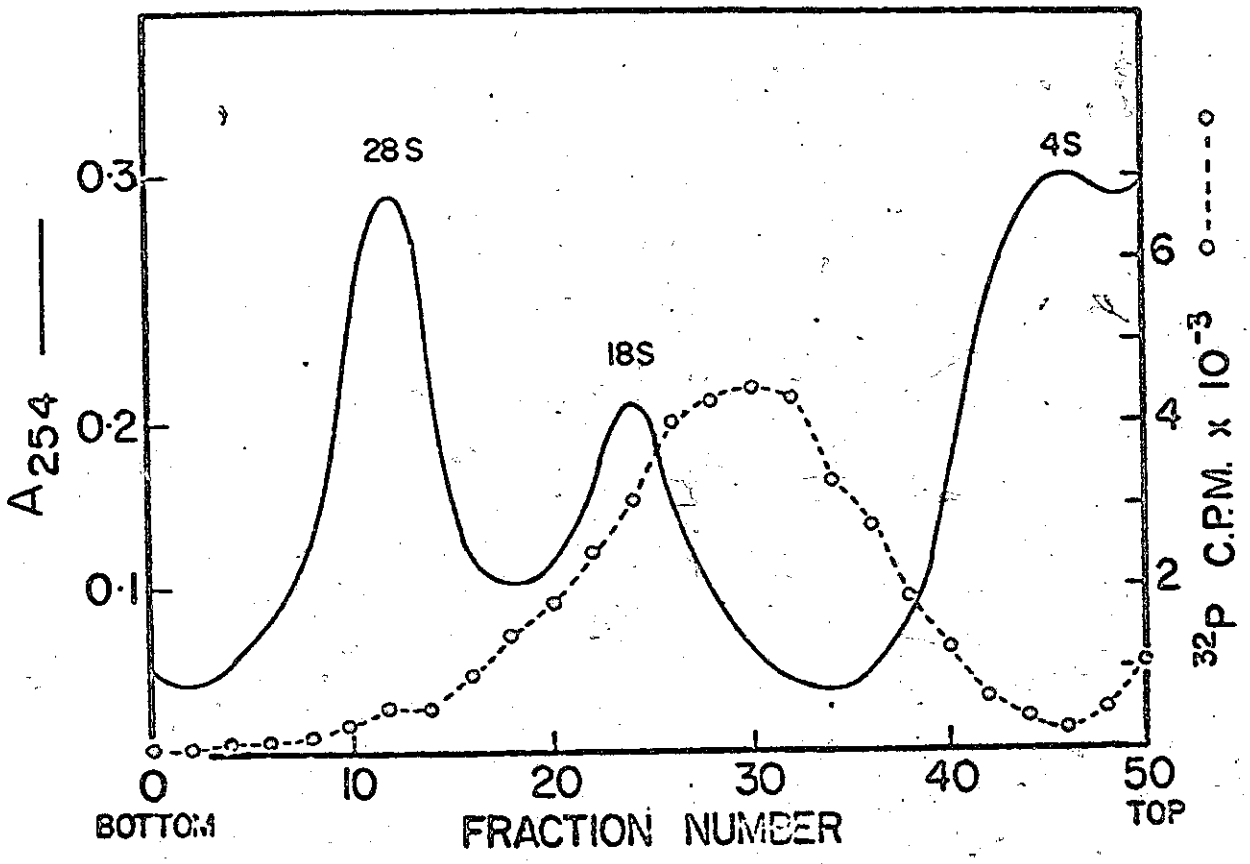


Figure 12. Isokinetic 15% to 30% sucrose gradient centrifugation of chromatin high molecular wt. RNA from L-cells. The 10 ml gradient was centrifuged for 17 hours in an IEC SB-283 rotor at 25,000 rpm at 25° C. A_{254} profile of unlabeled L-cell r-RNA and t-RNA markers. o——o ^{32}P CPM profile of labeled chromatin high molecular wt. RNA.

at 32,000 rpm in a Spinco SW 50 rotor at 25° C. Forty fractions were collected from each gradient. Fig. 7 shows the complete separation of DNA from the high molecular weight RNA. The RNA fraction was pooled and dialyzed against 0.01 M sodium acetate pH 4.5 after adding cold carrier L-cell r-RNA and t-RNA. The RNA was then collected by precipitation with ethanol. Sedimentation of the ^{32}P labeled RNA through a 15-30% sucrose gradient indicates that it has a broad molecular weight spectrum with a peak S value around 10S (Fig. 12). This was confirmed by electrophoresis of the ^{32}P labeled RNA on a 2% acrylamide-0.2% agarose gel (Fig. 13). The base composition of this type of RNA from KB cells is shown in table 4. (The base composition of this RNA from L-cells was not determined). This fraction will be termed the chromatin high molecular wt. RNA fraction (CHMW-RNA fraction).

The histone fraction

The supernatant of the solution from the high speed centrifugation step containing histones, nonhistone proteins and RNA was poured off and dialyzed for six hours against two changes of 100 volumes of 5 M urea, 0.01 M tris, 0.002 M EDTA, 0.002 M 2-mercaptoethanol pH 8.0. The dialyzed solution was passed through a DEAE-cellulose column (for a sample derived from 4×10^0 cells, a column 0.5 cm diameter x 12 cm is sufficient). Whatman microgranular preswollen DE 52 DEAE cellulose was used. The histone fraction passes through the column in the runoff peak. For small quantities of cells it is not possible to quantatively concentrate the protein in this fraction by precipitation with organic solvents (Johns, 1964). The runoff peak fraction was brought to pH 4.5 by addition of glacial acetic acid and the solution passed through a

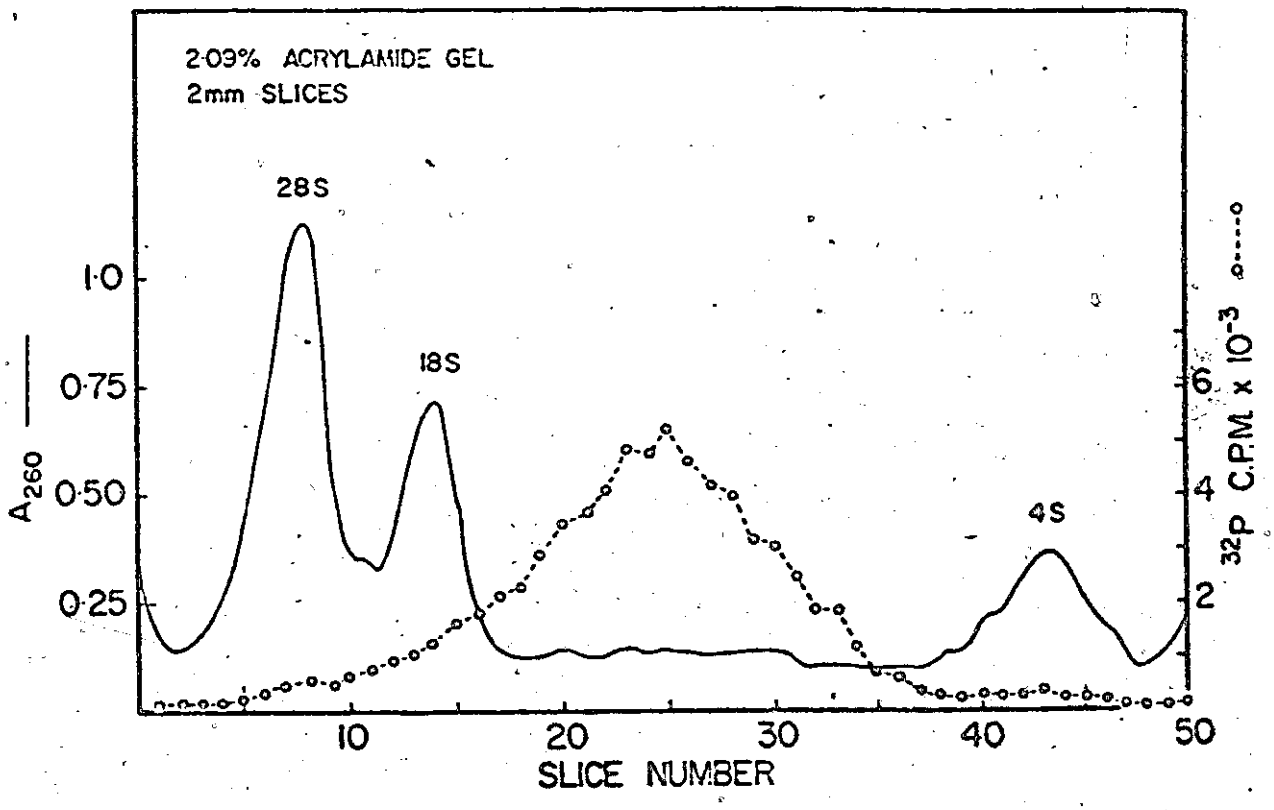


Figure 13. 2% polyacrylamide gel electrophoresis of chromatin high molecular wt. RNA from L-cells. A_{254} profile of unlabeled L-cell r-RNA and t-RNA markers. o——o ^{32}P CPM profile of labeled chromatin high molecular wt. RNA.

TABLE 4

BASE COMPOSITION OF CHROMATIN RNA

RNA TYPE	BASE			
	G	C	A	U
High molecular wt. RNA fraction	21·6	19·2	29·8	29·4
Low molecular wt. RNA fraction	24·1	25·3	19·6	31·0

CM-cellulose column (0.6 cm diameter x 8 cm). The column was washed with 0.05 M acetate buffer, pH 4.5 containing 0.005 M 2-mercaptoethanol. The total histone fraction was collected in a few drops by elution with 0.02 M HCl. [Subfractionation of the total histone fraction can be obtained using different elution buffers instead of 0.02 M HCl which elutes all the histones from the column together (Johns, Phillips, Simpson, Butler, 1960). This was not attempted here]. The histone fraction was lyophilyzed and characterized by acrylamide gel electrophoresis. Fig. 14 shows the histone profile obtained from a single monolayer of KB cells (12 x 10^6 cells) that were labeled with 200 µC ^{3}H-lysine and 200 µC ^{3}H-arginine in 10 ml of lysine and arginine free medium for four hours. 50 µg of L-cell unlabeled histones isolated as described in the methods and materials section were coelectrophoresed with the radioactive material. Five stained bands tentatively assigned to histone fractions F_2a1, F_2a2, F_2b, F_3 and F_1 (bottom to top of gel) were seen. The radioactive count profile of the sliced gel indicated that all the histone fractions were present in the CM-cellulose sample. Further, since there are no other major bands present, it would appear that this fractionation procedure yields little contamination of the histone fraction with the nonhistone proteins.

The NHP and low molecular weight RNA fractions

The NHP and low mol. wt. RNA components remained bound to the DEAE-cellulose column. They were eluted with a salt gradient of 0.0 M NaCl to 1.0 M NaCl in a buffer containing 5 M urea, 0.02 M tris, 0.002 M EDTA and 0.002 M 2-mercaptoethanol. A high protein or RNA concentration

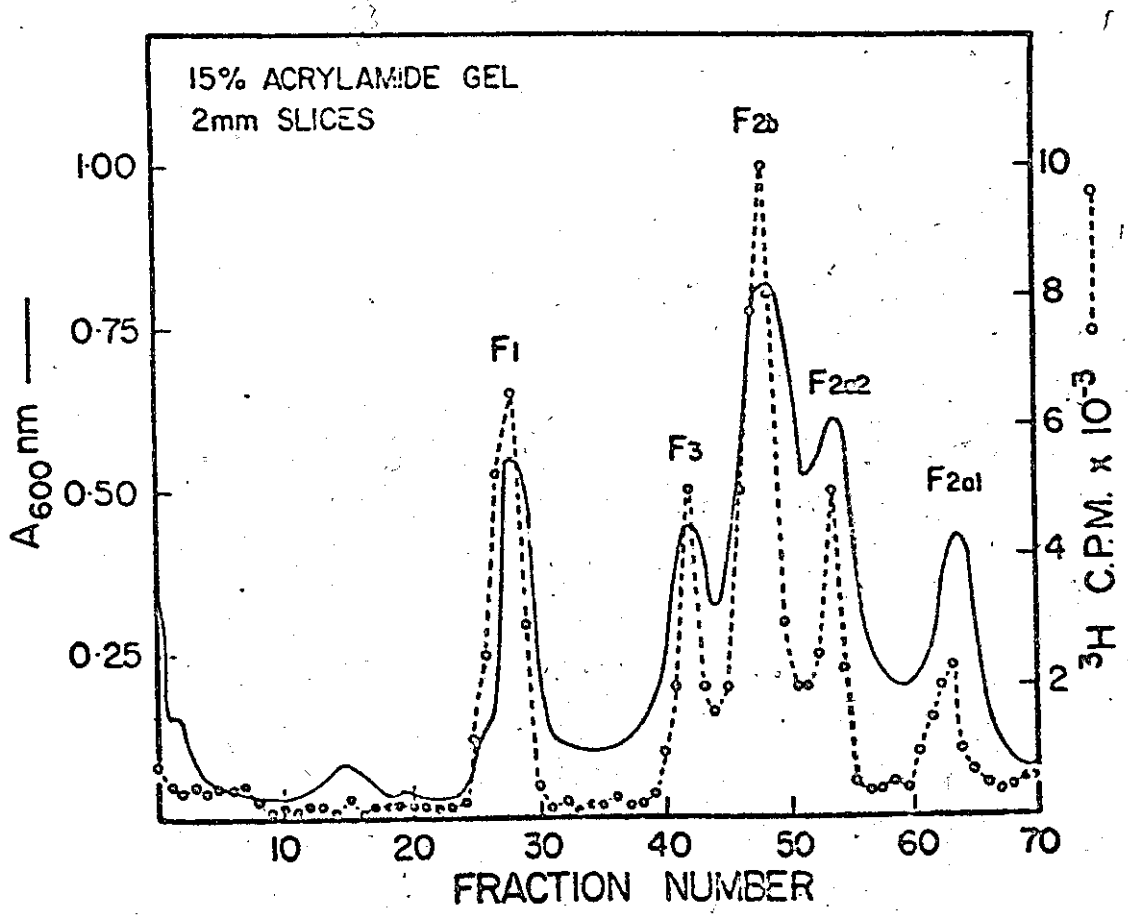


Figure 14. o------o Polyacrylamide gel electrophoresis ^{3}H CPM profile of histone fraction obtained from L-cell chromatin labeled with ^{3}H lysine and ^{3}H arginine. A_{600} profile of stained bands of unlabeled histone markers obtained directly from L-cell nuclei by acid extraction.

in a small volume of elution buffer facilitates the further analysis of these components with polyacrylamide gel electrophoresis. For this reason an accurate steep salt gradient was used. The gradient was made with a sucrose gradient maker (Buchler Instruments, North Lee, N.J.) using 40 ml of buffer in each reservoir. We have found that although the RNA components are relatively insensitive to the presence of urea in the eluting buffer the NHP fraction is sensitive. Complete absence of urea appears to allow the NHP to remain bound to the column at concentrations close to that required to elute the chromatin RNA components (Patel and Wang, 1964). Five M urea in the elution buffer allows almost complete separation of the NHP fraction and the RNA fraction. This critical dependence upon the presence or absence of urea is illustrated in figs. 15 and 16. Fig. 15 illustrates the A_{254} elution profile from the DEAE-cellulose column of the NHP and CLMW-RNA components when the elution buffer contains no urea. Two ml fractions were collected. This is a profile that was obtained from chromatin starting with 2×10^8 L-cells grown in the presence of ^{3}H-uridine 1 mC/100 ml and ^{14}C-leucine 50 µC/100 ml of medium for 6 hr. It can be seen that the ^{3}H uridine label material (RNA) coelutes from the column along with the ^{14}C leucine labeled material (protein). This in itself might not be a serious difficulty since little ^{3}H-uridine becomes incorporated into protein and visa versa for ^{14}C-leucine.

It would of course prevent the use of ^{32}P-H_3PO_4 to label CLMW-RNA fractions since the NHP is rapidly phosphorylated in the cell

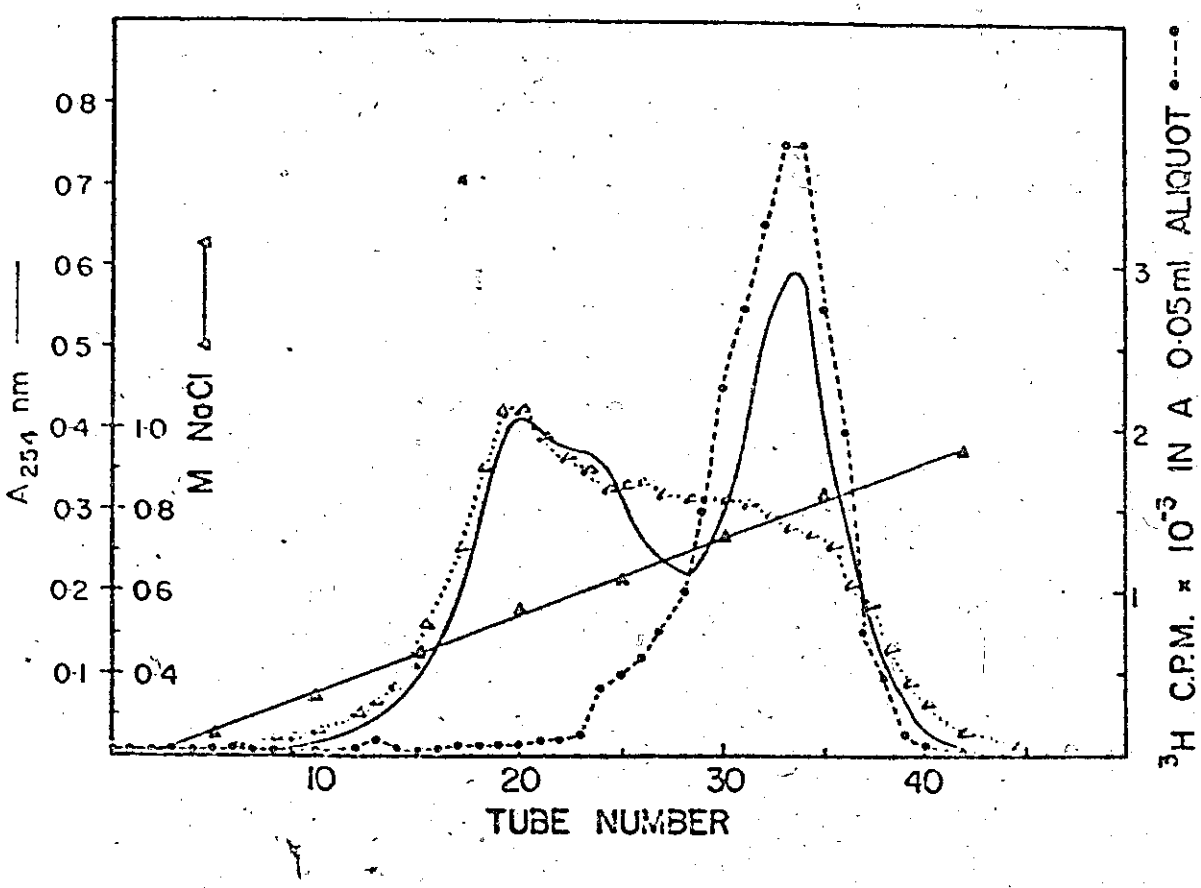


Figure 15. Elution profile of L-cell NHP and CLMW-RNA fractions from a 0.8 cm diameter x 8 cm DEAE cellulose column without 5 M urea in the elution buffer. Δ.....Δ elution profile of ^{14}C leucine material. o——o elution profile of ^{3}H uridine labeled material.

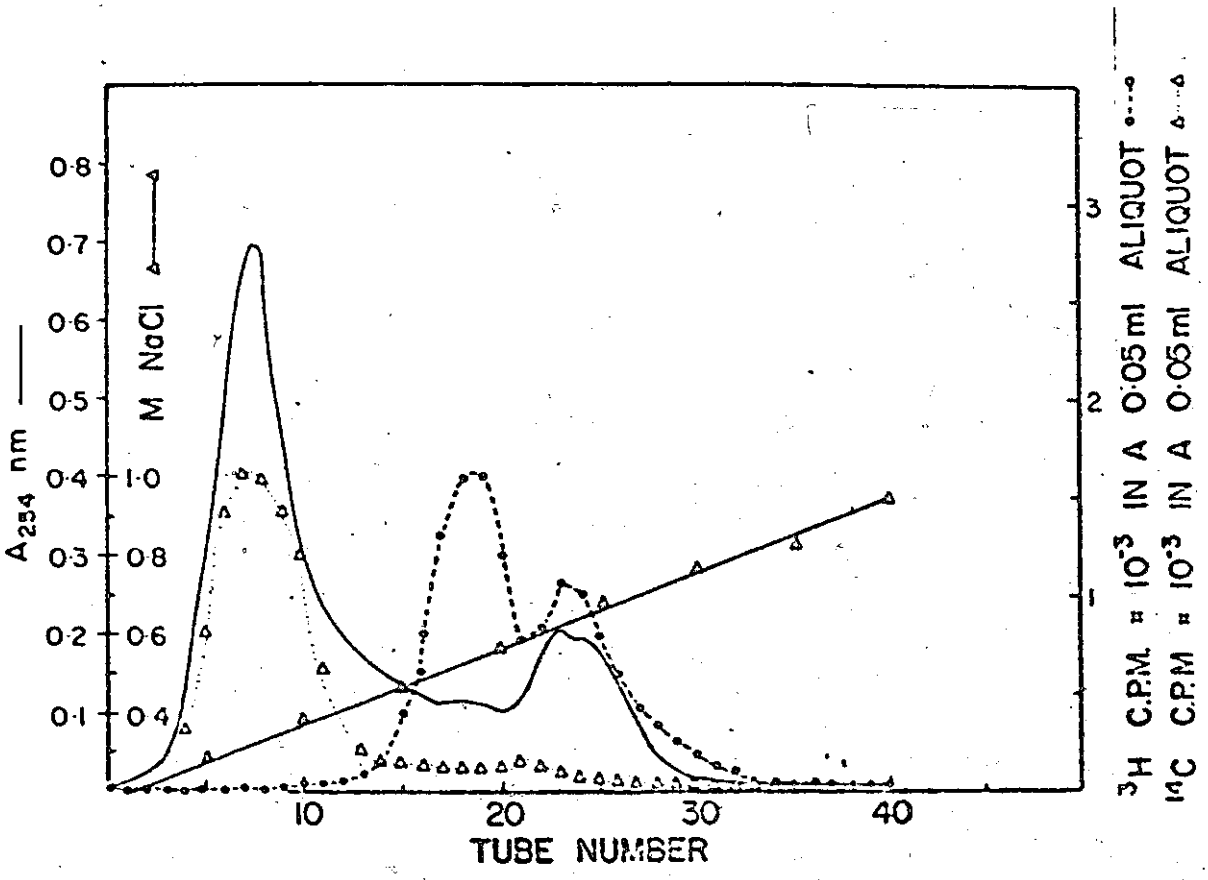


Figure 16. Elution profile of L-cell NHP and CLMM-RNA fractions from a 0.8 cm diameter x 8 cm DEAE cellulose column with 5 M urea in the elution buffer. Δ.....Δ elution profile of ^{14}C-leucine labeled material. o——o elution profile of ^{3}H-uridine labeled material.

(Yakeda, Yamamura and Ohga, 1971; Yeng, Yeng and Allfrey, 1970). One could, therefore, not easily distinguish between the NHP and the CLMW-RNA fractions during any subsequent fractionating procedures with ^{32}P-H_3PO_4 as a label.

There is a further problem with the type of profile obtained in fig.15 which is not immediately apparent. It was found that some ^{3}H labeled DNA is also eluted from the column at this position.. We shall discuss how this DNA may arise in the cell later. The presence of this DNA seriously impedes any resolution of the CLMW-RNA components on 12.5% acrylamide gels. Fig. 17 illustrates the type of profile obtained if the ^{3}H-uridine labeled peak fraction from fig. 15 is run on a 12.5% acrylamide gel. A large fraction of the counts just enter the gel. This, as we will see later, is DNA. The CLMW-RNA fractions travel fast into the gel, and while at least one major peak can be seen (a 5S RNA fraction) most of the CLMW-RNA fractions fail to stand out well against the background of DNA counts. Clearly it was necessary to in some way separate this DNA fraction from the CLMW-RNA fraction.

Addition of 5 M urea to the elution buffer for the DEAE cellulose column accomplishes this.

Fig. 16 shows the A_{254} elution profile from the DEAE-cellulose column of the NHP and CLMW-RNA components when the elution buffer contains 5 M urea. Two ml fractions were collected. This is a profile that was obtained from chromatin starting with 2×10^{9} L-cells grown in the presence of ^{3}H-uridine 1 mC/100 ml and ^{14}C-leucine 50 µC/100 ml of medium for 6 hr. Fractions 0 to 12 contain

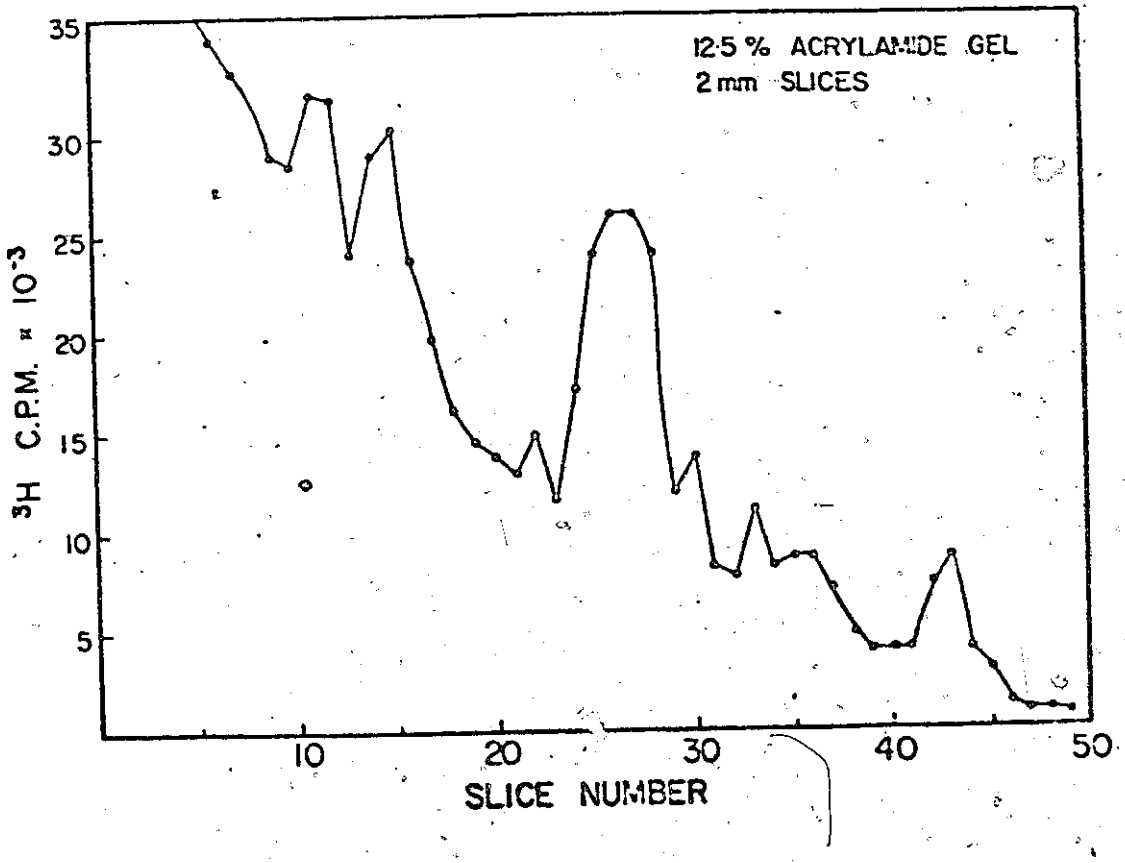


Figure 17. Polyacrylamide gel electrophoresis of ^{3}H CPM profile of L-cell ^{3}H uridine labeled material eluted from DEAE cellulose column in Fig. 15.

most of the ^{14}C leucine counts and will be termed the "NHP fraction". The Polyacrylamide gel electrophoresis (fig. 18) indicates that this is a very complex protein fraction. It has not been further characterized in this work. The ^{3}H uridine counts elute as two radioactive peaks; the first, fractions 15-20 we call peak A, the second fractions 22-27 we call peak B.

Peak A

Gel filtration of sephadex G-100 indicates that there are a number of RNA components of quite different chain lengths present in peak A. To illustrate this the fraction from the DEAE-cellulose column was pooled, unlabeled carrier 4S, 5S, 18S and 28S L-cell RNA isolated as described by Attardi, Parkas, Huang, Attardi (1966) were added. The RNA was precipitated by addition of 2 volumes of ethanol and 0.2 vols of 1 M sodium acetate pH 4.5 upon standing for 4 hours at -20° C. The precipitate was dissolved in two ml of the elution buffer for the G-100 column. A few drops of bromophenol blue dye and glycerol was added. The elution profile is seen in fig. 19. At least six distinct peaks can be seen. On this column 18S and 28S RNA come off in the void volume. Thus the first CLMW-RNA peak can contain components of almost any S value greater than about 6S. A distinct peak is seen coeluting with the unlabeled 5S RNA component. It appears that none of the CLMW-RNA fractions coelute exactly with the unlabeled 4S RNA fraction. Two CLMW-RNA fractions appear on this basis to have S values less than 4S.

Polyacrylamide gel electrophoresis using a 12.5% acrylamide separation gel gives a finer resolution of the CLMW-RNA fractions.

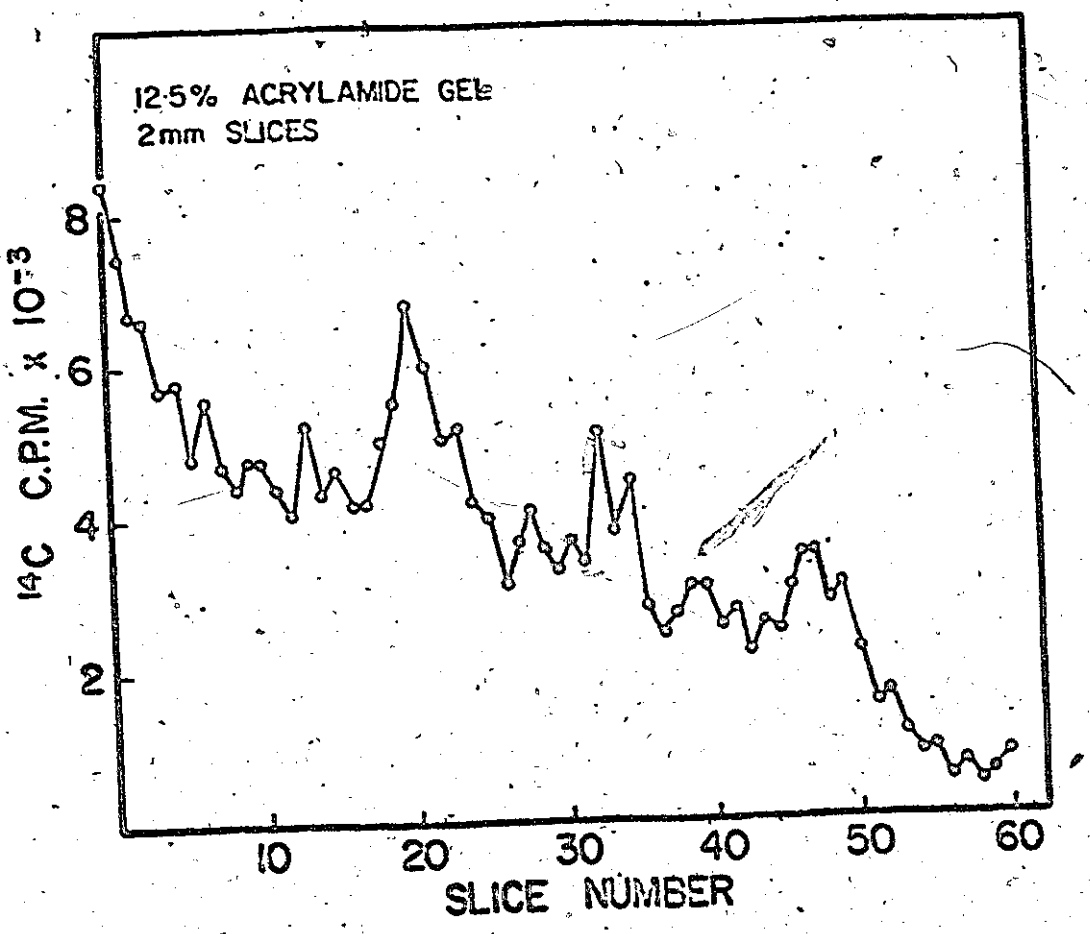


Figure 18 Polyacrylamide gel electrophoresis ^{14}C CPM profile of L-cell ^{14}C leucine labeled chromatin NMP fraction.

If peak A from the DEAE-cellulose column is run on the 12.5% acrylamide gel system, a radioactivity distribution in 2 mm slices is obtained as shown in fig. 20. Eleven distinct peaks can be seen.

While most of the ^{14}C leucine labeled material elutes in the NHP fraction from the DEAE cellulose column (fig. 16) there is a small proportion of the counts under peak A. This labeled material forms three distinct bands on a 12.5% acrylamide gel (fig. 20). It has not yet been ascertained if the first two bands are covalently bound to the RNA in peak C though this is a distinct possibility (Dahmus and McConnell, 1969; Dahmus and Bonner, 1970; Jacobson and Bonner, 1971). The three ^{14}C labeled peaks are pronase sensitive and unaffected by RN'ase or DN'ase.

In view of the difficulties some previous workers have had in preparing and characterizing some chromatin RNA fractions (Artman and Roth, 1971; Heyden and Zachau, 1971; Szeskak and Phil, 1972) it was important to show that the above CLMW-RNA fractions do not arise as a result of contamination of the chromatin with cytoplasmic RNA, also that they are not a result of degradation of higher molecular weight chromatin RNA components during the 24 hour high speed centrifugation.

A 1 litre batch of L cells was collected and resuspended in 200 mls of medium containing 5% dialyzed foetal calf serum. These cells were then divided into two 100 ml samples. ^{3}H-uridine 10 µC/ml was added to one sample, ^{14}C-uridine 0.5 µC/ml was added to the other. The cells were grown for six hours. The buffered homogenate containing the cellular post-nuclear fraction of the ^{14}C labeled

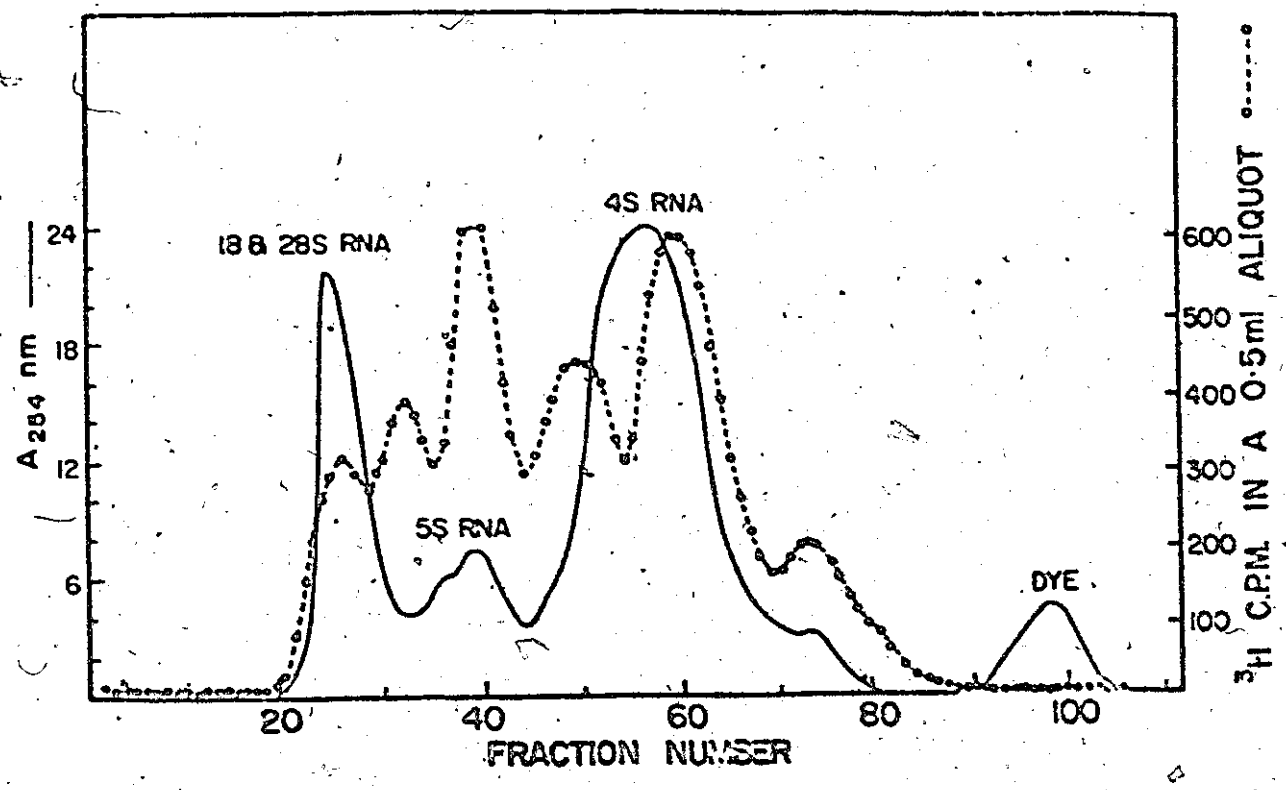


Figure 19. Elution profile of L-cell total CLMF-RNA fractions (peak A of DEAE-cellulose column) from a 1 cm diameter x 250 cm Sephadex G-100 column. o——o elution profile of the ^{3}H uridine labeled material. ——— A_{254} elution profile of marker rRNA, 5S and 4S RNA's and a dye bromophenol blue.

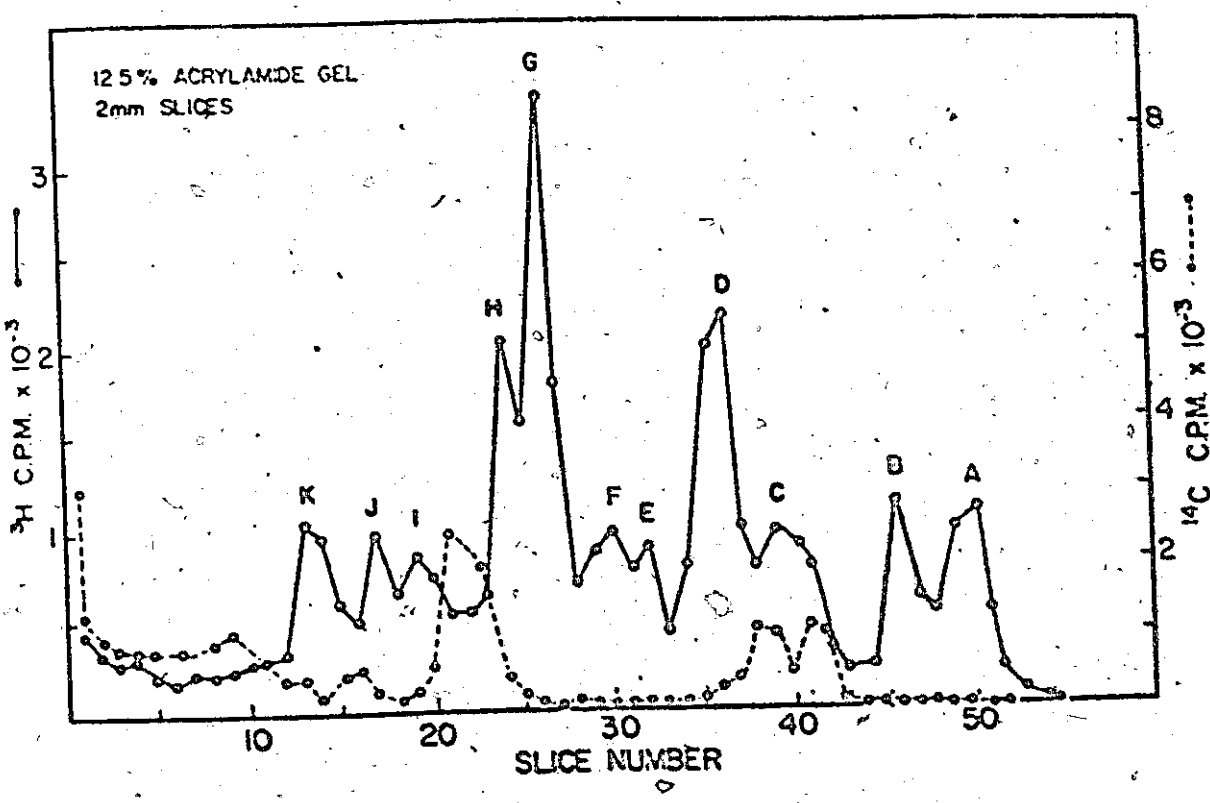


Figure 20. o——o Polyacrylamide gel electrophoresis ^{3}H CPM profile of L-cell ^{3}H uridine labeled chromatin low molecular wt. RNA. o——o ^{14}C CPM profile of ^{14}C leucine labeled material present in the chromatin low molecular wt. RNA preparation.

cells (no intact cells or nuclei present upon microscopic examination) was used to prepare nuclei from the ^{3}H labeled cells. Thus any ^{14}C labeled RNA in the final CLMW-RNA profile could only arise from some kind of aggregation of cytoplasmic ^{14}C-RNA to the ^{3}H-chromatin as the nuclei of the ^{3}H labeled cells are being purified.

In the 24 hour high speed centrifugation step 23S E. coli rRNA, ^{14}C labeled, was included (10 µg/ml, specific activity approx. 4000 CPM/µg) with the chromatin from the cells labeled with ^{3}H uridine. Any degradation of RNA should lead to fragments of ^{14}C labeled E. coli RNA in the supernatant and eventually they should appear on the acrylamide gel. Figure 21 shows that little ^{14}C counts appear to be present.

The CLMW-RNA profile on the 12.5% acrylamide gel is completely removed by RNase pretreatment (Fig. 22). It is uneffected by DNase or pronase treatment. The fact that the fractions D and C are unaltered with pronase treatment suggests that if they contain bound amino acid components these components must be small relative to the rest of the polynucleotide chain since pronase pretreatment does not alter their position relative to the other CLMW-RNA fractions.

The above CLMW-RNA fractions do not appear to be unique to L cells. Figure 23 shows the profiles obtained from KB cells and rat hepatoma cells labeled for 6 hours with 10 µc/ml of ^{3}H uridine. The RNA fractions were isolated as described for L cells. These CLMW-RNA components also appear in cells which are euploid. Figure 23 also illustrates the profile obtained with DON-C3

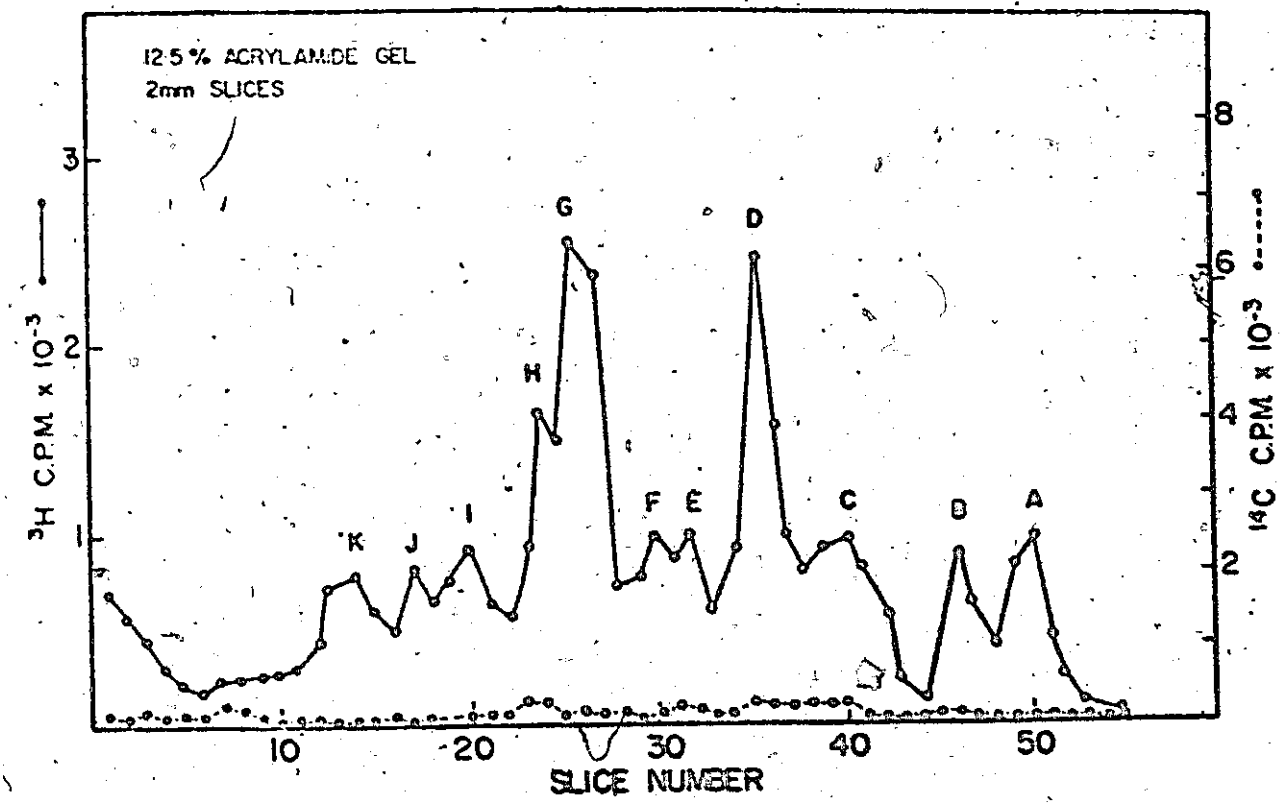


Figure 21. o——o Polyacrylamide gel electrophoresis of ^{3}H uridine labeled L-cell total CLEN-RNA fractions. o——o ^{14}C labeled contaminating material from either cytoplasmic RNA or degraded E. coli 23S r-RNA.

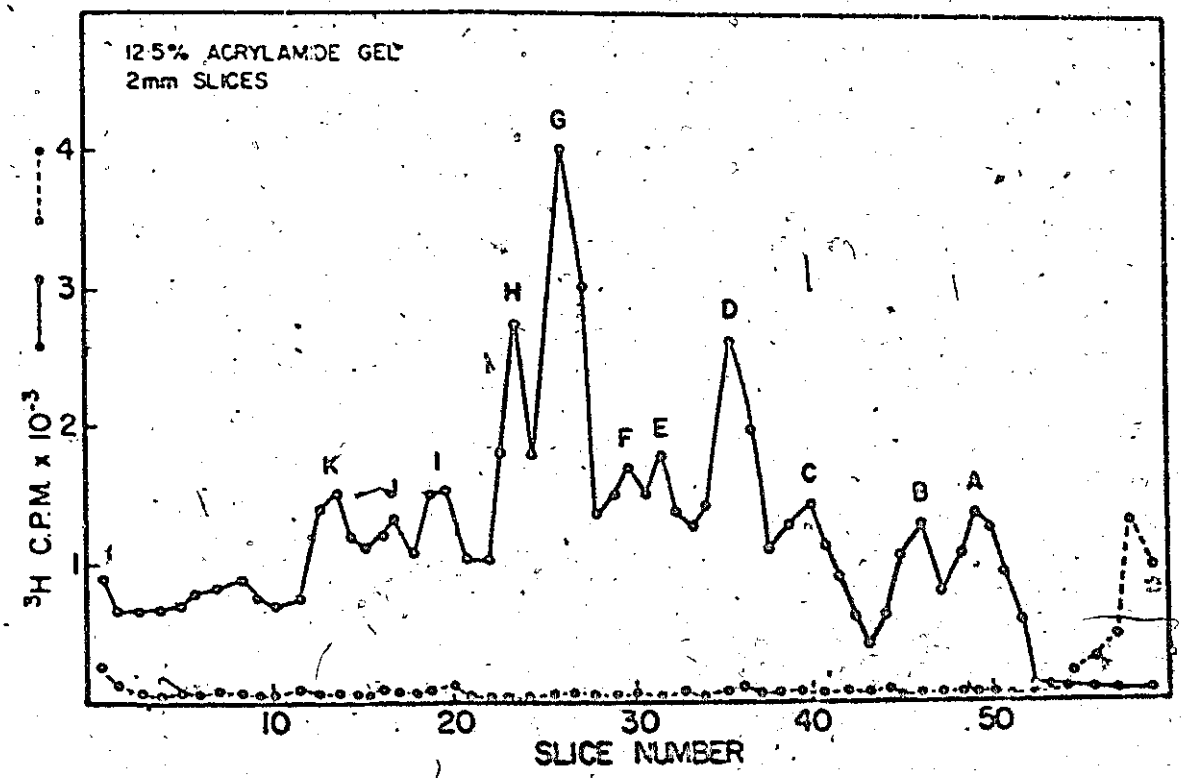


Figure 22. Polyacrylamide gel electrophoresis of L-cell the CLMV-RNA without o——o and with o- - - -o RN'ase pretreatment.

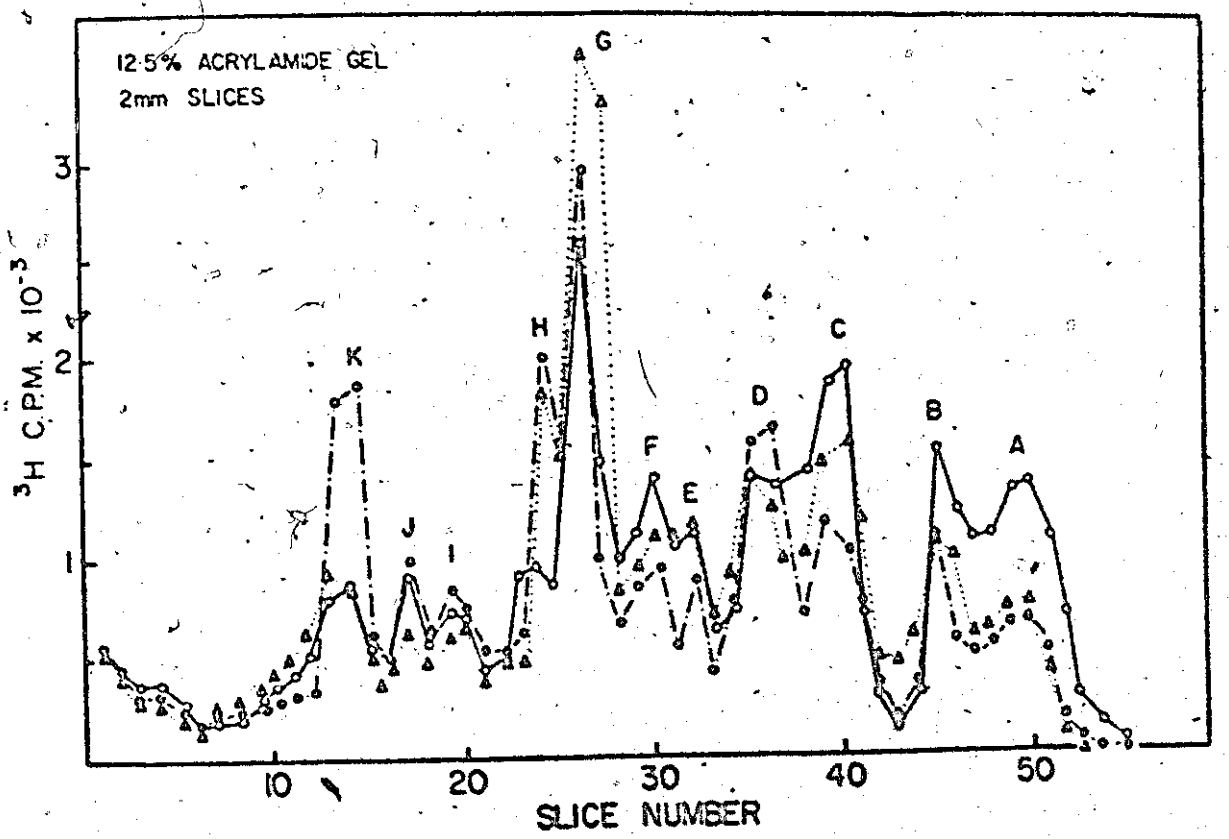


Figure 23. Polyacrylamide gel electrophoresis of the CLMH-RNA fraction from KB cells o·-·-o, DON-C3 cells o——o, Rat hepatoma cells Δ.....Δ. Three separate gels are illustrated together.

chinese hamster cells. It can be seen that all the cell lines appear to contain the eleven individual fractions though the relative amounts of labeled RNA in each fraction is different from one cell line to another.

Characterization of the CLMW-RNA fractions

A wide range of RNA components in this S value range (3S-10S) have been isolated from nuclei and from chromatin by a number of investigators using greatly differing procedures (Bonner, Dahmus, Fambrough, Huang, Marushige and Yuan, 1968; Kanehisa, Fujitani, Sano and Yanaka, 1971; Prestayko and Busch, 1968; Huang and Huang, 1969; Mayfield and Bonner, 1971; Kanehisa, Yanaka and Kano, 1972; Busch, Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi, and Mouritzen, 1971; Weinberg and Penman, 1968; De Filippes, 1970; Hogan and Gross, 1972; Egyhazi and Edstrom, 1972; Arnold and Young, 1972; Rein, 1971). It would be of interest to ascertain if some of these CLMW-RNA fractions are similar to the nuclear or chromosomal RNA preparations already described.

A preparation of L-cell ^{3}H-uridine labeled CLMW-RNA was fractionated by electrophoresis (fig. 24) with ^{14}C-uridine labeled rat hepatoma cell 4S, 5S and 5.5S RNA isolated as described by Penn, Knight and Darnell (1968) and characterized by Sy and McCarthy (1970). The approximate S values of the CLMW-RNA fraction was obtained using the ^{14}C labeled material as standards (Lewicki and Sinskey, 1970). The estimated S values are shown in table 5 . Fractions A, B and C appear to have S values less than 4S. There appears to be no

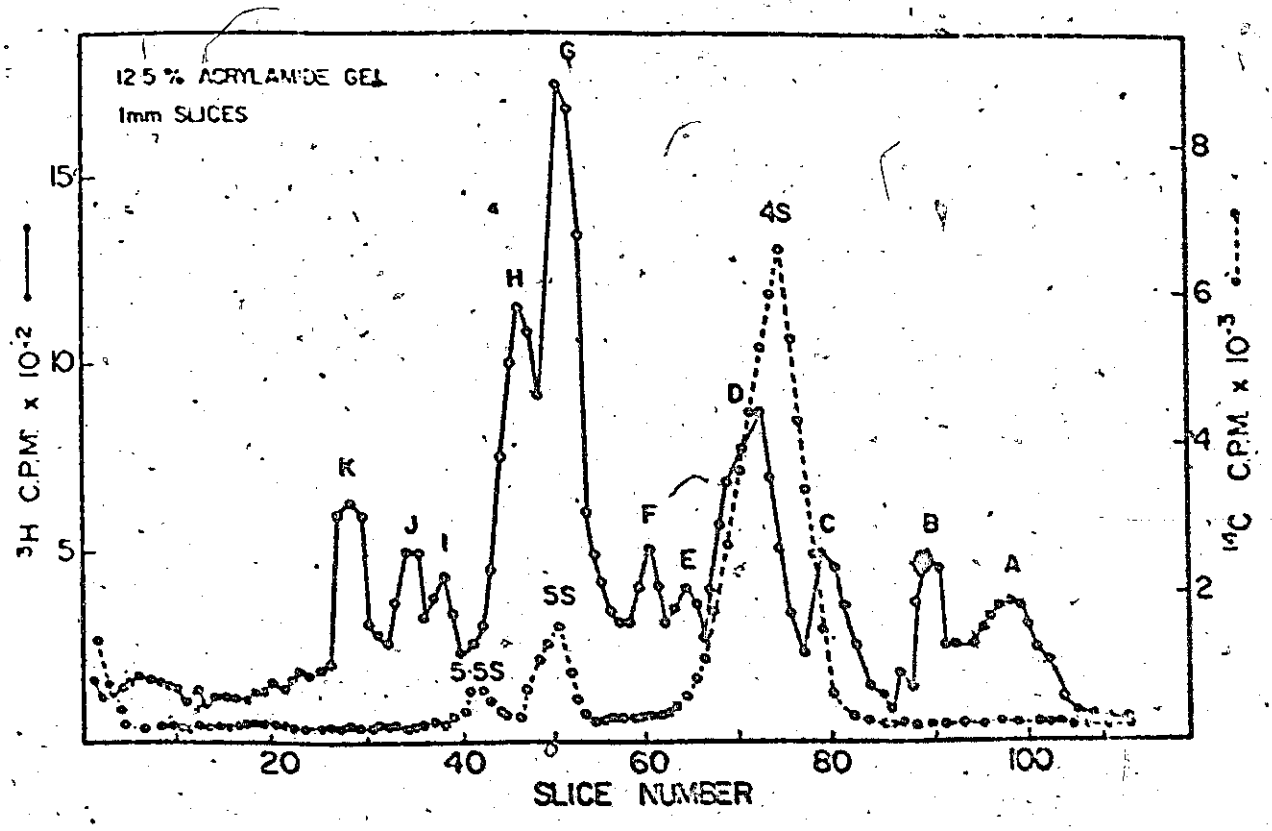


Figure 24. Coelectrophoresis of 4S, 5S and 5.5S ^{14}C labeled RNA o——o and L-cell CLM9-RNA ^{3}H labeled o——o on a polyacrylamide gel.

TABLE 5

S VALUES AND ^{32}P-NUCLEOTIDE COMPOSITIONS OF CLMW-RNA FRACTIONS FROM L-CELLS

CLMW-RNA	S VALUE	GMP	CMP	AMP	UMP
A	3·1	22·1	24·5	25·6	27·8
B	3·4	31·3	27·0	18·8	22·9
C	3·8	26·3	25·8	22·7	25·2
D	4·1	30·0	27·3	22·4	20·3
E	4·4	28·1	19·5	24·1	28·3
F	4·6				
G	5·0	25·3	19·8	27·1	27·8
H	5·2	29·1	25·0	20·3	25·6
I	5·7	30·8	23·1	19·9	25·2
J	5·9	28·3	23·2	22·3	26·2
K	6·2	28·1	21·4	23·1	27·4

EACH VALUE REPRESENTS THE MEAN OF THREE DETERMINATIONS

component with an S value of 4S.

The base composition of the CLMW-RNA fractions from L-cells was determined as described in the methods section, table 5. The cells were grown at a concentration of 2×10^7 cells/ml for 6 hrs in 100 mls of medium containing 100 μC/ml of carrier free ^{32}P-H_3PO_4. Using the two dimensional chromatography system described in the methods section the location of the nucleotide components present in the L-cell CLMW-RNA's was determined. We could not detect nucleotide components other than Ap, Gp, Cp, Up, pGp, pUp. In particular we could not detect any counts where dihydro-Up was located in any of the CLMW-RNA fractions.

The procedure described in the methods section should remove a considerable part if not all of the nucleolar material normally present in chromatin preparations (Wang, 1968). Of the low molecular wt RNA components present in the nucleus and in chromatin is one described by Busch, Ro-Chai, Prestayko, Shibata, Crooke, El-Khatib, Choi, and Muritzen (1971) called U3, which appears to be localized specifically to the nucleolus. When Novikoff hepatoma nucleolar 4-8S RNA, - a generous gift from Dr. H. Busch, Baylor College of Medicine, Houston - was fractionated by electrophoresis with the CLMW-RNA fraction (fig. 25), it can be seen that no component ran with the U_3 nucleolar RNA component. This suggests to us that our preparation is relatively free of nucleoli.

The polyacrylamide gel electrophoresis system used above will separate RNA molecules only in terms of their chain length. It does

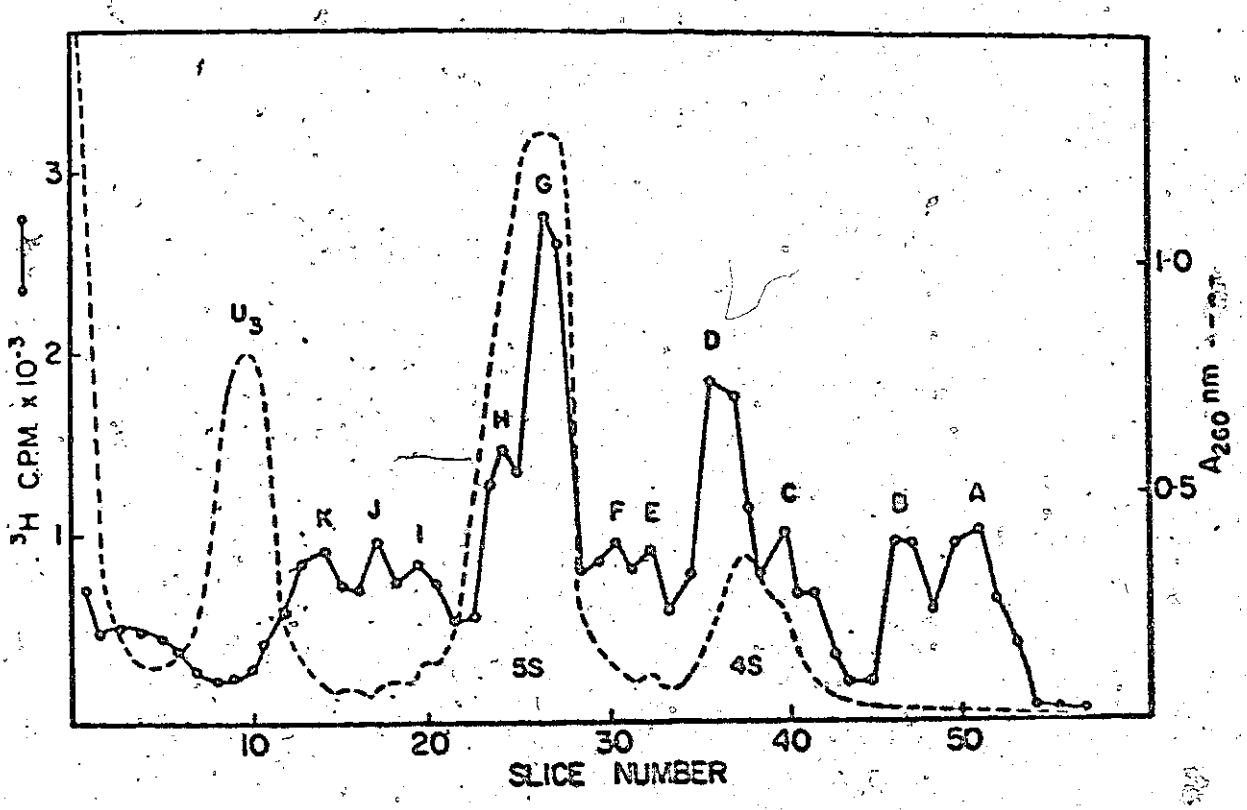


Figure 25. Coelectrophoresis of nucleolar 4-8S RNA o——o and L-cell CLMM-RNA ^{3}H labeled o——o on a polyacrylamide gel.

not give any indication how heterogenous an individual peak might be, for example t-RNA gives only one peak on this gel yet it contains at least 100 subspecies. Are there further subspecies within the CLMW-RNA fractions A to K?

This is not an easy question to answer. Any role in specific gene regulation would demand a very heterogenous population of RNA molecules.

Two-dimensional polyacrylamide gel electrophoresis of ^{32}P-labeled CLMW-RNA from L-cells does indicate that considerable heterogeneity exists in this fraction. Fig. 26 illustrates the profile obtained from L cell ^{32}P labeled cytoplasmic 4, 5 and 5.5S RNA's. Fig. 27 illustrates the profile obtained with the ^{32}P labeled L-cell CLMW-RNA fraction. Only 4 spots are seen in the case of the cytoplasmic components (itself a complex fraction) while at least 25 could be seen for the CLMW-RNA fraction. Only about 10 are seen in fig. 27 due to loss of minor spots in the photographic reproduction.

Peak B from DEAE cellulose column

This fraction is composed mainly of DNA with less than 2% RNA and no detectable protein. Coelectrophoresis of this fraction with ^{14}C labeled 4, 5 and 5.5S RNA's yields a single major peak at the top of the gel, fig. 28. This peak is sensitive to DN'ase and insensitive to RNase or pronase. It presumably arises via conversion of: -

^{3}H uridine → ^{3}H cytidine → ^{3}H deoxycytidine

since cold deoxycytidine and thymidine (2 µg/ml) partially inhibit incorporation of ^{3}H-uridine into this fraction (Martinez and Fuhs, 1971). This fraction was not further analysed.

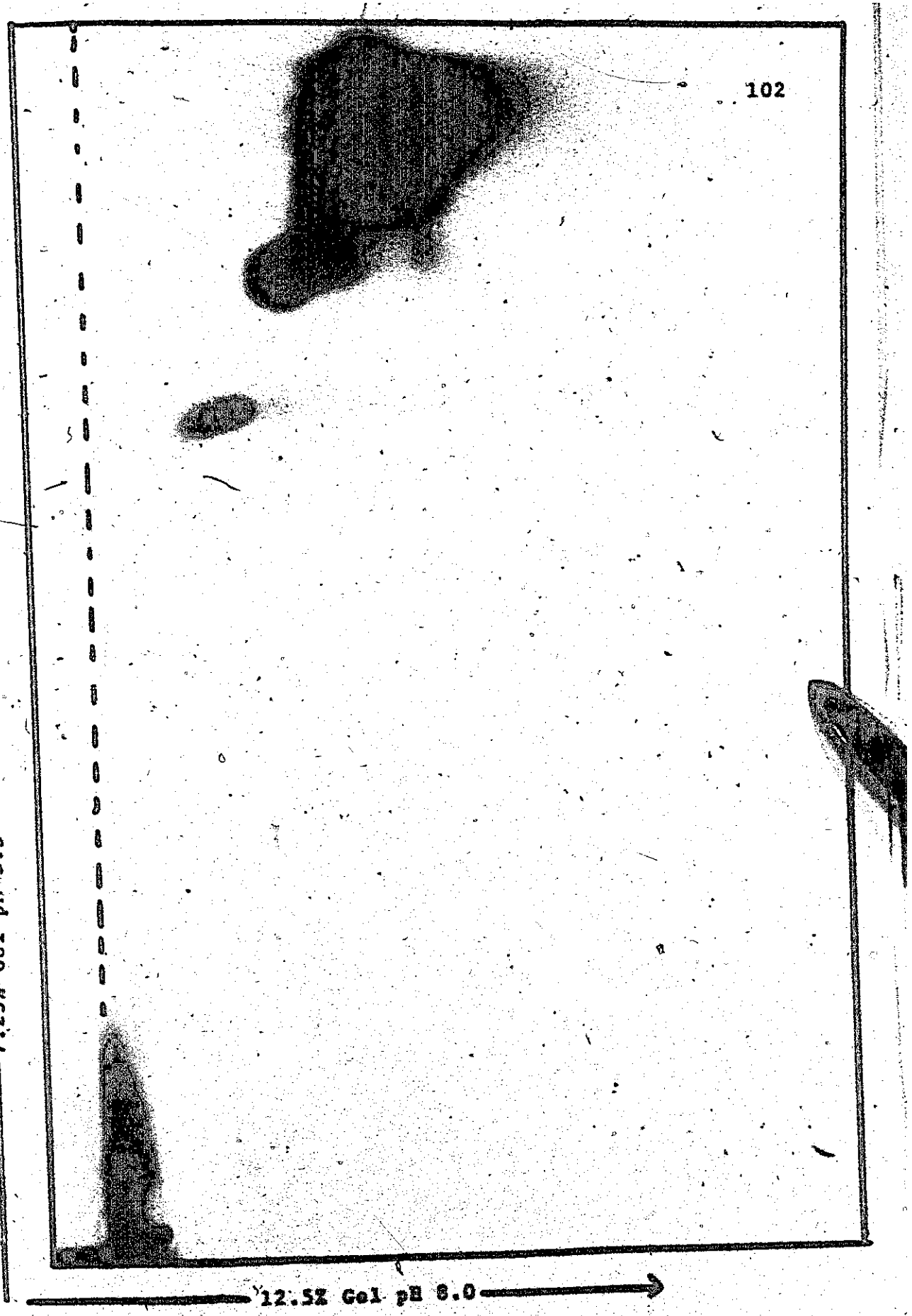


Figure 26. Two dimensional polyacrylamide gel electrophoresis of cytoplasmic 4s, 5s, and 5.5s RNA.

Figure 27. Two dimensional polyacrylamide gel electrophoresis of L-cell chromatin CLMW-RNA's.

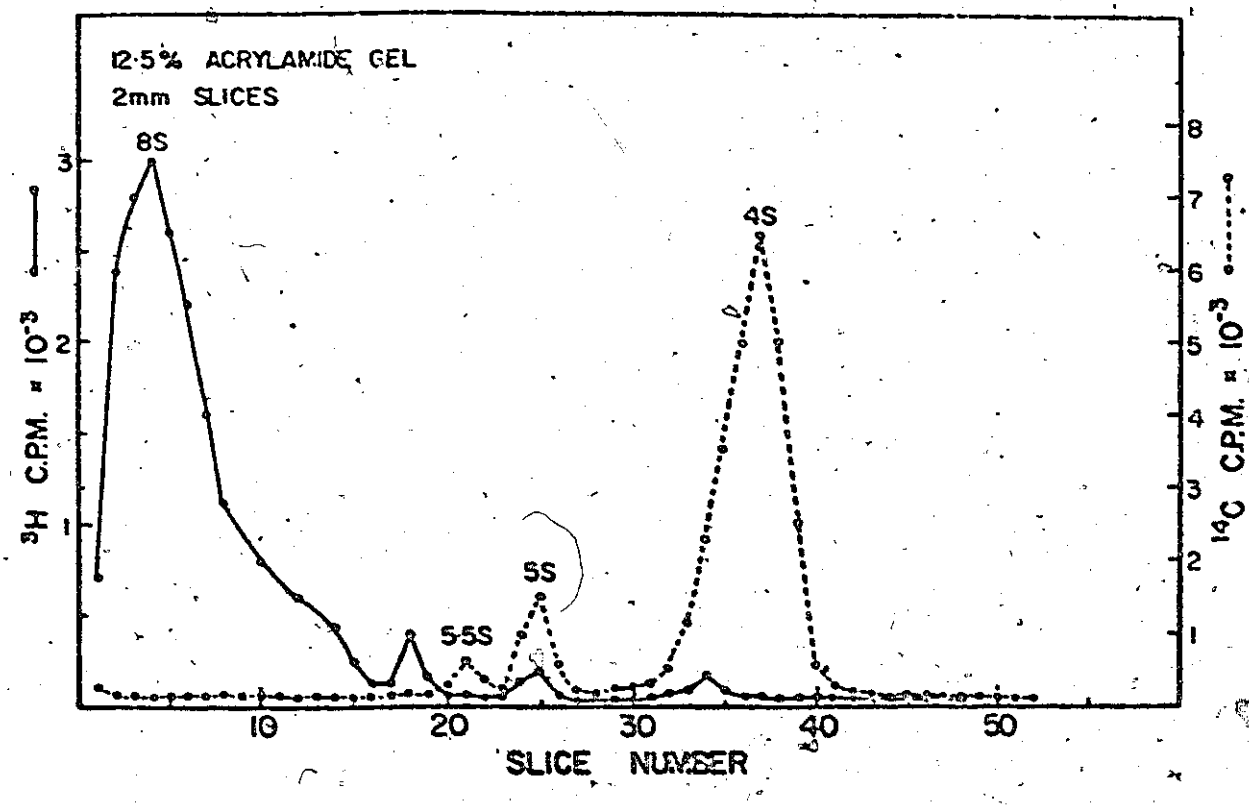


Figure 28. Coelectrophoresis of peak B from the DEAE cellulose col ——o——o, with 4S, 5S and 5.5S RNA ^{14}C labeled ——o——o.

IV DISCUSSION

Before looking at the overall significance of this work I would like to discuss first, some of the technical data that have evolved from it.

The procedure described to isolate and fractionate chromatin from tissue culture cells has in part been based on a combination and modification of previously described methods (Shoshana, Simpson and Sober, 1972; Dahmus and McConnell, 1969; De Filippes, 1970; Shaw and Huang, 1970; Shirey and Huang, 1969). In contrast to procedures used to isolate chromatin from some animal or plant tissue (Bonner et al., 1968) it was found to be advantageous to isolate first highly purified tissue culture cell nuclei before purifying the chromatin itself. If this precaution is not taken the chromatin RNA fraction becomes contaminated with cytoplasmic RNA components. Addition of bentonite to the buffer used to lyse the nuclei is necessary to prevent contamination of the chromatin low molecular weight RNA with fragments of degraded larger nuclear RNA's. The value of bentonite in this step has been observed previously by De Filippes (1970). Taking this precaution and taking care to remove lysosomes in the cytoplasm before addition of the Tween 40/deoxycholate detergent mixture (Penman, 1969) no RN'ase activity could be detected in the final chromatin preparation as assayed according to the method

described by Kalnitsky, Hummel and Kierks (1959).

The type of RNA components found in the L-cells chromatin is unaltered if cold carrier t-RNA and r-RNA are present in the isolation buffers and during the high speed centrifugation steps. This further confirms that these components do not arise as a result of degradation or aggregation of other RNA fractions.

Initially 0.05 M $NaHSO_3$ was added to all buffers to inhibit protease activity. At least for L, KB, HTC or DON-cells (though not so for calf thymus tissue) it makes little difference whether $NaHSO_3$ is present. This procedure has been omitted in order to eliminate the possibility of base modifications of the chromatin RNA components (Shapiro, Cohen, Servis, 1970). A step involving centrifugation of the 0.01 M tris washed chromatin through a 1.7 M sucrose solution (Bonner et al., 1968) was omitted since this procedure did not appear in any way to alter the profile of the final chromatin fractions, other than to reduce them all quantitatively. In previous methods during the high speed centrifugation step in buffers containing urea, NaCl, guanidine hydrochloride or SDS as much as 1 to 5% protein was found in the DNA pellet (Shirey and Huang, 1969; Shoshana, Simpson and Sober, 1972; Elgin and Bonner, 1970). With this procedure much lower values of protein are present (0.1% of the total chromatin protein). This can be attributed not only to the use of urea and NaCl to dissociate the chromatin components, but also to the presence of 0.002 M EDTA and 2-mercaptoethanol. Also it is possible that for some cases the protein present in the high speed centrifugation pellet arose

from material that was not of chromosomal origin, perhaps nucleolar material or nuclear membrane components (Wang, 1968).

From this work it appears that there are a number of CLMW-RNA fractions closely associated with chromatin. The two dimensional polyacrylamide gel electrophoresis perhaps best displays the complexity of CLMW-RNA fraction.

In view of the widely differing procedures used it is not easy to correlate the RNA fractions described in one method with that of another. The "chromosomal RNA" described by Bonner, Dahmus, Fambrough, Huang, Marushige and Tuan (1968); Dahmus and Bonner (1970; Huang and Huang (1969); may be involved in gene regulation (Bekhar, Kung and Bonner, 1969; Huang and Huang, 1969; Mayfield and Bonner, 1971). Do non-protein bound CLMW-RNA's take part in gene regulation? Recently it has become clear that at least two non-protein bound RNA components play a role in modifying the template activity of chromatin (Kanehisa, Fujitani, Sano and Tanaka, 1971).

It was felt that there was a need for a technique with which we could look at the non-protein bound low molecular wt RNA components present in chromatin. At the same time one had to avoid a procedure which would remove any protein bound RNA components such as phenolic extraction of the RNA (Prestayko and Busch, 1968). The procedure briefly described in the methods section could meet these needs.

The CLMW-RNA fractions A, B and C all appear to have S values less than that of tRNA. There have been a number of reports of similar low molecular wt RNA components in chromatin. "Chromosomal

RNA" yields only one band on a 14% polyacrylamide gel (Holmes, Mayfield, Sander and Bonner, 1972) and could thus only account for one of the above fractions. An S value of 3.2S has been obtained for Novikoff Ascites tumor "chromosomal RNA" (Dahmus and McConnell, 1969). This would suggest that CLMW-RNA fraction C could have some or all of its components in common with that of "chromosomal RNA". One difficulty with this is that we could not detect the presence of dihydrouridine in any CLMW-RNA fraction. It is possible that the degree of ^{32}P incorporation into the total CLMW-RNA fraction has not been sufficient to yield detectable amounts of dihydro-Up. Dihydropyrimidine bases could not be detected by other workers in a chromatin protein bound RNA (3.0-3.5S) isolated from rat liver (Arnold and Young, 1972). The procedure used to isolate "chromosomal RNA" containing dihydrouridine was used. The base composition of L-cell CLMW-RNA fraction C is similar (table 5) to that of this 3.0-3.5S RNA. The base composition of a 3-4S RNA component isolated from human leukemic leukocyte chromatin (Getz and Saunders, 1970) differs from that of CLMW-RNA fraction C. It is possible that these differences in base composition seen when RNA's characterized by one group of workers is compared with that of another is due simply to a real difference in the base sequences of these RNA components in the widely different cell types studied.

A component with an S value of 4.1S has not been previously reported to be present in chromatin. There are reports of 4S chromatin-RNA components (Kanehisa, Tanaka and Kano, 1972; Busch,

Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi and Muritzen, 1971; De Filippes, 1970) and a 4.2S RNA component from chicken liver chromatin (Kanehisa, Tanaka and Kano, 1972) but these components have not been examined on acrylamide gels containing concentrations of acrylamide greater than 12% with cytoplasmic tRNA. It may thus be possible that a small difference of 0.1S units could not be detected and that CLMW-RNA fraction D and the 4S chromatin-RNA components are the same species of RNA. A 4S RNA has been isolated from cell nuclei (Busch, Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi and Muritzen, 1971) it appears to be tRNA with amino acid acceptor activity. A nucleolar 4S RNA of quite different base composition and one-sixth the amino acid acceptor activity of that of nuclear 4S RNA has been characterized. The role of this RNA is unclear.

Is the CLMW-RNA fraction D the chromatin equivalent of the nucleolar 4S RNA component? We cannot say at present. The fact that there appears to be no modified bases in this RNA makes an amino-acid-acceptor activity unlikely. The possibility that fraction D is a precursor to t-RNA cannot be ruled out at present, although these species have been normally located in the cell cytoplasm (Burdon and Clason, 1969; Bernhardt and Darnell, 1969).

The CLMW-RNA fractions E and F do partially separate as two distinct bands on the analytical 12.5% acrylamide gel, however, the separation of the slab gels used for autoradioradiography did not give sufficient separation for an accurate base composition of the two individual fractions. A nuclear 4.5S RNA fraction containing

three distinct species has been located in the extranucleolar fraction of the nucleus (Ro-Choi, Reddy, Henning, Takano, Taylor and Busch, 1972). A 4.5S RNA component has been isolated from chick liver chromatin (Kanehisa, Tanaka and Kano, 1972). This fraction was found to modify the chromatin structure in its template activity for RNA synthesis. When this RNA was examined on a 16% acrylamide gel there appeared to be partial resolution of at least two components in this 4.5S fraction. The base composition of CLMW-RNA fractions E and F from L cells is comparable with that of the chicken liver 4.5S fraction.

There are numerous reports of 5S RNA components in nuclei, ribosomes, nucleoli, and chromatin (Weinberg and Penman, 1968; Clason and Burdon, 1969; Prestayko and Busch, 1968; Kanehisa, Tanaka and Kano, 1972). Two ribosomal 5S RNA components have been found in eucaryotic cells (Busch, Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi and Mauritzen, 1971).

A third 5S component having a base composition quite different than that of the ribosomal components has been found in whole nuclear RNA by these workers also. It is not in nucleolar or ribosomal RNA and is presumably associated with the chromatin fraction. A 5S RNA fraction has been isolated from chick liver chromatin which, like the 4.5S RNA component, appears to modify the chromatin template activity (Kanehisa, Tanaka and Kano, 1972).

Using the procedure described here, we also consistently obtain a CLMW-RNA component of slightly higher S value than that of 5S. This

is the CLMW-RNA fraction H. We can find no previous report of such an RNA component.

The CLMW-RNA fractions I and J together have base compositions similar to that of U1 nuclear RNA described by Busch, Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi, and Mauritzen (1971). U1 has been shown to contain at least 4 components, two of which are minor bands. The CLMW-RNA fraction K for the same reason may be similar to U2 nuclear RNA, both U1 and U2 appear to be localized to the extranucleolar portions of the nucleus.

It is thus possible to postulate that many of the nuclear low molecular wt RNA components already characterized are involved in some type of reversible binding to chromatin. This reversible binding may play some part in control of gene expression. Interestingly, some of the low molecular wt nuclear RNA's that have been studied in detail hybridize to a substantially large part of the DNA (Busch, Ro-Choi, Prestayko, Shibata, Crooke, El-Khatib, Choi, and Mauritzen, 1971). Clearly further work is required to determine what part these RNA fractions play in gene expression.

V A HYPOTHETICAL MODEL FOR GENE REGULATION IN EUCARYOTIC CELLS

As we have seen in the introduction, this thesis centers itself around the question of: How does regulation of gene transcription in eucaryotic occur? I now wish to propose a new model showing how the cell can exert its control of gene expression at the transcriptional level.

Unlike other models, it is suggested here that there are two major elements in controlling gene transcription in higher organisms: RNA turning genes on and repressor proteins turning them off. This model thus differs significantly from those of Scherrer and Marcaud (1968), Georgiev (1969a), Britten and Davidson (1969) and Wasserman (1972), where the major elements in the controlling process were either the NHP's or RNA.

Before discussing the model I would first like to introduce the following functional definitions.

OPERON: In using the term "operon" here we shall restrict our meaning to a part of the eucaryotic cell DNA that is transcribed by the RNA polymerase beginning at one region which we shall call the promoter region (P) and continuing through an undefined number of genes until some undefined terminator signal is reached. No further analogy with the procaryotic operon is implied. An inactive operon is surrounded by a protein layer. It is suggested that this layer can be divided into two main functional regions, a tightly bound (TB) protein zone and a loosely bound (LB) protein zone. When the TB protein zone is present alongside the DNA, the RNA polymerase cannot transcribe the operon. For transcription of the operon, the TB protein zone must first be removed. The remaining protein layer

on the operon, the LB protein zone, does not impede the passage of the polymerase along the DNA strand. It either falls away when the TB protein zone is removed, is pushed away by the polymerase, or is otherwise removed in an, as yet, unknown manner. It should be emphasized that the proposed TB and LB protein zones need not correlate directly with the known ease by which certain proteins can be dissociated from DNA by increases in ionic strength, changes in pH, or some other simple environmental change (Bonner et al., 1968). Rather, they are meant to distinguish between one region of the protein layer that must somehow be removed by the cell in order to make transcription of the operon possible and another region of the protein layer that in itself does not impede the transcription process.

It is proposed that the RNA transcribed from each operon is transcribed as one continuous polycistronic message. In Fig. 29 three such operons A, B and C are illustrated. In this model we will assume all genes are read from left to right. Each operon contains one of the following genes or any combination of two or more.

STRUCTURAL GENES ($^{S}G_{n}$). These contain the base sequences on the DNA which carry information for the synthesis of structural proteins or enzymes used by the cell. They do not contain information for proteins that have a regulatory role at the transcriptional level, although other levels of control within the cell are of course possible. All $^{S}G_{n}$ genes are coated by the loosely bound protein zone in the repressed state of the operon. The subscript "n" is used here to distinguish between different structural genes.

ACTIVATOR GENES ($^{A}G_{n}$). It is suggested that these genes are contained largely in the repetitive sequences of the DNA of the cell genome. These genes generally would be located immediately after

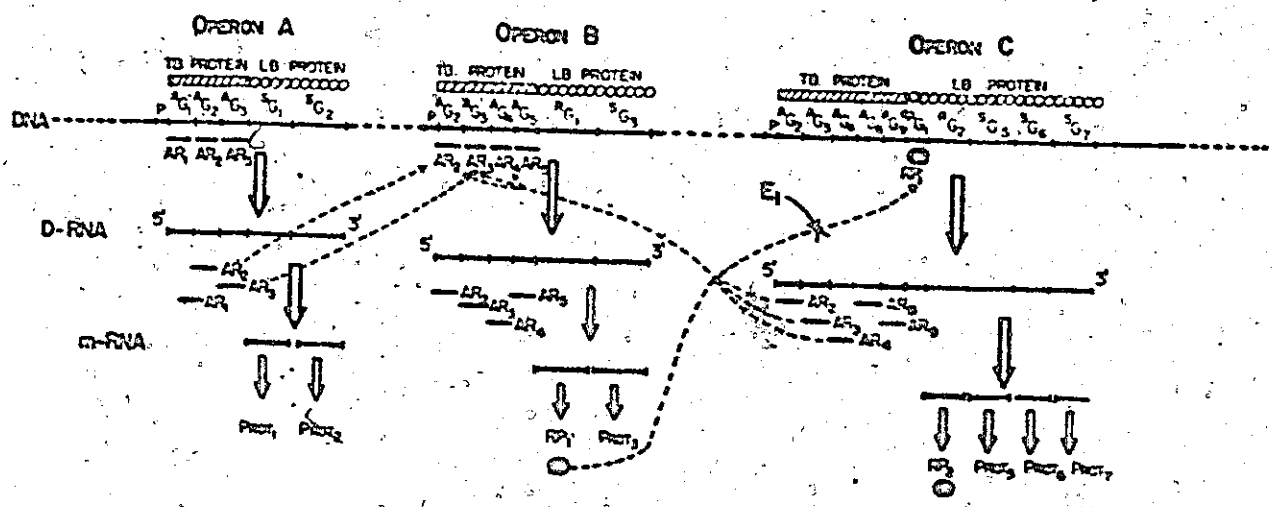


Figure 29. The suggested functional structures of three simple operons of a eucaryotic cell.

the promoter region of the operon. The RNA transcribed from each operon would thus have located on its 5' end RNA sequences complementary to these genes. In the process of breakdown of the giant operon RNA into monocistronic units of $^{R}G_{n}$ and $^{S}G_{n}$ gene products, it is proposed that short sequences of RNA complementary to the repetitive sequences of DNA on the $^{A}G_{n}$ genes would be formed. The $^{A}G_{n}$ genes are coated by the tightly bound protein zone when the genome is switched off. The subscript "n" is used to distinguish different activator genes.

ACTIVATOR RNA (AR_{n}). These are the fragments of RNA released after the breakdown of RNA from that part of the operon that contained the $^{A}G_{n}$ type genes and will thus contain sequences complementary to repetitive sequences in DNA.

The concentration of each activator RNA will vary throughout the life of the cell in an extremely complex way, since its concentration at any one time will depend on a balance between breakdown due to nuclease activity and supply from different operons, each producing different amounts of activator RNA's. An essential proposed feature of the activator RNA molecules is that they can displace those parts of the tightly bound protein zone of the operon to which they bind. Thus in operon A (Fig. 29' when AR_{1}, AR_{2} and AR_{3} are present simultaneously, all of the tightly bound protein zone proteins are removed from this operon; the RNA polymerase is thus free to transcribe the complete operon. Should there later be an absence of one or all the activator RNA's AR_{1-3} some or all of the tightly bound protein zone proteins would bind back to the DNA of operon A turning it off.

REPRESSOR GENES ($^{R}G_{n}$). These regions of the DNA carry information for synthesis of the repressor proteins (RP_{n}). The subscript "n" is used to distinguish different repressor genes or their proteins. These proteins will in the absence of effector (E) bind reversibly to a region of the DNA - the repressor protein acceptor gene ($^{RA}G_{n}$). In doing so they remove the tightly bound protein zone of the operon that they bind to; however, the polymerase cannot transcribe the whole operon since the repressor protein firmly bound to the DNA prevents its passage along the operon to the structural genes. In some cases these proteins can have their binding to the $^{RA}G_{n}$ type gene inhibited by the presence of an effector molecule (E_{1}) (for example, RP_{1} in Fig. 29). Under these conditions before the tightly bound protein zone can rebind, the polymerase can transcribe the operon at least once. Activator RNA molecules (AR_{n}) formed from the transcribed RNA will then bind back to the $^{A}G_{n}$ genes keeping the operon on, by displacing any of the tightly bound protein zone that had formed on the operon. It is also suggested that there are cases in which the repressor proteins are not acted upon by any effector molecules but always display a strong affinity for a $^{RA}G_{n}$ type gene. In doing so the further production of RNA along this operon is prevented for as long as the presence of this regulator protein is maintained within the nucleus. All $^{R}G_{n}$ genes are coated by the loosely bound protein zone when the operon is repressed.

REPRESSOR PROTEIN ACCEPTOR GENES ($^{RA}G_{n}$). These regions of DNA contain sequences which are recognized by the repressor proteins. The recognition is highly specific; for example the $^{R}G_{1}$ gene repressor

protein binds to only a $^{RA}G_1$ type repressor protein acceptor gene. In this model no essential function is assigned to the RNA transcribed from $^{RA}G_n$ type genes. Again, all $^{RA}G_n$ genes are coated by the loosely bound protein zone when the operon is switched off.

All other terms used in this article have the same meaning as those already in use.

THE MODEL

An essential feature of our model is that it has a series of interlocking elements. It is assumed that at any one time in the cell there are always some active genes. It is via complex interactions of these genes that the decision of which further genes will be turned on or off is made.

It is proposed that in the cell the passage of the RNA polymerase along the operon requires the presence of RNA sequences (AR_n) complementary to the base sequence of the DNA for those $^{A}G_n$ genes which are covered by the tightly bound protein zone. A number of low molecular weight RNA components have been isolated from nuclei which could have this property; one of these, "chromosomal RNA" (Huang and Bonner, 1965; Dahmus and McConnell, 1969; Getz and Saunders, 1970; Marsluff, Smith and Huang, 1972; Holmes, Mayfield, Sandor and Bonner, 1972) appears to fulfill many of these requirements. It has been shown to be essential for a sequence specific interaction of chromosomal proteins with DNA (Dekhor, Kung and Bonner, 1969; Huang and Huang, 1969). The chromosomal RNA appears to hybridize with a large fraction of the total cellular

DNA (Dahmus and Bonner, 1970; Mayfield and Bonner, 1971); further, the hybridization is preferentially to the repetitive sequences of DNA (Mayfield and Bonner, 1971).

The unique base composition of "chromosomal RNA" would require that the AR_n molecules be modified before binding to the ^AG_n genes.

Other RNA components have been isolated from chromatin (Benjamin, Levander, Gellhorn and DeBellis, 1966; Prestayko and Busch, 1968; Pelling, 1970; Gasaryan et al., 1971) as well as the CLMH-RNA's discussed in this thesis. While in most cases their structure and function has not been examined in detail, it is possible that our hypothetical activator RNA components are contained in some of the above RNA fractions.

One low molecular weight RNA component from chicken liver chromatin (Kanehisa, Fujitani, Sano and Tanaka, 1971) inhibited RNA synthesis in vitro with E. coli RNA polymerase and DNA as template - an effect common to many other types of cellular RNA's. However, unlike other RNA species tested when homologous tissue chromatin is used as template, the RNA synthesis is considerably stimulated. This stimulation appears to be due to a modification of the chromatin template availability by the RNA.

I do not wish to explain how the presence of the AR_n at the ^AG_n genes facilitates passage of the RNA polymerase along the operon, but it is possible that this is intimately involved with the removal of the F1 or F3 histones from this region (Georgiev, 1969b; Kozlov and Georgiev, 1971; Barker, 1971).

Operon A (Fig. 29), represents one of the simplest organizations of an operon: the polymerase passes through the $^{A}G_{n}$ genes, then transcribes the structural genes $^{S}G_{1}$ and $^{S}G_{2}$. A high molecular weight DNA-like RNA (D-RNA) molecule is formed in this process. From the proposed organization of the activator genes and structural genes it is suggested therefore that the 5' terminal end of the transcribed RNA will contain a number of repeated RNA sequences. In the process of the polycistronic D-RNA being broken down to individual cistrons of structural genes and sent out into the cytoplasm, it is suggested that by means of specific nucleases present in the nucleus activator RNA molecules are formed from the repetitive sequences of RNA that were transcribed from the $^{A}G_{n}$ genes. The evidence that leads to these suggestions is as follows: it appears that newly transcribed RNA in the nucleus has a much higher molecular weight than that of monocistronic messenger RNA in the cytoplasm. The D-RNA appears to be rapidly broken down within the nucleus (Harris, 1963; Penman, Scherrer, Becker and Darnell, 1963; Georgiev and Lerman, 1964; Samarina, 1964; Scherrer, Marcaud, Breckenridge, Attardi, Parnas, Ewang and Attardi, 1966; Church and McCarthy, 1967).

The D-RNA breakdown appears to begin from the 5' terminal end; while the 3' terminal end passes out into the cytoplasm (Georgiev, Ryskov, Coutelle, Mantieva and Avakyan, 1972). The repetitive sequences of DNA appear to be located mainly at the 5' region of RNA, the unique sequences mainly at the 3' end (Ryskov, Mantieva, Avakian and Georgiev, 1971; Georgiev et al., 1972). The fragmentation of the D-RNA 5' part would require the presence of nuclease within the nucleus capable of

breaking the D-RNA in a specific manner. This does not seem unreasonable if it is suggested that along the D-RNA there are linker base sequences between different $^{A}G_{n}$ genes, possibly giving a unique conformation to this region of the D-RNA (Cory, Spahr and Adams, 1970) or in some other way causing a preferential hydrolysis by the ribonuclease at these points. Cases of sequence specific breakdown of viral RNA (Green, 1970), ribosomal RNA (Greenberg and Penman, 1966; Jeanteur and Attardi, 1969) and even D-RNA are known (Aronson, 1972). However, while the model suggests a function for some of the repetitive sequences in DNA, it does not exclude the possibility that other repetitive sequences are present which have other functions, such as terminators or sequences recognized by specific protein protectors. From the arrangement of the $^{A}G_{n}$ gene sequences it might be expected that the repetitive sequences of DNA be arranged in a tandem arrangement and distributed throughout most of the DNA of the cell. This appears to be so (Britten and Kohne, 1968; Thomas, Hamkalo, Misra and Lee, 1970).

It is possible - although it is not essential for the model to work - that some of the low molecular weight RNA components present in relatively large amounts in cell nuclei (Weinberg and Penman, 1968; Moriyama, Hodnett, Prestayko and Busch, 1969) arise as a result of the release of common linker sequences between the AR_{n} components on the D-RNA as well as the individual AR_{n} components. The above nuclear RNA sequences could lend specific conformations to the D-RNA to insure that the nuclear nuclease activity will hydrolyze the D-RNA at the correct internal locations.

Operon A, illustrated in Fig. 29, once activated by the presence of activator RNA's AR_{1-3}, will thus continuously produce proteins 1 and 2 assuming that here there are no other post-transcriptional controls This is the kind of operon we might expect in a cell which has genes that are used in some fundamental biochemical pathway of the cell where continuous production of mRNA is required. An ever increasing level of activator RNA's will of course be prevented by the presence of nuclear nuclease activity (de Lamirande and Allard, 1959; Sierralta and Minguell, 1970). However, it is suggested here that at least some of the activator RNA's are present on the DNA long enough that the polymerase can produce more D-RNA and their further production is facilitated. At least in the case of avian nucleated red blood cells, it appears that the degraded D-RNA remains bound to the chromosome for a period of time before moving through the nucleus (Gasaryan, Lipasova, Kirjanov, Ananjanz and Ermakova, 1971).

An essential feature of any model accounting for regulation of gene transcription in eucaryotic cells is that different batteries of genes must be able to interact with each other (Davidson and Britten, 1971). It is suggested that an operon may interact with other operons in one of two ways. It can induce the production of D-RNA in another operon via the production of activator RNA's, or inhibit this formation of D-RNA by the production of repressor proteins. Let us consider each in turn.

Operon B of Fig. 29 could be activated by the combined effect of operon A which provides activator RNA's AR_2 and AR_3 and some other operons (not shown) which provide AR_4 and AR_5. It could also be

activated by an operon that provides AR_5 and operon C, when C itself is active, since it contains activator gene sequences for AR_{2-4}. As we will see later, C is normally only active in this diagram in the presence of an inducer E_1. It must be remembered that activation of an operon, for example operon B, by activators AR_{2-4} and AR_5 is not a single isolated event within the cell. The polymerase will have to wait until there is a sufficient build up in the concentrations of AR_{2-4} and AR_5 within the nucleus such that sufficient activator RNA's are available to simultaneously bind to the $^{A}G_{2-4}$ and $^{A}G_5$ genes. The polymerase at this operon will then be able to transcribe its D-RNA, which in turn gives rise to further species of AR_{2-4} and AR_5. These of course could bind back to the $^{A}G_{2-4}$ and $^{A}G_5$ genes facilitating their further production, diffuse to other operons, or be broken down by nuclease activity. A further complexity is that an operon containing one or more multiple identical activator genes will require not only a representative activator RNA for each $^{A}G_n$ gene it contains before transcription but will have to wait until a high concentration of the multiple $^{A}G_n$ gene activator RNA builds up in the nucleus. Once this happens it tends to maintain itself and also turn on other operons containing a high content of that $^{A}G_n$ gene.

Clearly a negative control element is required to repress the expression of certain genes once they have served their purpose for a period in the lifetime of the cell. This is the function it is proposed the repressor proteins serve.

Operon B (Fig. 29) differs from A in that it contains a regulator gene ($^{R}G_1$). Thus repressor protein RP_1 is formed when operon B is

activated by AR_{2-4} and AR_5. Operon C could be activated by the combined action of operon B which gives rise to activator RNA's AR_{2-4}, and some other operons (not shown) which provide AR_8 and AR_9 from amongst their $^{A}G_n$ genes. However, since in this case operon B is producing RP_1 this repressor protein will by binding to the $^{RA}G_1$ gene prevent the repressor gene $^{R}G_2$ and structural genes $^{S}G_{5-7}$ of operon C from being transcribed. This could be an essential process for the cell, if it wants to have operon B, and other operons containing $^{A}G_8$ and $^{A}G_9$ genes on all the time, but C turned off. If there was no $^{RA}G_1$ gene present in C the activator RNA's AR_{2-4}, AR_8 and AR_9 would have turned it on.

The degree of binding of RP_n molecules to $^{RA}G_n$ genes could vary considerably, some binding so strongly as to permanently turn off operons. For other RP_n molecules this may be quite reversible. At least some non-histone proteins turn over rapidly (Allfrey, Daly and Mirsky, 1955; Holoubek and Crocker, 1968). In such cases a way to turn on operon C without the preexistence of activator RNA's, would be to shut off RP_1 synthesis (for example via a reduction in AR_5 concentration in the nucleus). After the RP_1 concentration decreases there will be insufficient RP_1 to bind to $^{RA}G_1$. The polymerase will transcribe the operon at least once before the reformation of a tightly bound protein zone on operon C, giving rise to AR_{2-4}, AR_8 and AR_9, which in turn feedback and maintain the operon switched on.

There is another way the cell could turn on operons without the preexistence of activator RNA's to bind to its $^{A}G_n$ genes. As seen in the case of operon C, RP_1 could be a non-histone protein that has its conformation altered by some effector E_1 such that it can no longer

bind to the $^{RA}G_1$ gene, or binds in a manner that does not inhibit the action of the RNA polymerase. For the same reason as explained above, the absence of the inhibition effect of RP_1 on the RNA polymerase at the $^{RA}G_1$ gene will allow the operon C to be switched on.

As in the bacterial system (Jacob and Monod, 1961, Gilbert and Mueller-Hill, 1967) removal of E_1 will allow RP_1 to again inhibit the action of the RNA polymerase at the $^{RA}G_1$ and cause the operon to revert to a switched off state. It cannot be estimated what proportion of the non-histone proteins exist as effector-sensitive and the effector-nonsensitive types at this time.

This model can explain how a large series of gene transcription alterations occur, as for example when tissues are stimulated by hormones. Let us call the state of gene expression before the cells were stimulated, state A; that after stimulation, state B. Operons D and E (Fig. 30) could be the type of key linking operons between many operons in state A soon to be turned off and operons in state B to be turned on. Let us call these types of operons an "activator RNA amplifier operon combination" for reasons that will become clear below.

Let us say operons of major importance in state A require a high concentration of activator RNA's AR_4 and AR_5, while operons in state B require in particular activator RNA's AR_8 and AR_9. Amongst the operons expressed in state A is operon D (Fig. 30). This operon is activated by AR_2, AR_4, AR_5 and AR_7, and will give rise to the repressor protein RP_3 which will bind to the DNA sequence of the $^{RA}G_3$ gene in operon E. The tightly bound protein zone will be removed from the $^{A}G_n$ genes of this operon; however, because the RP_3 protein remains bound to

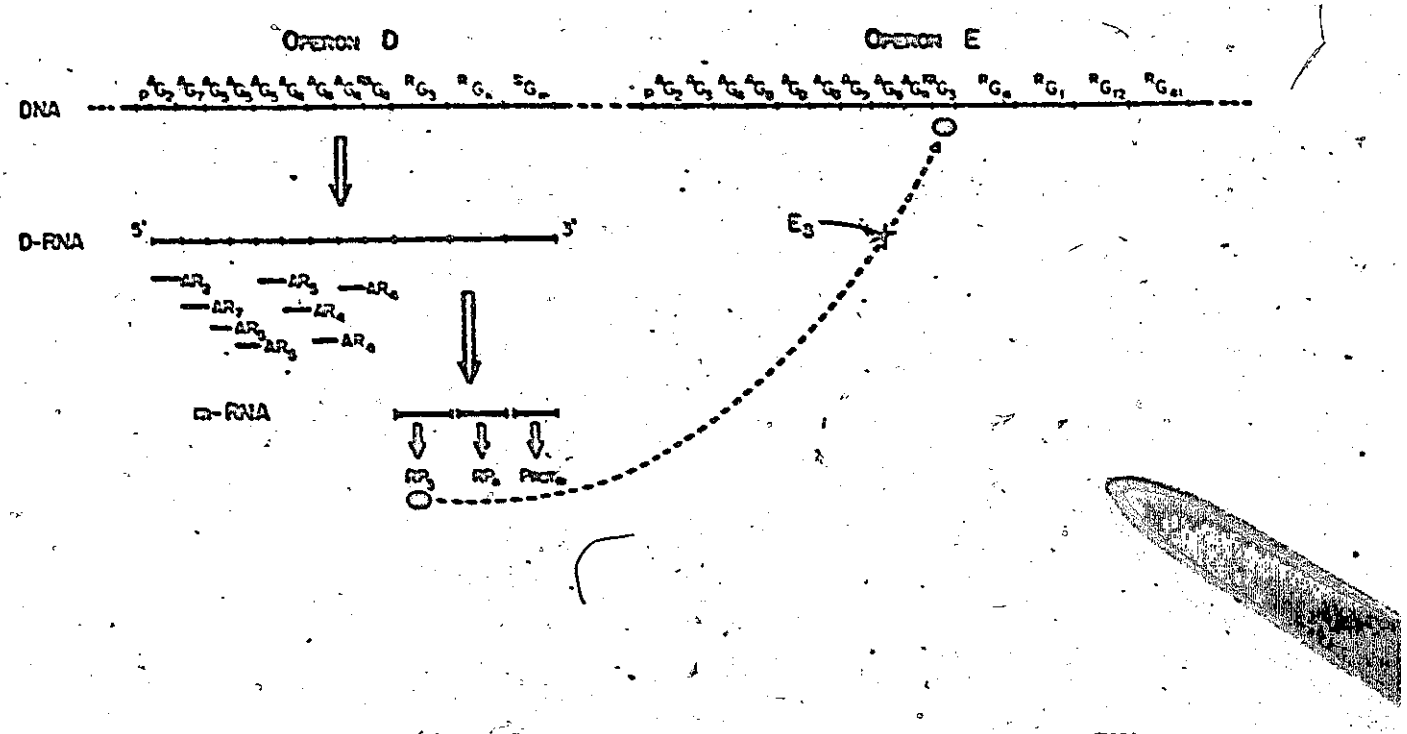


Figure 30. The suggested functional structure of an activator RNA amplifier operon combination.

the DNA the operon is not transcribed into RNA. The RP_3 protein bound to the $^{RA}G_3$ sequence prevents the RNA polymerase from transcribing the operons. It is suggested that an effector (E_3) capable of causing transformation of cell state A to state B can, by binding either directly to RP_3 or via some other intermediary protein (Steggles, Spelsberg and O'Malley, 1971), inhibit or alter the binding of RP_3 to the $^{RA}G_3$ gene. The RNA polymerase can then transcribe operon E, giving rise to new activator RNA's, AR_8 and AR_9. Also in the case of this example, a new repressor protein RP_4 is formed. The RP_4 will in this case bind to the $^{RA}G_4$ on operon D closing it down. The newly formed activator RNA's per se will be capable of starting up other operons within the cell. (Recent work (Congote and Trachewsky, 1972) has shown that in the case of at least one effector, aldosterone, acting on rat kidney cortex tissue, an early effect of hormone treatment was a specific increase in nuclear RNA capable of hybridizing with repetitive DNA sequences). The new operons switched on could be like operons A, B or C. But it is possible that AR_8 and AR_9 could bring about expression of many other operons that the cell at this time does not wish to express. Therefore, operons D and E would contain a number of $^{R}G_n$ type genes to ensure that such operons are not expressed. For example, the $^{R}G_1$ gene on operon E of Fig. 30 would prevent the expression of operon C of Fig. 29. We would thus expect the appearance of new non-histone proteins in the nucleus of cells involved in such an effector-dependent change from state A to state B.

I would like to cite some of the experimental evidence for

the existence of such repressor proteins. The repressor proteins are suggested to be contained in the non-histone protein (NHP) fraction of chromatin. The amounts of NHP present in isolated chromatin varies markedly from one cell type to another (Frenster, Allfrey and Mirsky, 1963; Commings, 1967; Marushige and Ozaki, 1967). In general there appears to be more NHP in template active chromatin than template inactive chromatin (Bonner and Huang, 1963; Frenster, 1965; Marushige and Dixon, 1969; Helmsing and Berendes, 1971). This we would expect from our model since, as already explained, the further production of many new activator RNA species in template active chromatin necessitates the specific repression of other operons that would otherwise be turned on as well. The NHP fraction shows strong tissue specificity (Loeb and Creuzet, 1969; Platz, Kish and Kleinsmith, 1970; Teng, Teng and Allfrey, 1970; Kostraba and Wang, 1970; MacGillivray, Carroll and Paul, 1971). At least some NHP's interact specifically with DNA (Paul and Gilmour, 1968; Kleinsmith, Heidema and Carroll, 1970; Salas and Green, 1971). In contrast to histones which turn over at a low rate (Gallwitz and Mueller, 1969; Hancock, 1969) at least some NHP's turn over rapidly in the cell (Allfrey, Daly and Mirsky, 1955; Holoubek and Crocker, 1968). Also their synthesis is, unlike many other proteins of the cell, maintained at mitosis (Stein and Baserga, 1970b). The model clearly requires that proteins bind specifically to DNA, and that some at least be tissue specific. A rapid turnover of these proteins is in agreement with a highly flexible differential expression and repression of some operons. The NHP composition appears to be altered during the process

of differential gene expression, i.e. when cells undergo a state A to state B type transition. A wide range of effectors have been suggested as being involved in these processes: estrogens (Yeng and Hamilton, 1970; Mester and Baulieu, 1972; Barker, 1971); cortisol (Deisseroth, 1969; Buck and Schauder, 1970; Shelton and Allfrey, 1970); progesterone (O'Malley et al., 1972); insulin (Buck, Schauder and Weser, 1970); thyroxin (Tata, 1966); isoproterenol (Stein and Baserga, 1970a); β-ecdyson (Helmsing and Berendes, 1971); and unknown effectors (LeStourgeon and Rusch, 1971). Clearly these changes in NHP compositions are in line with the effector dependent changes of NHP we would expect from out model.

It has been suggested that the binding of the repressor proteins RP_n to their $^{RA}G_n$ genes brings about removal of the tightly bound protein zone of each operon. Sufficient data is not yet available to suggest what components are involved in this process or how it takes place. At least one study has indicated removal of the tightly bound F3 arginine-rich histone by a specific NHP after stimulation of uterine tissue with estradiol (Barker, 1971). There are numerous reports of NHP increasing template activity of chromatin (Wang, 1969; Spelsberg and Hnilica, 1969; Gilmour and Paul, 1969; Wang, 1970; Kamiyama and Wang, 1971; Kostraba and Wang, 1972) as well as reports of increased RNA transcribed from chromatin which has an increase in its content of NHP (O'Malley and McGuire, 1969; Helmsing and Berendes, 1971; O'Malley et al., 1972). It might thus appear contradictory to suggest a repressor protein role for the non-histone proteins. However,

careful analysis of the model reveals no major contradiction with the experimental results reported. Increased RNA transcribed from chromatin could arise when a few NHP's have their binding to the DNA inhibited by one or more effectors, as in the case of operon E (Fig. 30) already discussed. As already pointed out, formation of many new activator RNA's also requires formation of new repressor proteins to turn off unwanted operons that would otherwise be activated by the new profile of activator RNA's. The increase in template activity of chromatin seen in vitro when NHP is added could be due to exposure of the DNA previously hidden by the tightly bound protein zone but now available to the procaryote polymerase for transcription (Kamiyama and Wang, 1971; Kostraba and Wang, 1972). This observed increase in template activity of DNA appears to lie in the repetitive sequences of the DNA (Kostraba and Wang, 1972). Such increases in template activity seen in vitro have been suggested to bring about an increase in the length of the transcribed RNA rather than to expose new initiation sites on the DNA (Kamiyama and Wang, 1971). This is interpreted to mean that once the RP_n has bound to the $^{RA}G_n$ gene further RP_n molecules only increase the removal of the TB protein zone within this operon and not at the many other repressed operons within the cell.

The operations presented above are in many cases too simple to account for the sophisticated control of gene expression that appears to take place in eucaryotic cells. It is felt, however, that similar gene arrangements and interactions, containing multiple $^{A}G_n$, $^{R}G_n$, $^{RA}G_n$ and $^{S}G_n$ genes in such operons, would be in many ways sufficient to

explain many of the known experimental facts of the eucaryotic chromosome, its components and their functions.

VI SUMMARY

A general method for the isolation and fractionation of chromatin into its four major components, DNA, RNA, histones and non-histone proteins, is described. The procedure avoids the use of strongly acidic or alkaline conditions, the use of ionic detergents or phenol. As few as 1.4×10^6 cells can be used. The chromatin components are dissociated in a 3 M NaCl, 5 M urea solution containing 2-mercaptoethanol and EDTA. The DNA and high molecular weight RNA are collected by high speed centrifugation and DNA is separated from the RNA by means of Cs_2SO_4 equilibrium density centrifugation. The histones, non-histone proteins and low molecular weight RNA's are fractionated using DEAE cellulose column chromatography and polyacrylamide gel electrophoresis. The heterogeneity of the RNA fraction is further illustrated using two dimensional polyacrylamide gel electrophoresis.

A new hypothesis to explain gene expression in eucaryotic cells at the transcriptional level is described. Previous models for regulation of gene transcription in eucaryotic cells have suggested batteries of genes giving rise to "activator RNA" type molecules which may interact with other operons turning them on. In other models a giant "polycistronic D-RNA" was suggested to be made up of an "informative part" at the 3' end containing genes that are translated in the cytoplasm, and a "non-informative part"

at the 5' end which is degraded in the nucleus. No apparent function was assigned to the "non-informative part" of the D-RNA.

In this model it is suggested that the RNA in the "non-informative" part of the D-RNA is instrumental in turning other genes on, behaving in some respects like "activator RNA's". It is proposed genes are repressed in a specific manner by repressor proteins. For some genes this repression can be inhibited either through a reduction in the production of the appropriate repressor protein, or via the interaction of the repressor protein with an effector molecule.

Thus, unlike other models, we suggest that there are two major elements in controlling gene transcription in higher organisms: RNA turning genes on and repressor proteins turning them off.

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