To the Graduate Council:

I am submitting herewith a dissertation written by Tim M. Townes entitled "Evaluation of Nascent RNA as a Criterion of Chromatin Fractionation." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

W. Stuart Riggsby, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

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EVALUATION OF NASCENT RNA AS A CRITERION OF CHROMATIN FRACTIONATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Tim M. Townes

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ABSTRACT

Separation of chromatin into transcriptionally active and inactive fractions is a goal which has been pursued for many years. Many methods have been proposed and have been shown to be successful based on one or more criteria for activity. One of the most widely applied criterion has been the presence of nascent RNA chains in certain fractions following pulse-labeling with labeled RNA precursor. The applicability of this criterion has been questioned by the finding that exogenously added RNA binds selectively to a chromatin fraction which co-sediments with putatively nascent RNA in sucrose gradient fractionation schemes. Chapters I and II of Part I describe the partitioning of non-nascent RNA in two other fractionation schemes, the DNAse II-$MgCl_2$ and ECTHAM-cellulose procedures, respectively.

The results presented in Chapter I demonstrate that free RNA, which is either added exogenously or generated endogenously, binds to the same fraction that is in fact enriched in actively transcribed DNA sequences. Therefore, although this fraction may indeed by enriched in transcriptionally active chromatin, the "nascent RNA" assay is not a reliable indicator of such activity.

The results presented in Chapter II demonstrate that free RNA does not selectively bind to "active" chromatin generated by the ECTHAM-cellulose procedure. However, free RNA itself binds to the resin and elutes at the same pH as chromatin which has many of the structural features expected for an active fraction. Therefore, the nascent RNA assay is also an unreliable indicator of activity for the ECTHAM-cellulose procedure.

The work described in Part I, Chapters I and II grew out of a project in which chromatin fractionation was to be used as the first step in the isolation of molecules which regulate transcription. Although the project as originally proposed was not completed, Chapters I, II and III of Part II describe the preparation of reagents which are essential in assays for specific regulatory activity.

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INTRODUCTION

PART I. FRACTIONATION OF CHROMATIN INTO ACTIVE AND INACTIVE SEGMENTS

Although every cell of a particular metazoic organism contains the same basic genetic complement (Gurdon, 1962[1]), only a small portion of the genome of any individual cell type is frequently transcribed (Brown and Church, 1972[2]; Grouse, Chilton, and McCarthy, 1972[3]). Several lines of evidence indicate that the structure of actively transcribed regions is dramatically different from relatively inactive regions. First of all, autoradiographs of thymocyte nuclei labeled in vitro with [^{3}H]uridine demonstrate that RNA synthesis is associated with diffuse, extended regions of interphase chromosomes rather than condensed, compact regions (Littau, 1964[4]). Secondly, electron micrographs of meiotic prophase chromosomes obtained from developing amphibian oocytes reveal that the extended loops of the lampbrush chromosomes are extremely active in transcription (Miller, 1965[5]).

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[1] J.B. Gurdon. 1962. Dev. Biol. 4, 256.

[2] I.R. Brown and R.B. Church. 1972. Dev. Biol. 29, 73.

[3] L. Grouse, M.D. Chilton, and B.J. McCarthy. 1972. Biochemistry 11, 798.

[4] V.C. Littau, V.G. Allfrey, J.H. Frenster and A.E. Mirsky. 1964. Proc. Natl. Acad. Sci. USA 51, 93.

[5] O.L. Miller. 1965. Natl. Cancer Inst. Monogr. 18, 79.

Thirdly, autoradiographs of Dipteran polytene chromosomes after in situ hybridization with labeled mRNA indicate that the extended regions known as "puffs" are much more active in RNA synthesis than condensed regions (Berendes, 1973[6]). And finally, hybridization studies demonstrate that DNA sequences known to be actively transcribed in a particular cell type are selectively degraded when nuclei are exposed to DNAse I or micrococcal nuclease (Weintraub and Groudine, 1976[7]; Wu, Wong and Elgin,1979[8]). Experiments involving nuclease digestion have been particularly useful in obtaining information regarding the structure of actively transcribed sequences.Apparently, DNAse I and micrococcal nuclease cleave DNA in the more extended chromatic regions but can not cleave sequences which are protected from the enzymes by extensive secondary chromatin structure. Direct proof of this assumption was recently obtained by S.R. Elgin and her collaborators (Wu, Wong, and Elgin, 1979[9]; Wu, et al. 1979[10]). In Drosophila a specific set of genes is expressed when the organism is grown at 35 rather than 25°C. Although the function of proteins encoded by these genes is not known, the de novo expression of the genes following a simple stimulus provides a direct system for studying mechanisms

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[6]H. Berendes. 1973. Int. Rev. Cytol. 35, 61.

[7]H. Weintraub and M. Groudine. 1976. Science 93, 848.

[8]C. Wu, Y.-C. Wong, and S.C.R. Elgin. 1979. Cell 16, 807.

[9]Ibid

[10]C. Wu, P.M. Bingham, K.J. Livak, R. Holmgren and S.C.R. Elgin. 1979. Cell 16, 797.

involved in differential gene expression. The new expression of these sequences can be followed microscopically and biochemically. On the microscopic level, new puffs in the salivary gland polytene chromosomes are observed when flies are grown at 35°C. That these puffs mark the location of new sites of transcription is demonstrated by in situ hybridization experiments which show that RNA extracted from flies grown at 35 but not 25°C hybridize to these specific chromosomal sites.

A more detailed view of the structural changes associated with new expression of these sequences is demonstrated on the biochemical level when nuclei are obtained from flies grown at 25°C and are digested with DNAse I or micrococcal nuclease. Sequences which code for heat shock proteins remain in tact. The preservations of these sequences is demonstrated in the following way. After nuclease digestion, DNA is extracted and electrophoresed on agarose gels. In the case of micrococcal nuclease digestion, the smallest band of DNA observed is a sequence of 200 base pairs. This band represents DNA protected from the nuclease at the most basic level; that is, the level of a nucleosome. Bands which are multimers ofthe basic 200 base pairs unit are also observed. These bands result from cleavage of DNA at both ends of a series of 2 to 8 monosomes. Above the level of an octomer, no general repeating unit is observed. However, if DNA from the gel is transferred to nitrocellulose by the method of Southern (1975[11]) and hybridized to a cloned heat shock gene, bands of greater than 2,000 base pairs are observed following autoradiography. Moreover, the banding pattern observed is different when different cloned heat shock genes are used as

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[11]E.M. Southern. 1975. J. Mol. Biol. 98, 503.

probes. These results indicate that chromatin is organized into higher order structures (that is structures of order higher than nucleosomes) and that these structures are specific for individual genes.

When nuclei are obtained from flies or cultured Drosophila cells which have been exposed to heat shock for various times (5-30 min), the same general banding pattern as described above for the 25°C incubation is observed after micrococcal nuclease digestion. However, when specific DNA sequences are examined by hybridization of cloned heat shock genes to Southern blots, hybridization to bands derived from chromatin packaged into higher orders is inversely proportional to the time of heat shock; that is, the longer the flies are exposed to 35°C, fewer sequences encoding heat shock proteins are available for hybridization. However, higher order bands derived from sequences not induced by heat shock are unaffected by incubation at 35°C. These results seem to indicate that heat shock triggers a change in chromatin structure. Perhaps this change involves the unfolding of chromatin and this extension of chromatin fibers permits DNAse I and micrococcal nuclease to cleave DNA which was previously protected.

In summary, although there is no experimental evidence to distinguish between the alternatives that chromatin structure is changed after rather than before transcription, the results demonstrated conclusively that the structure of actively transcribed chromatin is indeed different from infrequently transcribed sequences.

Since actively transcribed chromatin differs structurally from inactive chromatin, it should be possible to develop techniques which take advantage of these differences in order to isolate fractions enriched in active sequences. If separation could be achieved, the structure of active chromatin could be studied in more detail.

The first attempts to fractionate chromatin into active and inactive sequences involved sedimentation of fragmented chromatin on sucrose and glycerol gradients (Frenster, et al. 1963[12]). This method was originally devised to take advantage of the more extended structure of active chromatin; active chromatin should have a lower sedimentation coefficient than condensed, inactive chromatin. Indeed, slowly sedimenting chromatin does display several characteristics expected for active chromatin. It is enriched in nascent RNA and has a 6°C lower melting temperature than rapidly sedimenting chromatin. Slowly sedimenting fractions are also highly sensitive to DNAse I. However, Howk, et al. (1975[13]) have demonstrated that slowly sedimenting chromatin is not enriched in DNA sequences known to be transcribed in the tissue of origin. Therefore, although structurally distinct chromatin fractions are separated by sedimentation on sucrose and glycerol gradients, active chromatin is not distinguished from inactive chromatin. In 1972 several frctionation procedures were introduced. Janowski, et al. (1972[14]) reported that chromatin could be fractionated into active and inactive fractions by gel filtration chromatography. After shearing, chromatin is applied to a column of agarose beads and eluted with

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[12]J.H. Frenster, V.G. Allfrey and A.E. Mirsky. 1963. Proc. Natl. Acad. Sci. USA 50, 1026.

[13]R.S. Howk, A. Anisowicz, Y. Silverman, W.P. Parks, and E.M. Scolnick. 1975. Cell 4, 321.

[14]M. Janowski, D.S. Nasser and B.J. McCarthy. 1972. Karolinska Symp. 5, 112.

low salt. Fractions which elute late from the column are enriched in nascent RNA chains, in in vitro template activity for E. coli polymerase and in hormone receptor complexes in chromatin from hormone target tissue. All of these characteristics of late eluting chromatin seem to indicate that thesefractions contain sequences actively transcribed in vivo. However, in 1976, Kreig and Wells[15] demonstrated that putative active chromatin obtained by chromatography of sheared chicken erythrocyte chromatin was not enriched for globin genes and that both active and inactive fractions contained approximately equal amounts of keratin sequences which are not actively transcribed in chicken erythroid cells. Therefore, this procedure also appears to be ineffective in separating active and inactive chromatin.Also in 1972, McConaughy and McCarthy[16] described a procedure which fractionated chromatin by thermal chromatography. Reasoning that ex tended active chromatin should have a lower melting temperature than compact, inactive chromatin, these investigators sheared chromatin, applied it to a hydroxyappatite column under conditions in which double but not single stranded DNA binds, and eluted chromatin with increasing temperature. DNA extracted from chromatin eluting at the lowest temperatures was enriched for sequences known to be transcribed in the tissue of origin. Moreover, DNA extracted from chromatin eluting at higher

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[15] P. Kreig and J.R.E. Wells. 1976. Biochemistry 15, 4549.

[16] B. McConaughy and B.J. McCarthy. 1972. Biochemistry 11, 998.

temperatures was enriched for sequences known not to be transcribed in the tissue of origin. Although thermal chromatography on hydroxyappatite is apparently capable of separating active and inactive chromatin, the disadvantage of this technique is that chromatin structure is destroyed by the high temperatures and, therefore, intact active chromatin can not be obtained for further studies.

In order to fractionate chromatin under mild conditions so that the structure of active and inactive chromatin could be preserved, Simpson and co-workers (1972[17]) developed a procedure involving chromatography and sheared chromatin on a weak anion exchange resin, ECTHAM-cellulose. The resin was prepared by coupling tris(hydroxymethyl)aminoethane to cellulose with epichlorohydrin at a capacity of 0.1 - 0.15 milliequivalents per gram of cellulose (Peterson and Kuff, 1969[18]). Since the resin is prepared at such low capacity and the ionizing groups have relatively low pK's, chromatin can be bound and differentially eluted in a pH range of 7.2 to 9.2 in 10 mM NaCl. These conditions are sufficiently mild to allow the recovery of structurally intact chromatin fractions.

Sheared chromatin is loaded on the column equilibrated in 0.01 M Tris pH 7.2 and eluted with 0.01 M Tris (base) - 0.01 M NaCl. Loosely bound fractions are eluted early from the column and then more strongly bound molecules are eluted as the ionizing groups are titrated. When all of the groups are titrated, the pH increases quickly to around 9.2 and, consequently,

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[17]G.R. Reeck, R.T. Simpson and H.A. Sober. 1972. Proc. Natl. Acad. Sci. USA 69, 2317.

[18]E.A. Peterson and E.K. Kuff. 1969. Biochemistry 8, 2916.

the most tightly bound chromatin fractions are eluted. Fractions which elute late from ECTHAM-cellulose have many of the properties expected for actively transcribed chromatin. Late-eluting chromatin contains 40-50 times more RNA polymerase binding sites than early eluting chromatin (Simpson, 1974[19]) and also has a Tm which is markedly lower than chromatin in early fractions (Reeck, Simpson, and Sober, 1972[20]). Circular dichroism (Polacow and Simpson, 1973[21]) and sedimentation studies (Simpson, 1975[22]) indicate that late eluting chromatin has a more extended structure than early eluting chromatin, and the greater susceptibility to nuclease attack of late chromatin also indicates a less condensed structure (Simpson and Polacow, 1973[23]). Chromatin in late fractions contains more non-histone and less histone H1 protein than early chromatin (Simpson and Reeck, 1973[24]; Reeck, Simpson and Sober, 1974[25]) and also contains significantly less satellite DNA than chromatin in early fractions (Simpson, 1975[26]). It has also been reported that late eluting fractions contain 2 to 3 times more nascent RNA

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[19]R.T. Simpson. 1974. Proc. Natl. Acad. Sci. USA 71, 2740.

[20]Reeck, G.R., et al. 1972.

[21]I. Polacow and R.T. Simpson. 1973. Biochem. Biophys. Res. Commun. 52, 202.

[22]R.T. Simpson. 1975. Biochem. Biophys. Res. Commun. 65, 552.

[23]R.T. Simpson and I. Polacow. 1973. Biochem. Biophys. Res. Commun. 55, 1078.

[24]R.T. Simpson and G.R. Reeck. 1973. Biochemistry 12, 3853.

[25]G.R. Reeck, R.T. Simpson, and H.A. Sober. 1974. Eur. J. Biochemistry 49, 407.

[26]R.T. Simpson. 1975.

than early eluting fractions (Simpson, 1974[27]). All of these characteristics of chromatin eluting late from ECTHAM-cellulose seem to indicate that these fractions contain active chromatin. However, Howk, et al. (1975[28]) have demonstrated that DNA extracted from late eluting chromatin is not enriched in sequences known to be actively transcribed in the tissue of origin. Therefore, although the ECTHAM-cellulose procedure like the sucrose and glycerol gradient procedures does separate structurally distinct chromatin fractions, it apparently does not distinguish between truly active and inactive chromatin.

Another chromatin fractionation procedure which is mild enough to yield structurally intact chromatin is the DNAse II-$MgCl_2$ procedure. This procedure was originally developed by Billing and Bonner (1972[29]) and was designed to take advantage of the greater susceptibility to nuclease attack of extended chromatin. Chromatin is prepared by standard methods except that it is not solubilized by sonication. Instead, the chromatin sample is swollen by overnight dialysis in 25 mM sodium acetate (pH 6.6), dispersed by gentle homogenization and digested with DNAse II (250 units/mg DNA) for 5 minutes. Putatively active sequences are then separated from the bulk of chromatin by differential solubility in $MgCl_2$. Specifically, the DNAse II digested sample is made 2 mM $MgCl_2$ and stirred for 20 minutes at 4°C.

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[27]R.T. Simpson in Current Topics in Biochemistry. 1974. C.B. Anfinsen and A.N. Schechter, eds., Academic Press, p135.

[28]Howk et al. 1975.

[29]R.J. Billing and J. Bonner. 1972. Biochim. Biophys. Acta 281, 453.

Under these conditions, extended active chromatin remains soluble, whereas condensed, inactive chromatin aggregates and precipitates. The soluble fraction is enriched in non-histone protein (Gottesfeld and Bonner, 1978[30]) and in nascent RNA (Billing and Bonner, 1972[31]). The soluable fraction also melts at a significantly lower temperature than inactive fractions (Bonner, et al. 1975[32]). Most importantly, however, DNA extracted from soluble chromatin is enriched for sequences known to be actively transcribed in the tissue of origin and is depleted in sequences known not to be expressed in the tissue of origin (Gottesfeld and Partington, 1977[33]). Therefore, the DNAse II-$MgCl_2$ procedure appears to be an effective method for preparing active chromatin.

The most recent chromatin fractionation procedure described involves the mild digestion of nuclei with micrococcal nuclease (Bloom and Anderson, 1978[34]). Like the DNAse II procedure, chromatin is initially fragmented by mild digestion with nuclease. However, in this procedure chromatin is digested in intact nuclei. Following digestion, nuclei are pelleted and the

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[30]J.M. Gottesfeld and J. Bonner in The Molecular Biology of the Mammalian Genetic Apparatus. 1977. P. Ts'O, ed., Elsevier/North-Holland Biomedical Press, Amsterdam, p381.

[31]R.J. Billing and J. Bonner, 1972.

[32]J. Bonner, J.M. Gottesfeld, W. Garrard, R. Billing, and L. Uphouse, in Methods in Enzymology 40. 1975. B.W. O'Malley and J.G. Hardman, eds., Academic Press, p97.

[33]J.M. Gottesfeld and G.A. Partington. 1977. Cell 12, 953.

[34]K.S. Bloom and J.N. Anderson. 1978. Cell 15, 141.

supernatant which contains monomer sized chromatin particles released from nuclei, is designated as the first supernatant fraction (1SF). Pelleted nuclei are lysed and centrifuged again to yield a pellet (P) and a second supernatant fraction (2SF). When the DNA of 1SF, 2SF and P is analyzed for sequences known to be actively transcribed in the tissue of origin, 1SF is 5 to 6 fold enriched and 2SF is 5 to 6 fold depleted in these sequences compared to undigested DNA. DNA from the pellet (P) contains the same amount of specific sequence per microgram of DNA as the undigested control. Apparently micrococcal nuclease, like DNAse I, preferentially cleaves DNA in the extended euchromatic regions of chromatin and, therefore, effects the release of actively transcribed sequences from the bulk of chromatin. Monomer sized particles then penetrate the nuclear membrane and can be recovered as a fraction enriched for active sequences. The advantage of this procedure is that chromatin does not have to be prepared before fractionation; therefore, changes in chromatin structure resulting from preparative procedures are minimized. However, the major disadvantage of the technique is that only monomer sized particles are obtained in the active fraction. This fact precludes studies of active chromatin structure above the level of the mononucleosome and diminishes the information which can be obtained by transcribing active fractions in vitro. However, the procedure may still be useful as an enrichment step in the isolation of putative regulatory components which may be associated with actively transcribed sequences.

In summary, many procedures have been devised to separate chromatin into active and inactive fractions; each procedure has advantages and disadvantages. Many assays have also been developed for determining the activity of a particular fraction. Although the putative active fraction obtained from each procedure yields positive results in some of the assays, no procedure produces a fraction which satisfies all of the criteria for active chromatin.

As indicated above, one of the most widely applied criterion has been the presence of nascent RNA chains in certain fractions, following pulse-labeling with labeled RNA precursor. The applicability of this criterion has been questioned by the finding that exogenously added RNA binds selectively to a chromatin fraction which co-sediments with putatively nascent RNA in sucrose gradient fractionation schemes (Seidman and Cole, 1977[35]). Chapters I and II describe the partitioning of non-nascent RNA in two other fractionation schemes, the DNAse II-$MgCl_2$ and ECTHAM-cellulose procedures, respectively.

PART II. PREPARATION OF REAGENTS FOR IN VITRO TRANSCRIPTION EXPERIMENTS

The work described in Part I, Chapters I and II, grew out of a project in which chromatin fractionation was to be used as the first step in the isolation of molecules which regulate transcription. The original protocol for this project is depicted in Figure 1. Chromatin is prepared from fetal liver

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[35]M.M. Seidman and R.D. Cole. 1977. J. Biol. Chem. 252, 2630.

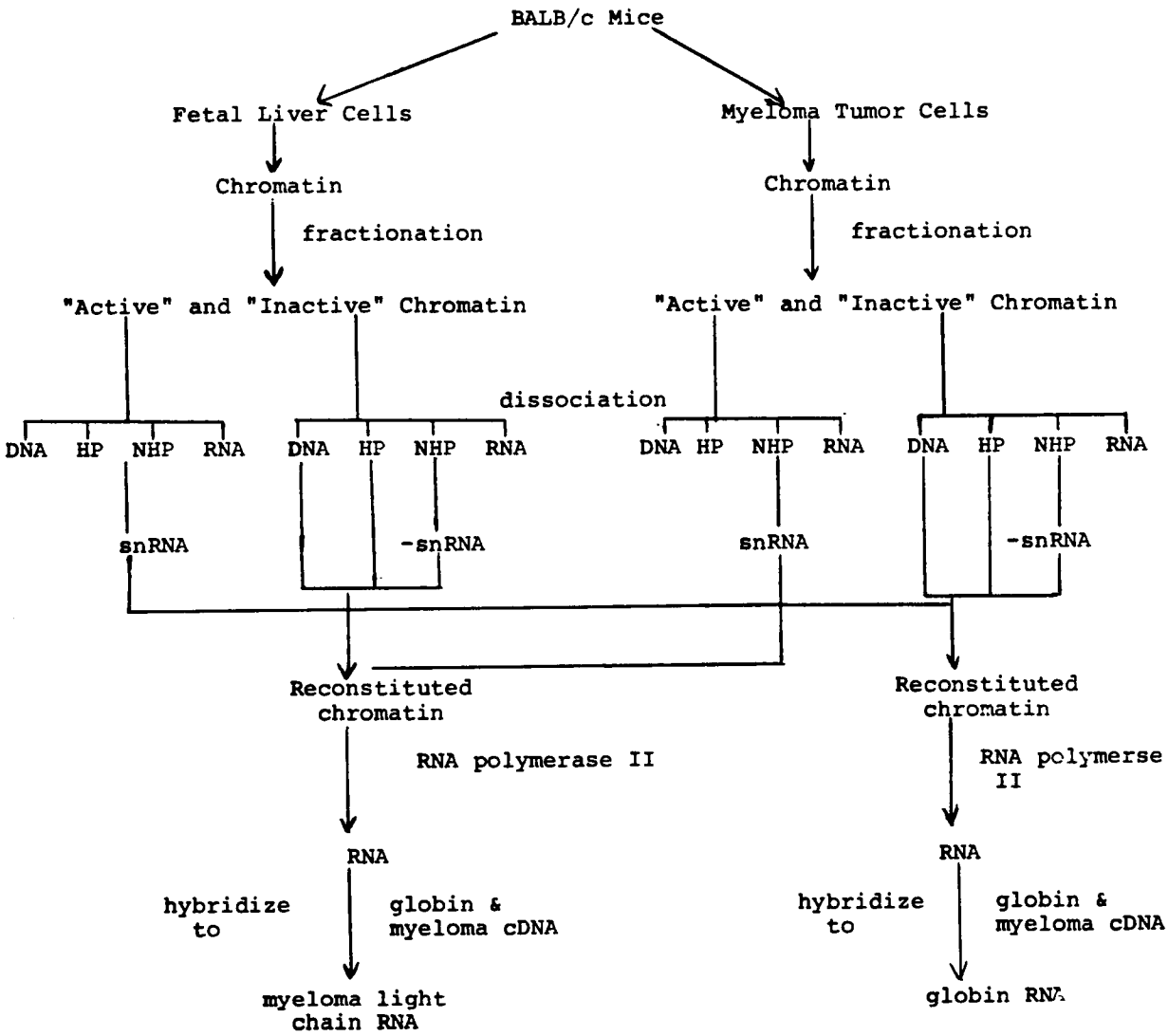

Figure 1. Protocol for chromatin reconstitution experiments

cells which are actively synthesizing globin protein and from myeloma tumor cells which are actively producing specific light chain protein. The chromatin is fractionated into active fractions by the DNAse II-$MgCl_2$ or ECTHAM-cellulose procedures. If the fractionation procedure is successful, the active fraction from fetal liver cells will contain the globin gene; the inactive chromatin from fetal liver cells should contain the myeloma light chain gene which is not actively transcribed in those cells but is nonetheless present in the genome of BALB/c mice. On the other hand, the active fraction obtained from myeloma chromatin should contain the light chain gene and inactive fractions should contain the globin genes.

Active and inactive fractions are then dissociated into three various components - DNA, histone proteins (HP), non-histone proteins (NHP), and RNA (Bekhor, et al. 1969[36]; Gilmour and Paul[37]; Paul and More, 1972[38]; Spelsberg and Hnilica, 1970[39]; Kleiman and Huang, 1972[40]).Several groups had suggested that NHPs controlled the transcription of specific genes (Gilmour and Paul, 1968[41]; Paul and Gilmour, 1968[42]; Gilmour and Paul,

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[36]I. Bekhor, G.M. Kung and J. Bonner. 1969. J. Mol. Biol. 39, 351.

[37]R.S. Gilmour and J. Paul. 1968. J. Mol. Biol. 40, 137.

[38]J. Paul and I. More. 1972. Nature New Biol. 239, 134.

[39]T.C. Spelsberg and L.S. Hnilica. 1970. Biochem. J. 120, 435.

[40]L. Kleinman and R.C. Huang. 1972. J. Mol. Biol. 64, 1.

[41]R.S. Gilmour and J. Paul. 1968.

[42]J. Paul and R.S. Gilmour. 1968. J. Mol. Biol. 34, 305.

1970[43]; Spelsberg, et al. 1971[44]; Kostraba and Wang, 1973[45]; Paul, et al. 1973[46]), however, none of the groups could exclude the possibility that small RNAs which contaminated their NHP preparations were actually the molecules responsible for the transcription of specific genes in reconstituted chromatin. Therefore, RNAs from active fetal liver chromatin would be reconstituted with DNA histones and non-histones from inactive myeloma chromatin. Small nuclear RNAs (snRNAs) in myeloma non-histone proteins would be removed by digestion with RNAse (Bekhor, et al. 1969[47]). The reciprocal reconstitution would also be done.

At this point, reconstituted chromatin would be transcribed in vitro with an RNA polymerase, and the RNA examined for both globin and myeloma light chain transcripts. If an active snRNA is obtained from fetal liver cells, globin mRNA should theoretically be transcribed from myeloma chromatin. Myeloma light chain RNA should not be produced. On the other hand, an active snRNA from myeloma cells should stimulate the synthesis of myeloma light chain mRNA from fetal liver chromatin. Globin mRNA should not be produced.

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[43]R.S. Gilmour and J. Paul. 1970.

[44]T.S. Spelsberg, L.S. Hnilica and A.T. Ansevin. 1971. Biochem. Biophys. Acta 228, 550.

[45]N.C. Kostraba and T.Y. Wang. 1973. Exptl. Cell Res. 80, 291.

[46]J. Paul, R.S. Gilmour, N. Affara, G. Birnie, P. Harrison, A. Hill, S. Humphries, J. Windass, and B. Young. 1973. Cold Spring Harbor Symp. Quant. Biol. 38, 885.

[47]I. Bekhor, et al. 1969.

The most critical reagents involved in the in vitro transcription assay are the two cDNAs which are used in hybridization reactions to detect specific transcripts. In fact, the whole project as diagrammed in Fig. 1 hinges on the production of these two highly specific probes.

Chapters I and III describe the isolation of globin and myeloma light chain mRNAs and the synthesis of the cDNA copies of these messengers. Several problems were encountered in the preparation of these reagents and these problems are enumerated at the end of Chapter III. Chapter II describes the preparation of the eukaryotic polymerase which was to be used in in vitro transcription experiments.

PART I

FRACTIONATION OF CHROMATIN INTO ACTIVE AND INACTIVE SEGMENTS

CHAPTER I

NON-NASCENT RNA BINDS SELECTIVELY TO THE SOLUBLE FRACTION IN CHROMATIN SEPARATED BY THE DNASE II-$MgCl_2$ PROCEDURE

INTRODUCTION

Several criteria have been used to evaluate the effectiveness of chromatin fractionation procedures (Savage and Bonner, 1978). One of these criteria, the presumed association of nascent RNA with actively transcribed DNA sequences in chromatin, is based on the assumption that transcripts which have not been completed at the time of chromatin isolation are bound to their template by RNA polymerase and, therefore, serve as markers for actively transcribed genes. The validity of this assumption has recently been questioned by Seidman and Cole (1977). They have demonstrated that pulse-labeled RNA which is associated with putative active chromatin obtained from sucrose gradients is not necessarily nascent. The most persuasive evidence reported by these authors was derived from a mixing experiment in which pulse-labeled, purified RNA was added to unlabeled chromatin. When this chromatin was fractionated in sucrose gradients, labeled RNA was recovered in association with the same slowly-sedimenting fraction of chromatin which had previously been shown to contain RNA pulse-labeled in vitro.

We wished to determine whether this artifact would also be observed when chromatin is fractionated by a method which has been more firmly established as separating transcriptionally active and inactive chromatin.

The experiments reported here were designed to determine whether free pulse-labeled RNA preferentially binds to putative active chromatin generated by the DNAse II fractionation procedure (Marushige & Bonner, 1971). This fractionation procedure was chosen because of the evidence (Gottesfeld, et al. 1974b; Gottesfeld, et al. 1976; Gottesfeld and Partington, 1977; Hendrick, et al. 1977) that the soluble (SII) fraction obtained in this procedure is enriched in DNA sequences which are actively transcribed in the cells from which they are derived.

METHODS

Cells - Livers were removed from fifty BALB/c mouse fetuses at the fifteenth day of gestation and minced with scissors in Delbecco's minimal essential medium (D-MEM) supplemented with 10% fetal calf serum and penicillin. Single cell suspensions were prepared by several gentle passages of minced tissue through a 20 ml syringe without a needle. The cells were washed once with D-MEM and resuspended in the same medium.

Labeling Cells - For single label experiments [^{3}H] uridine (Amersham, 25 Ci/mMol) or [^{14}C]uridine (Amersham, 56 mCi/mMol) was added to a 10 ml culture (10^{8} cells/ml) to a final concentration of 10 μCi/ml or 2.5 μ Ci/ml, respectively. The cells were incubated at 37°C for 10 min and then washed immediately with 2 volumes of ice-cold medium. Two subsequent washes with

ice-cold medium were performed before nuclei were isolated. (After each wash the cells were pelleted in a Sorvall GLC-1 centrifuge for 5 min at 2,500 rpm.)

For double labeling experiments 100 μCi of [^{3}H] uridine were added to a 10 ml culture (10^8 cells/ml), incubated for 10 min at 37°C and washed three times at room temperature with warm medium (37°C). Cells were resuspended in 100 ml fresh, warm medium and incubated for 60 min at 37°C. Following this incubation the cells were pelleted at room temperature and resuspended in 10 ml of warm medium; [^{14}C] uridine was added to a final concentration of 2.5 μCi/ml, and the cell suspension was incubated for 10 min at 37°C. Immediately after the 10 min incubation the cells were pelleted and washed with ice-cold medium; two subsequent washes with ice-cold medium were performed before isolation of nuclei.

Isolation of Nuclei - Cells were resuspended in nine volumes of Triton buffer (0.3 M sucrose, 2 mM Mg acetate, 3 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), 1.0 mM phenylmethylsulfonylfluoride (PMSF), 0.5% Triton X-100 and 10 mM Tris-HCl, pH 8.0) (Marzluff, et al. 1973) and lysed by five gentle strokes in a 40 ml siliconized glass Wheaton homogenizer with the B pestle. Nuclei were pelleted by centrifugation for 10 min at 1,000 x g, washed in Triton buffer, and then sedimented through 2.0 M sucrose as described by Gilboa et al. (1977). Seven and one-half ml of suspension (about 2 x 10^7 cells/ml) was mixed with an equal volume of 2.0 M sucrose buffer (2.0 M sucrose, 5 mM Mg acetate, 0.5 mM DTT, 10 mM Tris-HCl, pH 8.0), layered onto a 15 ml cushion of 2.0 M sucrose buffer in a 30 ml Corex tube and centrifuged for 60 min at 7,000 rpm in a Beckman JS-13 rotor. (Four tubes accomodated the whole sample.) The pellets were resuspended in Triton

buffer and washed twice in the same buffer by gentle resuspension in a glass homogenizer.

Chromatin Isolation - Chromatin was isolated by washing nuclei in buffers of decreasing ionic strength essentially as described by Shaw and Huang (1970). Briefly, nuclei were washed three times in saline-EDTA (0.075 M NaCl, 0.024 M EDTA), two times in 0.05 M Tris-HCl, pH 8.0, two times in 0.01 M Tris-HCl, pH 8.0, and once in 0.001 M Tris-HCl, pH 8.0. (All buffers contained 1 mM PMSF.) Following the final wash, swollen nuclei were resuspended in 15 ml of 0.001 M Tris-HCl, pH 8.0, lysed by homogenization in a siliconized, glass-teflon homogenizer and diluted with 0.001 M Tris-HCl, pH 8.0 to 10 A_{260} units/ml. (Absorbance at 260 nm was determined in 0.9 N NaOH; 1 mg of DNA corresponds to 27 A_{260} units) (Gottesfeld, et al. 1974).

DNAse II Fractionation - DNAse II fractionation was performed as described by Gottesfeld (Gottesfeld, et al. 1974b; Gottesfeld, et al. 1974). Briefly, chromatin was dialyzed overnight against 25 mM sodium acetate, pH 6.6. DNAse II (Worthington) was added to a final concentration of 100 units/ml, and the sample was digested for 5 min at 24°C. Digestion was terminated by the addition of 1.0 M Tris-HCl, pH 8.0 to a final concentration of 50 mM. Insoluble chromatin was pelleted by centrifugation at 27,500 x g for 20 min and designated PI. The supernatant was made 2 mM with respect to $MgCl_2$ and stirred for 30 min at 2-4°C. Precipitated material was pelleted by centrifugation at 27,500 x g for 20 min and designated PII. Chromatin recovered in the supernatant fraction at this step was designated SII.

Sucrose Gradient Sedimentation - Linear 5-20% sucrose gradients in 10 mM Tris-HCl, pH 8.0 were formed over a 3.0 ml cushion of 70% sucrose in

10 mM Tris-HCl, pH 8.0. Chromatin was loaded onto gradients, and the gradients were centrifuged for 14 hours at 27,000 rpm in the 27.1 buckets of a Beckman SW 27 rotor. The temperature was set at 4°C. The gradients were collected from the top by pushing Flourinert (ISCO, 1.7 gm/ml) through the bottom of the nitrocellulose tubes. Fractions indicated in figure legends were pooled and optical densities at 260 nm were determined. Aliquots from each pool were mixed with an equal volume of 10% trichloroacetic acid (TCA). After 10 min on ice, the resulting precipitates were collected on membrane filters. Radioactivity collected on the filters was counted in a Toluene-based scintillation fluid and specific activities (cpm/A_{260}) were calculated.

Preparation of ^{3}H-RNA - Chromatin was prepared from fetal liver cells pulse-labeled for 10 min with [^{3}H] uridine as described above. RNA was extracted by a modification of the procedure of Brawerman (1974). The chromatin suspension was made 0.1 M Tris-HCl, pH 9.0, and 1.0% sodium dodecyl sulfate (SDS), and digested for 30 min with proteinase K (Merck) at 50 µg/ml. The mixture was extracted once with redistilled phenol (water saturated) and twice the phenol mixture (phenol:chloroform:isoamyl alcohol/50:50:1). The final aqueous phase was made 0.2 M sodium acetate (pH 5.5) and two volumes of 95% ethanol were added. After overnight precipitation at -20°C, nucleic acids were pelleted and resuspended in 10 mM Tris-HCl, pH 7.0. DNAse I was added to a final concentration of 20 µg/ml. After digestion for 30 min at 37°C the sample was extracted twice with phenol mixture and precipitated with ethanol. RNA was resuspended in 10 mM Tris-HCl, pH 7.5, 1.0% SDS, and passed through a 0.9 x 50 cm Sephadex G-50 column equilibrated with the same buffer. Material recovered in the void volume was precipitated with ethanol, pelleted, and finally resuspended in water and stored at -70°C.

Calculation of Specific Activities of Sucrose Gradient Fractions - Phenol extracted, pulse-labeled RNA ("free RNA"), when centrifuged alone in sucrose gradients, had a peak sedimentation coefficient of less than 4S. In all such RNA preparations, more than 90% of the RNA was recovered in fractions 1-6. In order to correct for free RNA which co-sedimented with chromatin in the rest of the gradient (fractions 7-16), the following procedure was used. The activity (cpm) recovered in tubes 1-6 was divided by f, the fraction of phenol-extracted, pulse-labeled RNA which, when centrifuged alone in a parallel gradient, was recovered in fractions 1-6. This quotient represents the total cpm of free RNA in the gradient. This total activity was multiplied by (1-f), the fraction of phenol-extracted, pulse-labeled RNA which, when centrifuged alone, was recovered in fractions 7-16, to obtain the cpm of free RNA in fractions 7-16. This number was then substracted from the total cpm recovered in fractions 7-16 to yield cpm of chromatin-bound RNA. Values of f for each RNA preparation are given in the respective tables. Counts per minute of bound RNA were divided by the A_{260} of pooled fractions to calculate specific activity.

Specific activities rather than cpm alone are compared because a fraction which is genuinely enriched for active chromatin does not necessarily contain more total cpm of nascent RNA than an inactive fraction. For example, if the DNAse II procedure separates only 20% of actively transcribed sequences from the bulk of chromatin then 80% of the active sequences will cofractionate with inactive fractions. If the marker for active chromatin is pulse-labeled RNA, then only 20% of the total cpm will be found in the active fraction. However, if relatively little of the inactive chromatin (less than 10%) contaminates the active fraction the cpm per unit of chromatin will be higher in the active than the inactive fraction.

The comparison of specific activities in the example cited above is analogous to the comparison of $Cot_{½}$ values obtained when a specific cDNA is hybridized to SII and PI DNA (Gottesfeld & Partington, 1977). The $Cot_{½}$ values are a measure of the amount of specific gene present per A_{260} unit of DNA, not a measure of the total mass of specific sequence in SII and PI fractions.

RESULTS

Table 1 lists the results obtained from DNAse II fractionation of chromatin extracted from fetal liver cells pulse-labeled for 10 minutes with ^{14}C uridine. Nine and one-half percent of the digested material was recovered in the SII fraction, and the specific activity of the fraction was approximately six times greater than the specific activity of the PI fraction. These results are consistent with data published by Billing and Bonner (1972) and appear to indicate that the SII fraction is enriched for actively transcribed chromatin.

In order to determine whether SII chromatin is able to bind to free RNA, the following mixing experiment was performed. Pulse-labeled RNA which had been phenol-extracted from fetal liver chromatin was mixed with unlabeled chromatin and fractionated by the DNAse II procedure. The results of this experiment are shown in Table 2. Again the specific activity of the SII chromatin fraction was greater (79-fold) than PI chromatin. However, because much of the difference was most likely due to added labeled RNA which was not bound to chromatin, aliquots of these fractions were sedimented through 5-20% sucrose gradients in order to separate free RNA fromchromatin-bound RNA. The profiles of these gradients are depicted in Fig. 1; the specific activities of chromatin which sediments in fractions 7-16

Table 1. Association of pulse-labeled RNA with DNAse II-fractionated chromatin[a]

Fraction	Percent of total chromatin present in fraction	Specific Activity ^{14}C cpm/A_{260}
PI	82.6	1,061
PII	7.0	3,458
SII	9.5	6,836

[a]Fetal liver cells were pulse-labeled for 10 min at 37°C with [^{14}C]uridine. Chromatin was extracted and fractionated by the DNAse II-$MgCl_2$ procedure. Aliquots of subsequent fractions were precipitated with TCA and specific activities determined.

Table 2. Association of exogenous RNA with DNAse II-fractionated chromatin[a].

Fraction	Percent of total chromatin present in fraction	Specific Activity ^3H cpm/A_{260}
PI	76.6	43
PII	10.4	175
SII	13.0	3,408

[a]RNA was extracted from chromatin obtained from pulse-labeled fetal liver cells and mixed with unlabeled chromatin. The preparation was fractionated by the DNAse II-$MgCl_2$ procedure and the specific activity of each fraction was determined.

Fig. 1 Sucrose Gradient Sedimentation of Chromatin Fractions. An aliquot of each fraction described in Table 2 was sedimented through a 5-20% sucrose gradient. Total A_{260} units and cpm, respectively, in fractions 1-6 were as follows: PI, 0.0, 138; PII, 0.0, 321; SII, 2.36, 11,019. Total A_{260} units and cpm, respectively, in fractions 7-16 were as follows: PI, 0.974, 24; PII, 3.15, 170; SII, 1.12, 850. Sedimentation was from right to left.

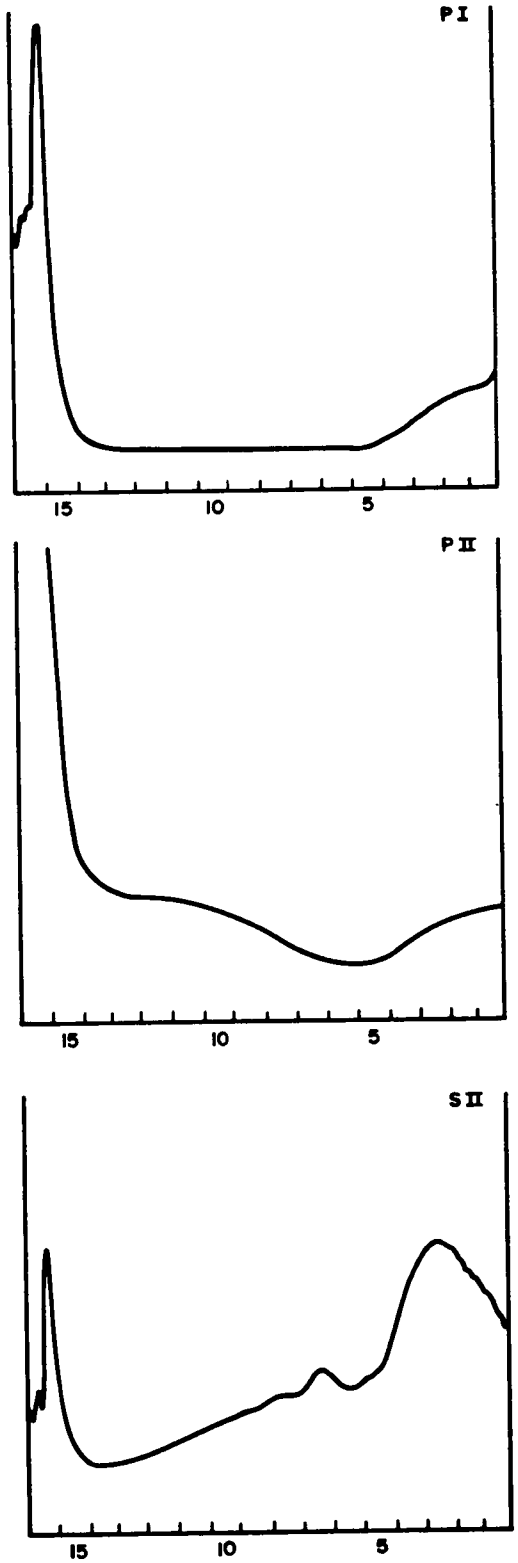

Figure 1

are shown in Table 3. The amount of bound RNA per A_{260} unit of chromatin was 14 times greater in the SII fraction than in the PI fraction. These results demonstrate that free RNA is able to bind to SII chromatin and suggest that the relatively high specific activity of SII chromatin observed in the in vivo labeling experiments may merely reflect the capacity of this chromatin to bind free RNA.

In order to assay for the binding of endogenous, free RNA to fetal liver chromatin, a pulse-chase experiment was performed. Fetal liver cells were pulse-labeled for 10 min with [^{3}H] uridine, chased for 60 min to generate naturally-occurring, intracellular, free RNA and pulse-labeled a second time for 10 min with [^{14}C] uridine. After a 60 min chase, most of the ^{3}H-labeled RNA should represent completed chains which have been released from their chromatin template (Greenburg and Penman, 1966; Darnell, 1968); ^{14}C-labeled RNA should mark nascent chains. In order to ascertain continued synthetic ability during the chase, the following control was carried out. The kinetics of [^{3}H]uridine incorporation during the 60 min chase was measured, and is shown in Fig. 2; little incorporation of [^{3}H] uridine is observed. However, synthesis of RNA continues during this period as indicated by the incorporation of labeled uridine for 60 min in a separate culture of fetal liver cells.

Table 3. Association of exogenous RNA with DNAse II-fractionated chromatin after sucrose gradient sedimentation[a]

Fraction	Specific Activity (^{3}H cpm/A_{260})
PI	17
PII	48
SII	241

[a]Aliquots of fractions described in Table II were sedimented through 5-20% sucrose gradients. Fractions 7-16 of each gradient were pooled and specific activities determined. Corrections for the amount (cpm) of unbound RNA which cosedimented with chromatin to fractions 7-16 were as described in Methods (f = .95).

Fig. 2 Kinetics of [^{3}H]uridine incorporation by fetal liver cells. The ordinate represents cpm incorporated per 100 µl aliquots: closed circles, fetal liver cells (10^7 cells/ml) were incubated at 37°C with [^{3}H] uridine (10 µCi/ml); open circles, fetal liver cells (10^8 cells/ml) were pulse-labeled for 10 min at 37°C with [^{3}H]uridine (10 µCi/ml), washed, resuspended in fresh medium (10^7 cells/ml) and incubated at 37°C.

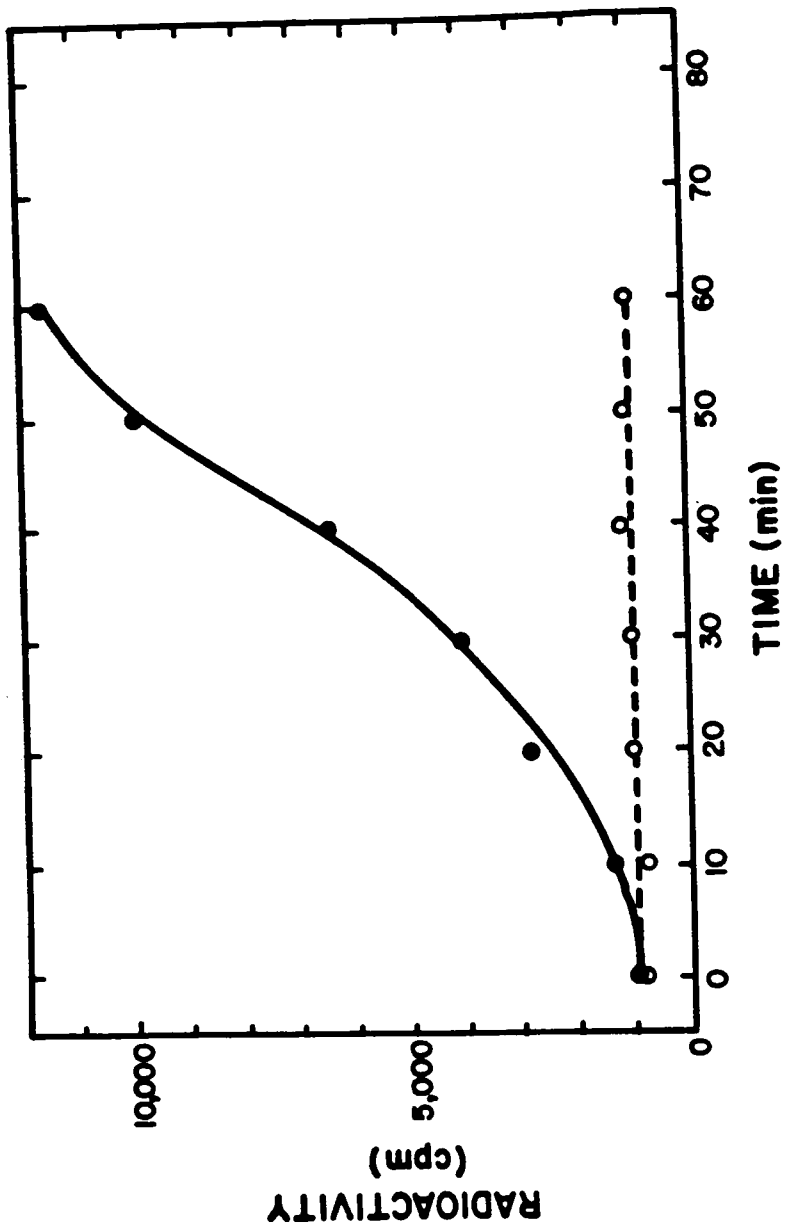
RADIOACTIVITY
(cpm)
10,000
5,000
0
0
10
20
30
40
50
60
70
80
TIME (min)

Figure 2

Chromatin was extracted from doubly-labeled cells and fractionated by the DNAse II method. Following fractionation aliquots of PI, PII, and SII chromatin were sedimented through 5-20% sucrose gradients. The specific activities of each fraction before sucrose gradient centrifugation are shown in Table 4. The amount of ^{3}H-labeled and ^{14}C-labeled RNA per A_{260} unit of chromatin was 5.6 and 4.0 times higher in the SII fraction than in the PI fraction. More importantly, even after sedimentation through sucrose, the specific activity (^{3}H-cpm/A_{260}) of SII chromatin was higher (2.3 times) than PI chromatin (Table 5). Therefore, endogenous, free RNA is also able to bind to SII chromatin and increase the specific activity of this fraction.

Greater specific activity of SII chromatin both before and after sucrose gradient centrifugation was also observed when specific activity was calculated from ^{14}C-cpm (Tables 4 and 5). Before sedimentation the amount of ^{14}C-cpm per A_{260} unit of SII chromatin was four times greater than PI chromatin. After sedimentation the specific activity of SII chromatin was 2.6 times greater than PI chromatin. Since both exogenous and endogenous free RNA preferentially bind to SII chromatin, these results suggest that ^{14}C-labeled RNA may not be nascent but may represent chains which have been cleaved from active chromatin and subsequently bound to chromatin recovered in the SII fraction. This interpretation is strengthened by the observation that greater than 70% of both ^{3}H- and ^{14}C-labeled RNA in the SII fraction is recovered in the top of the sucrose gradient; apparently not bound to chromatin. Therefore, much of the ^{14}C-labeled material is free RNA.

Table 4. Association of ^{3}H- and ^{14}C-labeled RNA with DNAse II-fractionated chromatin[a]

Fraction	Percent of total chromatin present in fraction	Specific Activity (^{3}H cpm/A_{260})	Specific Activity (^{14}C cpm/A_{260})
PI	77.6	442	512
PII	13.0	1,139	832
SII	9.4	2,495	2,068

[a]Fetal liver cells were pulse-labeled for 10 min with [^{3}H]uridine, chased for 60 min, and pulse-labeled again for 10 min with [^{14}C]uridine. Chromatin was extracted and fractionated by the DNAse II-$MgCl_2$ procedure. Aliquots of subsequent fractions were precipitated with TCA and specific activities determined.

Table 5. Association of ^{3}H- and ^{14}C-labeled RNA with DNAse II-fractionated chromatin after sucrose-gradient sedimentation[a]

Fraction	Specific Activity (^{3}H cpm/A_{260})	Specific Activity (^{14}C cpm/A_{260})
PI	642	466
PII	732	656
SII	1,526	1,192

[a]Aliquots of fractions described in Table IV were sedimented through 5-20% sucrose gradients. Fractions 7-16 of each gradient were pooled and specific activities determined. Total A_{260} units, cpm in ^{3}H, and cpm in ^{14}C, respectively, in fractions 1-6 were as follows: PI, 0.0, 129, 176; PII, 0.0, 530, 566; SII, 2.1, 6761, 8148. Total A_{260} units, cpm in ^{3}H, and cpm in ^{14}C, respectively, in fractions 7-16 were as follows: PI, 1.2, 782, 575; PII, 7.0, 5172, 4644; SII, 1.6, 3030, 2616. Corrections for the amount of unbound RNA which cosedimented with chromatin to fractions 7-16 were as described in Methods (f = .92).

DISCUSSION

Separation of chromatin into transcriptionally active and inactive fractions is a goal which has been pursued for many years. Many methods have been proposed and have been shown to be successful based on one or more criteria for activity. The most widely studied methods have been sedimentation of sheared chromatin on sucrose gradients (Frenster, et al. 1963; Chalkey and Jensen, 1968; McCarthy, et al. 1973; and Murphy, et al. 1973), differential solubility in $MgCl_2$ of DNAse II-generated fragments (Marushige and Bonner, 1971; Gottesfeld, et al. 1974b; Gottesfeld, et al. 1976; Gottesfeld and Partington, 1977; and Hendrick, et al. 1977), and chromatography of sonicated chromatin on ECTHAM-cellulose (Reeck, et al. 1972; Reeck, et al. 1974; and Simpson, 1974).

The criteria used to demonstrate transcriptional activity have been both direct and indirect. In principle, it should be possible to demonstrate the presence of actively transcribing chromatin (whether separated from inactive chromatin or not) by a direct measurement of transcription. One popular assay for active chromatin has been the ability of the chromatin fraction tested to act as a template for RNA polymerase (Frenster, et al. 1963; Murphy, et al. 1973; Simpson, 1974; Berkowitz, et al. 1975; and Bonner, et al. 1973). However, confidence in this method was substantially impaired when Zasloff and Felsenfeld (1977) demonstrated that under the conditions commonly used for such assays, E. coli polymerase preferentially uses RNA rather than chromatin as a template. Therefore, the assay may simply reflect the presence of RNA in the preparation, and is unrelated to de novo synthesis from chromatin.

A second technique for assessing transcriptional activity of chromatin attempts to identify an active fraction on the basis of the presence of newly synthesized RNA in fractions of chromatin isolated from cells labeled with an RNA precursor; this criterion is the subject of the present communication. This assay has been used as a fractionation criterion in all of the procedures described above: sucrose gradient sedimentation (McCarthy, et al. 1973; Murphy, et al. 1973; and Berkowitz, et al. 1975), DNAse II-$MgCl_2$ fractionation (Billing and Bonner, 1972), and ECTHAM-cellulose chromatography (Simpson, 1974). Seidman and Cole (1977) have shown that this "nascent RNA" assay is not a reliable indication of sedimentation behavior of transcriptionally active chromatin. We have shown here that this indicator is also unreliable in identifying transcriptionally active chromatin fractionated by the DNAse II-$MgCl_2$ procedure. We will report elsewhere (T.M. Townes and W.S. Riggsby, unpublished data) experiments which indicate that the "nascent RNA" criterion is also unreliable as an indication of active chromatin in ECTHAM-cellulose chromatography.

The present concensus appears to be that the most reliable criterion for identifying active chromatin is the presence of DNA sequences known to be transcribed in the tissues of origin. To our knowledge, the only preparative fractionation procedures which have yielded positive results as judged by that criterion involve nuclease digestion of the chromatin, and subsequent recovery of a soluble fraction (Gottesfeld and Partington, 1977 and Bloom and Anderson, 1978). The most extensively documented procedure

is the DNAse II-$MgCl_2$ technique (Gottesfeld, et al. 1974b; Gottesfeld, et al. 1976; Gottesfeld and Partington, 1977; Hendrick, et al. 1977; and Wallace, et al. 1977). Applications of this criterion to the sucrose sedimentation and ECTHAM-cellulose fractionation procedures has yielded negative results (Savage and Bonner, 1978; Howk, et al. 1975; and Krieg and Wells, 1976). It thus appears that there is a chromatin fraction which selectively binds free RNA and which is physically similar, but not identical, to transcriptionally active chromatin. The structures of these two subfractions are sufficiently similar that they appear in the same fraction in the DNAse II-$MgCl_2$ procedure (Gottesfeld and Partington, 1977 and this report), but sufficiently different that they do not behave the same way on sucrose gradients (Seidman and Cole, 1977 and Krieg and Wells, 1976). The physical properties of chromatin which contains RNA (formerly thought to be entirely nascent RNA) have been studied in great detail (Chalkley and Jensen, 1968; Reeck, et al. 1972; Berkowitz and Doty, 1975; Gottesfeld, et al. 1975; Polacow and Simpson, 1973; Simpson and Reeck, 1973; and Duerksen and McCarthy, 1971). However, the physical properties of truly active chromatin are much less well known, and further characterization of the latter will be required before the similarities and differences in two subfractions can be established.

It is quite possible that the physical structure that allows the separation of an "active" chromatin fraction by the DNAse II-$MgCl_2$ method is destroyed by the protein rearrangements known to occur during the shearing process (Noll, et al. 1975 and Sollner-Webb and Felsenfeld, 1975) which is used to prepare chromatin for fractionation on sucrose gradients.

CHAPTER II

NON-NASCENT RNA BINDS NON-SELECTIVELY TO ALL CHROMATIN FRACTIONS OBTAINED BY ECTHAM-CELLULOSE CHROMATOGRAPHY

INTRODUCTION

When sonicated chromatin is fractionated on ECTHAM-cellulose, late-eluting fractions exhibit several properties which are expected for actively transcribing chromatin. Late eluting chromatin contains 40-50 times more RNA polymerase binding sites than early eluting chromatin (Simpson, 1974) and also has a Tm which is markedly lower than chromatin in early fractions (Reeck, Simpson and Sober, 1972). Circular dichroism (Polacow and Simpson, 1973) and sedimentation studies (Simpson, 1975) indicate that late eluting chromatin has a more extended structure than early eluting chromatin and the greater susceptibility to nuclease attack of late chromatin also indicates a less condensed structure (Simpson and Polacow, 1973). Chromatin in late fractions contains more non-histone and less histone H1 protein than early chromatin (Simpson and Reeck, 1973; Reeck, et al., 1974) and also contains significantly less satellite DNA than chromatin in early fractions (Simpson, 1975).

It has also been reported that late eluting fractions contain 2 to 3 times more nascent RNA than early eluting fractions (Simpson, 1974). However, the proposed enrichment in nascent RNA of late chromatin is based on pulse-labeling experiments. Seidman and Cole (1977) and Townes and Riggsby (see Chapter I) have demonstrated that the association of pulse-

labeled RNA with putatively active chromatin generated by the sucrose gradient and DNAse II procedure does not mark actively transcribing chromatin but merely marks a fraction of chromatin which binds free RNA. The experiments described in this chapter were designed to determine whether free, pulse-labeled RNA preferentially binds to putative active chromatin prepared by the ECTHAM-cellulose procedure.

METHODS

Isolation and labeling of cells, isolation of nuclei, preparation of chromatin, preparation of ^{3}H-RNA, sucrose gradient sedimentation, and calculation of specific activities of sucrose gradient fractions were all as described in the Methods of Chapter I.

ECTHAM-Cellulose Fractionation - ECTHAM-cellulose was prepared by the method of Petterson and Kuff (1969). Chromatography was performed essentially as described by Reeck, et al. (1972). Briefly, chromatin (10 ODS/ml) was sonicated with a Branson sonifier (5 x 30-second blasts) at a setting of 2 (45 watts) and dialyzed overnight against 0.01 M Tris pH 7.2. The sample (ca. 200 A_{260} units) was loaded at a rate of 1 ml/5 min onto a column (2 x 8 cm) prepared from 4 gm of ECTHAM-cellulose (capacity, 90 ODS/gm), equilibrated in 0.01 M Tris pH 7.2. The column was washed with 50 ml 0.01 M Tris pH 7.2; chromatin was eluted with 0.01 M Tris, 0.01 M NaCl (not pHed). Fractions (1.2 ml) eluting at pH 8.5 or above were pooled and designated as late fractions. (See figure legends for fractions designated early and middle).

RESULTS

Figure 3 illustrates the elution profile from ECTHAM-cellulose of sonicated chromatin extracted from cells pulse-labeled for 10 min with ^{3}H uridine. As indicated in the figure, the counts per min per A_{260} unit of eluted chromatin increased with increasing pH. These results are consistent with the data of Simpson, et al. (1974a) and appear to indicated that the late-eluting fractions are enriched for actively transcribed chromatin.

In order to determine whether late-eluting chromatin is able to bind exogenously-added, free RNA, the following mixing experiment was per formed. Pulse-labeled RNA which had been phenol-extracted from fetal liver chromatin was mixed with unlabeled chromatin and fractionated on ECTHAM-cellulose. The elution profile from the column and specific activities of pooled fractions are shown in Fig. 4 and Table 6, respectively. The specific activity of the late fraction was approximately 17 times greater than the activity of the early fraction. However, because much of the difference could be due to added labeled RNA which was not bound to chromatin, aliquots of these fractions were sedimented throught 5-20% sucrose gradients in order to separate free RNA from chromatin-bound RNA. The profiles of these gradients are depicted in Fig. 5; the specific activities of chromatin which sedimented in fractions 5-15 are shown in Table 7. As indicated in the table, none of the labeled RNA was bound to late-elulting chromatin. Therefore, the relatively high specific activity of this fraction before sucrose gradient sedimentation was due to the late elution of free RNA which also bound to ECTHAM-cellulose. Most of the chromatin-bound RNA was recovered in early and middle frations.

Fig. 3 Elution from ECTHAM-cellulose of sonicated chromatin obtained from pulse-labeled fetal liver cells. Fetal liver cells (10^8 cells/ml) were pulse-labeled for 10 min at 37°C with [^{3}H]uridine 10 μCi/ml). Chromatin was prepared, sonicated, and chromatographed on ECTHAM-cellulose.

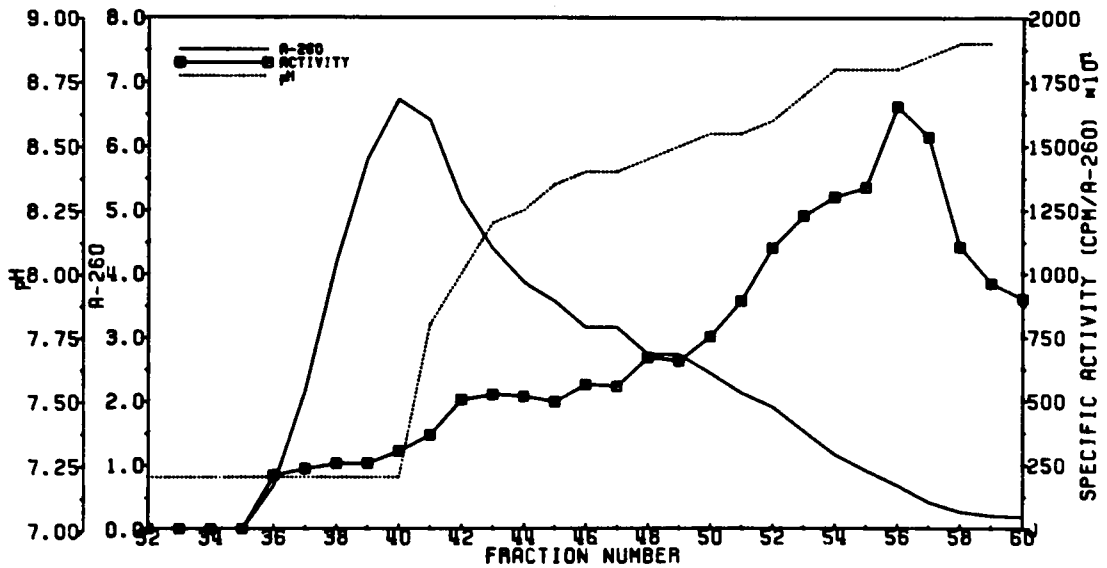
A-280
ACTIVITY
pH
9.00
8.75
8.50
8.25
8.00
7.75
7.50
7.25
7.00
pH
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
A-260
2000
1750
1500
1250
1000
750
500
250
SPECIFIC ACTIVITY (CPM/A-260) x10³
32
34
36
38
40
42
44
46
48
50
52
54
56
58
60
FRACTION NUMBER

Figure 3

Fig. 4 Elution from ECTHAM-cellulose of unlabeled, sonicated chromatin mixed with ^{3}H-RNA. Fetal liver cells were pulse-labeled as described in Fig. 1, and chromatin was prepared. RNA was phenol-extracted and mixed with unlabeled, sonicated fetal liver chromatin. The mixture was subsequently chromatographed on ECTHAM-cellulose.

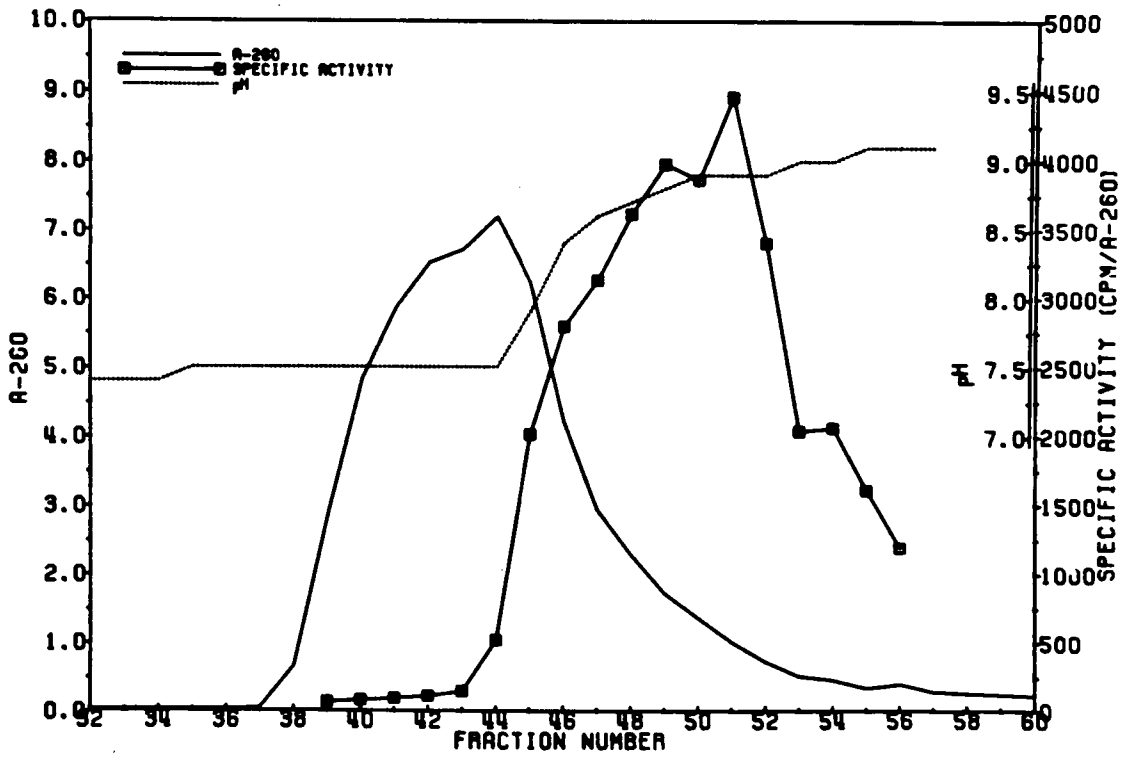
A-260
SPECIFIC ACTIVITY
pH
A-260
pH
SPECIFIC ACTIVITY (CPM/A-260)
FRACTION NUMBER
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
9.5
9.0
8.5
8.0
7.5
7.0
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
32
34
36
38
40
42
44
46
48
50
52
54
56
58
60

Figure 4

Table 6. Association of exogenous RNA with ECTHAM-cellulose-fractionated chromatin[a]

Fraction	Percent of total A_{260} present in fraction	Specific Activity (^3H cpm/A_{260})
Early[b]	63	333
Middle[c]	24	3,222
Late[d]	13	5,743

[a]Fractions of Fig. 2 were pooled; aliquots of each fraction were TCA precipitated and specific activities determined.

[b]Fractions 39-44 from Fig. 2.

[c]Fractions 45-47 from Fig. 2.

[d]Fractions 48-52 from Fig. 2.

Fig. 5 Sucrose gradient sedimentation of chromatin fractions eluting early, middle and late from ECTHAM-cellulose. An aliquot of each fraction described in Table 1 was sedimented through a 5-20% sucrose gradient. Total A_{260} units and cpm, respectively, in fractions 1-4 were as follows: early, 0.0, 944; middle, 0.30, 9,241; late, 0.70,19,541. Total A_{260} units and cpm, respectively, in fractions 5-15 were as follows: early 3.90, 593; middle, 3.07, 1,510; late, 3.52, 1,914. Sedimentation was from right to left.

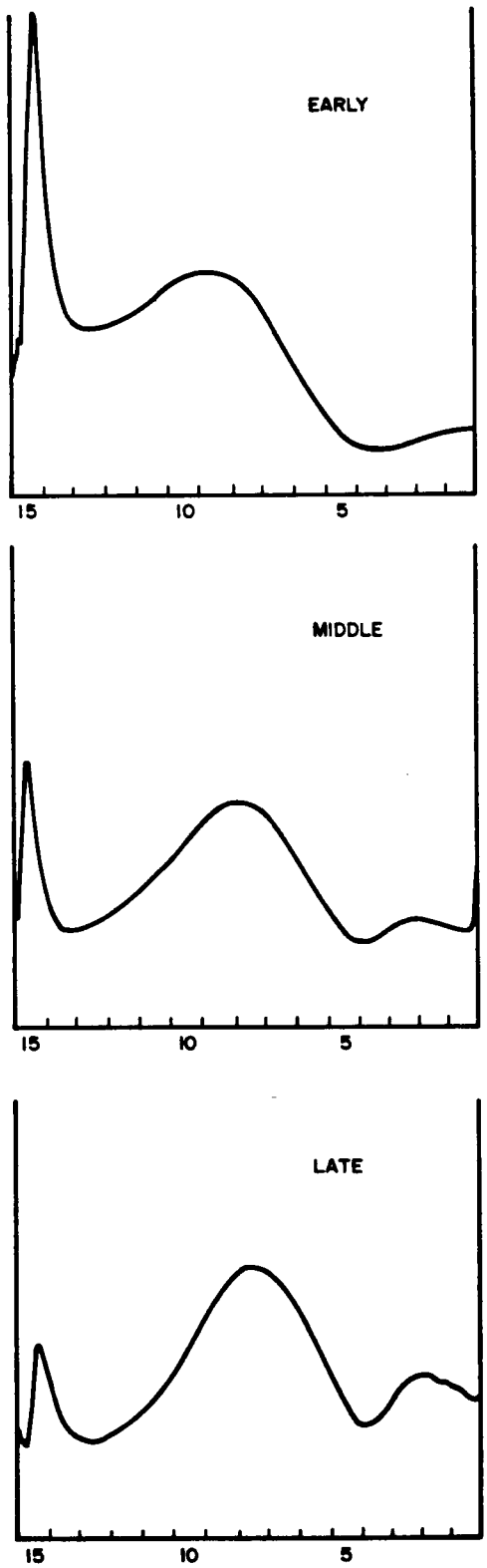

Figure 5

Table 7. Association of exogenous ^{3}H- RNA with ECTHAM-cellulose fractions after sucrose gradient sedimentation[a]

Fraction	Specific Activity (^{3}H cpm/A_{260})
Early	125
Middle	157
Late	0

[a]Fractions 5-15 of Fig. 3 were pooled; aliquots were TCA precipitated and specific activities determined. Correction for the amount of non-chromatin-bound RNA in fractions 5-15 were as described in Methods. The value of "f" for the control gradient equaled 0.90.

In order to assay for the binding of endogenous, free RNA to fetal liver chromatin and to determine if putative nascent RNA was indeed bound to chromatin, a pulse-chase experiment was performed. Fetal liver cells were pulse-labeled for 10 min with [^{3}H]uridine, chased for 60 min in order to generate naturally-occurring, intracellular, free RNA and pulse-labeled a second time for 10 min with [^{14}C]uridine. Chromatin was then extracted, sonicated and fractionated on ECTHAM-cellulose. The elution profile from the column and specific activities of pooled early, middle and late fractions are shown in Fig. 6 and Table 8, respectively. The quantity of ^{3}H-labeled RNA per A_{260} unit of chromatin was higher (2.4 times) in the late fractions than in the early fractions. However, after sedimentation through sucrose gradients, little difference was observed in the specific activities (^{3}H cpm/A_{260}) of early, middle, or late eluting chromatin fractions (Table 9). Therefore, endogenous, free RNA appears not to bind preferentially to any of the three chromatin fractions obtained from ECTHAM-cellulose.

In addition, before sucrose gradient sedimentation, the amount of ^{14}C-labeled RNA per A_{260} unit chromatin was 2.7 times greater in the late fraction than in the early fraction (Table 8). However, after sedimentation, little difference in specific activity (^{14}C cpm/A_{260}) was observed in early, middle or late eluting chromatin (Table 9). This surprising result indicates that the relatively large amount of ^{14}C-labeled RNA present in the late fraction before sedimentation was not bound to chromatin and, therefore, did not represent nascent RNA. Apparently, nascent RNA is sheared from its template

Fig. 6 Elution from ECTHAM-cellulose of chromatin extracted from fetal liver cells pulse-labeled with [^{3}H] and [^{14}C]uridine. Fetal liver cells were pulse-labeled for 10 min at 37°C with [^{3}H]uridine (10 μCi/ml), washed, incubated for 60 min at 37°C, and labeled again for 10 min with [^{14}C]uridine (2.5 μCi/ml) for 10 min. Chromatin was prepared, sonicated and chromatographed on ECTHAM-cellulose.

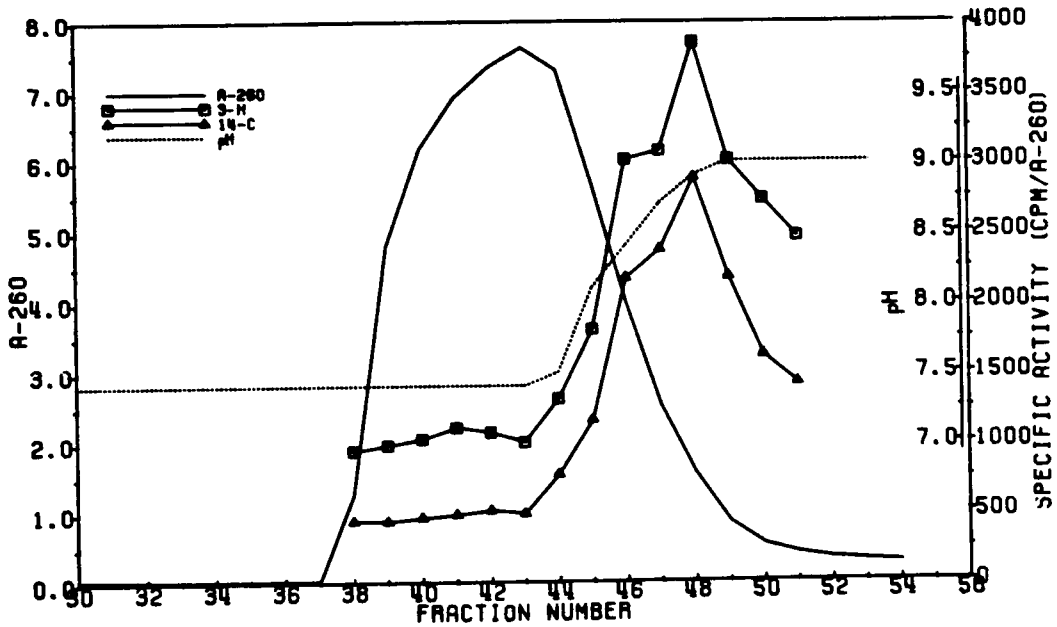
A-260
3-H
14-C
pH
A-260
pH
SPECIFIC ACTIVITY (CPM/A-260)
FRACTION NUMBER
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
9.5
9.0
8.5
8.0
7.5
7.0
4000
3500
3000
2500
2000
1500
1000
500
0
30
32
34
36
38
40
42
44
46
48
50
52
54
56

Figure 6

Table 8. Association of ^{3}H- and ^{14}C-labeled RNA with ECTHAM-cellulose Fractionated Chromatin[a]

Fraction	Percent of total chromatin fraction	Specific Activity (^{3}H cpm/A_{260})	Specific Activity (^{14}C cpm/A_{260})
Early[b]	60	3,301	1,756
Middle[c]	30	4,474	2,862
Late[d]	10	7,826	4,674

[a]Fractions of Fig. 4 were pooled; aliquots of each fraction were TCA precipitated and specific activities calculated.

[b]Fractions 39-44 of Fig. 4.

[c]Fractions 45-47 of Fig. 4.

[d]Fractions 48-51 of Fig. 4.

Table 9. Association of ^{3}H and ^{14}C-labeled RNA with ECTHAM-cellulose Fractions After Sucrose Gradient Sedimentation[a]

Fraction	Specific Activity (^{3}H cpm/A_{260})	Specific Activity (^{14}C cpm/A_{260})
Early	3,696	1,664
Middle	3,882	1,646
Late	4,063	1,834

[a]Aliquots of fractions described in Table IV were sedimented through 5-20% sucrose gradients. Fractions 5-15 of each gradient were pooled and specific activities determined. Total A_{260} units, cpm in ^{3}H, and cpm in ^{14}C, respectively, in fractions 1-4 were as follows: early, 0.00, 767, 462; middle, 0.06, 5,341, 3,309; late, 0.50, 15,037, 10,280. Total A_{260} units, cpm in ^{3}H and cpm in ^{14}C, respectively, in fractions 5-15 were as follows: early, 3.49, 12,965, 5,847; middle, 3.37, 13,545, 5,835; late, 2.98, 13,417, 6,359. Corrections for the amount of unbound RNA which cosedimented with chromatin to fractions 5-15 were as described in Methods (f = .92).

during chromatin preparation, binds to ECTHAM-cellulose, and elutes in the late fraction. Coelution with late eluting chromatin then leads to the calculation of an artifically high specific activity for this fraction.

DISCUSSION

One of the most widely used criteria for evaluating the activity of fractions derived by various chromatin fractionation procedures has been the association of nascent RNA with actively transcribed sequences. The presumed active fractions obtained from sucrose-gradients (Frenster, et al. 1963; Murphy, et al. 1973; and Berkowitz and Doty, 1975), differential precipitation after DNAse II digestion (Billings and Bonner, 1972), and ECTHAM-celllulose chromatography (Simpson, 1974) are enriched for pulse-labeled RNA. However, Seidman and Cole (1977) and Townes and Riggsby (1980) have demonstrated that the association of pulse-labeled RNA with a particular chromatin fraction is not an accurate index of transcriptional activity. In both the sucrose gradient (Seidman and Cole, 1977) and DNAse II (Townes and Riggsby, 1980) fractionation procedures, free RNA preferentially associates with the same fraction to which RNA pulse-labeled in vivo is bound. Therefore, the association of pulse-labeled RNA with a particular chromatin fraction does not necessarily mark transcribed sequences but may merely mark a fraction of chromatin which preferentially binds RNA that has been cleaved from its template during chromatin preparation and/or fractionation.

In the DNAse II fractionation procedure, the fraction of chromatin which binds free RNA (Townes and Riggsby, 1980) is coincident with the fraction (SII) which has been shown by Gottesfeld, et al. (1977) to contain DNA sequences actively transcribed in the tissue of origin. It, thus, appears that there is a chromatin fraction which selectively binds free RNA and which is physically similar to transcriptinally active chromatin.

The structure of RNA-binding chromatin is, however, sufficiently different from actively transcribing chromatin that the two subfractions can be partially separated by sedimentation of sheared chromatin on sucrose gradients. In this fractionation procedure, chromatin which binds free RNA is recovered exclusively in slowly sedimenting fractions (Seidman and Cole, 1977); whereas, chromatin which contains actively transcribed DNA sequences is recovered throughout the gradient (Krieg and Wells, 1976).

As indicated in the Results, endogenous free RNA binds almost equally to all three fractions of chromatin generated by chromatography of sheared chromatin on ECTHAM-cellulose. Howk, et al. (1975) have found that all three fractions also contain relatively equal amounts of DNA sequences know to be transcribed in the tissue of origin. Therefore, although the ECTHAM-cellulose procedure does separate structurally distinct chromatin fractions (Reeck, et al. 1972; Simpson, 1974; Simpson and Reeck, 1973; Polacow and Simpson, 1973; Simpson, 1974; and Simpson, 1975), it does not distinguish between RNA binding and actively transcribing chromatin.

The lack of binding of exogenously added RNA to late chromatin was unexpected since endogenous free RNA bound equally well to all three chromatin fractions. It is possible that the binding of RNA to late chromatin is concentration dependent and that the concentrations of free RNA and chromatin in the mixing experiment are sufficiently different from in vivo conditions that binding to late chromatin does not occur. Whatever the case, neither exogenous nor endogenous free RNA bind selectively to putative active chromatin. Therefore, the greater specific activity observed before sucrose gradient sedimentation in late eluting chromatin as opposed to early and middle eluting chromatin results exclusively from the binding of free RNA to ECTHAM-cellulose and from the elution of this RNA in late fractions. This property of free RNA leads to the incorrect interpretation of results obtained when pulse-labeled RNA is used to mark actively transcribing chromatin.

The elution of free RNA in late fractions could also explain the observation that E. coli RNA polymerase is more active in RNA synthesis on late fractions than on early or middle fractions (Simpson, 1974). Zasloff and Felsenfeld (1977a and 1977b) have demonstrated that under the conditions commonly used for in vitro transcription assays, E coli polymerase preferentially uses RNA rather than chromatin as a template. Therefore, the relatively large amount of synthesis supported by fractions eluting late from ECTHAM may simply result from the synthesis of RNA from RNA templates.

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PART II

PREPARATION OF REAGENTS FOR IN VITRO TRANSCRIPTION EXPERIMENTS

CHAPTER I

ISOLATION AND CHARACTERIZATION OF GLOBIN mRNA

INTRODUCTION

Globin mRNA was the first eukaryotic mRNA to be isolated and characterized by its ability to direct the synthesis of a specific protein in vitro (Lockard and Lingrel, 1969; Evans and Lingrel, 1969). Consequently, it is one of the most well-studied mRNAs in eukaryotic organisms. Relatively large quantities of this molecule can be isolated from circulating reticulocytes; approximately ninety percent of the mRNA in reticulocyte polysomes is globin mRNA. Furthermore, large quantities of reticulocytes can be obtained from the blood of animals in which severe anemia has been induced with phenylhydrazine. Therefore, the procedure for isolating globin mRNA begins with the induction of anemia. Animals are then bled, reticulocytes lysed and polysomes isolated. RNA is extracted from polysomes and fractionated on poly(U) Sepharose which selectively binds poly(A) containing mRNA. Polyadenylated RNA is sedimented on sucrose gradients until a relatively pure population of 10S molecules is obtained. Ten S RNA is then assayed for its ability to direct the synthesis of globin protein in a cell-free translation system. If the 10S RNA proves to be functional in the cell-free system, a DNA copy of this mRNA is synthesized with reverse transcriptase. The ultimate assay for mRNA purity is then hybridization of cDNA back to its template RNA. If the kinetics of the reaction fit the kinetics predicted by mathematical equations which describe the hybridization reaction, the mRNA preparation is pure.

METHODS

Induction of Reticulocytosis

BALB/c mice were given six daily intraperitoneal injections (0.1 ml) of a neutralized aqueous solution of 0.9% phenylhydrazine - HCl. On the seventh day, samples of blood were taken from several mice by eye puncture and stained with Cresyl brilliant blue. Mice were considered anemic when 80-90% of the red cell population were reticylocytes.

Isolation of Polysomes

Anemic mice were injected with 0.1 ml of heparin (1000 U/ml). After one hour, each mouse was bled from the neck into ice-cold isotonic medium (0.14 m NaCl, 5 m$\underline{M}$ $MgCl_2$, 5 m$\underline{M}$ KCl), containing heparin (1000 U/ml). The cells were washed three times in isotonic medium plus heparin and then lysed in two volumes of hypotonic medium (10 m$\underline{M}$ Tris-HCl, pH 7.4, 2 m$\underline{M}$ $MgCl_2$ 10 m$\underline{M}$ KCl) by vigorous shaking for two minutes in the cold. Immediately after shaking, the NaCl concentration was increased to 0.15 $\underline{M}$ in order to stabilize leukocytes which were not lysed in the hypotonic buffer. Intact leukocytes and debris were then pelleted by centrifugation (12,000 x g in a Beckman JS-13 rotor for 10 minutes at $4^{o}C$). The lysate was layered onto a 7 ml cushion of 36% sucrose containing 0.1 $\underline{M}$ Tris-HCl, pH 7.4, 15 m$\underline{M}$ KCl and 5 m$\underline{M}$ magnesium acetate and centrifuged for 3 hours at 35,000 rpm in a Beckman 42.1 rotor. The resulting pellet contained reticulocyte polysomes (Evans and Lingrel, 1969).

Extraction of RNA

The polysomal pellet was resuspended in water at 20 ODS/ml and digested with Proteinase K (50 µg/ml; E.M. Laboratories, Inc.) in 1.0% SDS, 10 mM Tris-HCl, pH 7.5 for 30 minutes at 0°C. RNA was then extracted as described in Chapter I.

Poly(U) Sepharose Chromatography

A 1 x 12 cm poly(U) Sepharose column was prepared by swelling 2.5 gm of poly(U) Sepharose 4B (Pharmacia) in 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.5 for five minutes and packing in a water-jacketed column. The column was then equilibrated with high salt buffer (0.5 M NaCl, 0.5% SDS, 0.01 M Tris, pH 7.5).

The RNA sample was diluted with glass-distilled water to 20 ODS/ml, heated at 65°C for five minutes and quick cooled. The solution was made 0.5 M NaCl, 0.5% SDS, and 0.01 M Tris-HCl, pH 7.5 and loaded onto the column at 25°C at a rate of 1.0 ml/5 minutes. After loading, the column was washed with high salt buffer until all unbound RNA was eluted. The column was then washed with a low salt buffer (0.1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, pH 7.5) to remove RNA which was not tightly bound to the resin. Finally, poly(A)-containing RNA was eluted by washing the column with water at 50°C.

Sucrose Gradient Sedimentation

Five to twenty percent sucrose gradients containing 0.01 M Tris-HCl pH 7.5 were prepared with an Isco gradient maker. RNA was resuspended in water, heated at 65°C for five minutes, quick cooled and then layered onto the gradient. The gradients were then centrifuged either at 27,000 rpm for 24 hours or 41,000 rpm for 14 hours as indicated in the Results.

Wheat Germ Cell-Free System

The preparation of the wheat germ extract was as described in Roberts and Patterson (1973) with the following modification. The S-30 fraction was preincubated for 30 minutes at 30°C. This step was critical in reducing background synthesis.

The standard protein synthesis assay was performed in a volume of 100 µl which contained 25 µl S-30, 20 mM Hepes-HCl (pH 7.6), 1 mM dithiothrietol, 1 mM ATP, 20 µM GTP, 8 mM creatin phosphate, 40 µg/ml creatin phosphokinase, 25 µM of the appropriate unlabeled amino acids, 80 mM KCl, 3.0 mM Mg acetate, 50 µM spermine, 75 µg/ml wheat germ tRNA, 5 µCi ^{3}H-leucine (60 Ci/mmol, NEN) and globin RNA at 33.75 µg/ml.

The system was calibrated for use in this laboratory with brome mosaic virus RNA as indicated in Figures 1 and 2. The optimum Mg^{++} and K^{+} concentrations were 3.5 and 90 mM, respectively.

Analysis of Products Synthesized in Wheat Germ System

Fifty microliters of the reaction were immediately added to 250 µl of dialysis buffer (50 mM β-mercaptoethanol, 0.1 M $NaPO_4$, pH 7.0) and dialyzed against 2 one liter volumes of the same buffer for 10-12 hours. The sample was mixed with 1-2 mg of carrier mouse hemolysate (0.3 ml) and globin was prepared as described by Clegg, et al. (1966). The *in vitro* product was analyzed by CMC-23 chromatography as described by Popp and Baliff (1973).

Fig. 1 Titration of wheat germ cell-free translation system for optimum Mg^{++} concentration. Three micrograms of purified brome mosaic virus RNA were translated in the wheat germ system at various Mg^{++} concentrations. After 90 minutes of incubation, protein was precipitated with trichloroacetic acid and the amount of labeled amino acid incorporated into protein was determined.

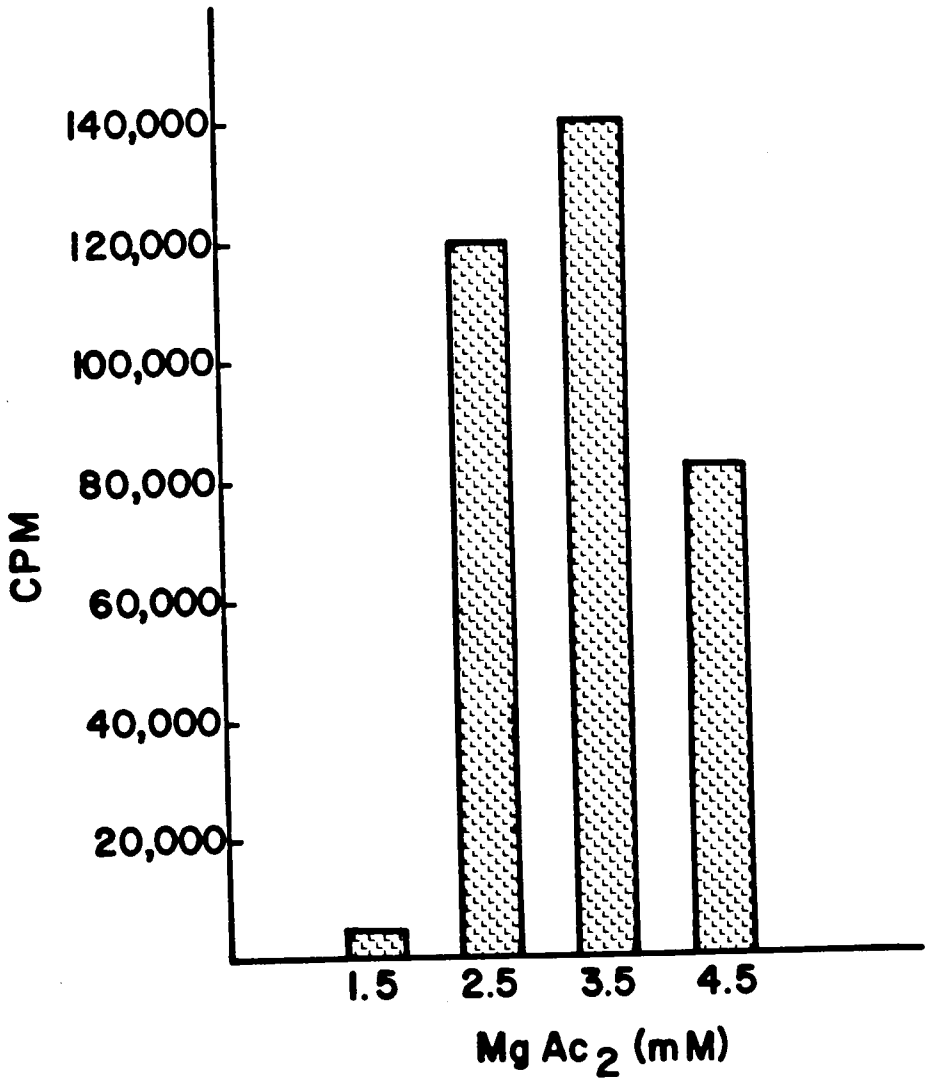
140,000
120,000
100,000
80,000
60,000
40,000
20,000
CPM
1.5
2.5
3.5
4.5
Mg Ac 2 (mM)

Figure 1

Fig. 2 Titration of wheat germ cell-free translation system for optimum K^+ concentration. Three micrograms of purified brome mosaic virus RNA were translated in the wheat germ system at various K^+ concentrations. After 90 minutes of incubation, protein was precipitated with trichloroacetic acid and the amount of labeled amino acid incorporated into protein was determined.

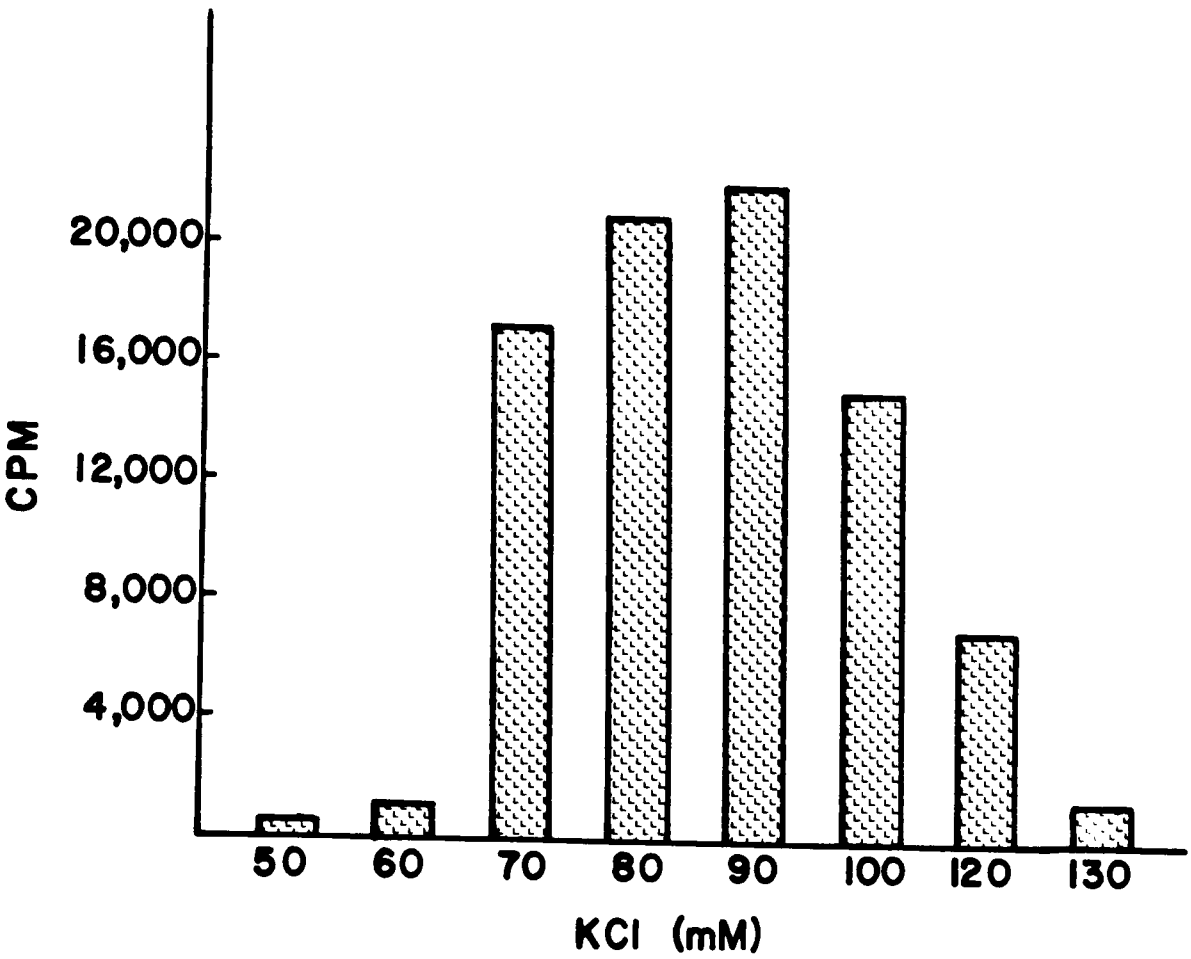
20,000
16,000
12,000
8,000
4,000
CPM
50
60
70
80
90
100
120
130
KCl (mM)

Figure 2

Synthesis of Globin cDNA

Globin cDNA was synthesized essentially as described by Myers, et al. (1977). The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1.0 mM dithiothrietol, 40 mM KCl, 0.2 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM [^{3}H]dCTP (20 Ci/mmol), 4 mM sodium pyrophosphate, 10 g/ml oligo dT_{10}, 60µg/ml mRNA and 45 U/ml of AMV reverse transcriptase.

After 120 minutes of incubation at 37°C the reaction was stopped by the addition of SDS to a final concentration 0.5%. The mixture was made 0.2 M NaOH and treated for 10 minutes at 70°C to hydrolyze RNA. The solution was then neutralized with HCl and buffered with Tris-HCl at a final concentration of 0.1 M, pH 7.0. One hundred and fifty micrograms of yeast carrier RNA was added per ml of solution and the mixture was passed over a 0.9 x 50 cm G-50 column equilibrated in water. Aliquots of each column fraction were counted in a liquid scintillation counter, peak void volume fractions were pooled, and cDNA was ethanol precipitated overnight at -20°C.

After pelleting, cDNA was resuspended in 100-200 µl of 0.9 M NaCl, 0.2 M NaOH and 1 mM EDTA, heated for 10 minutes at 70°C. The sample was then layered onto a 12 ml, 5-20% sucrose gradient containing 0.9 M NaCl, 0.2 M NaOH and 1 mM EDTA and centrifuged for 19 hours at 41,000 rpm in a Beckman SW41 rotor. The gradient was fractionated in 8 drop fractions. Each fraction was neutralized by the addition of an equal volume of 0.2 M HCl and an aliquot of each fraction was counted. The peak tube was pooled with fractions farther down the gradient and the sample was ethanol precipitated.

Hybridization of Globin cDNA and mRNA

Hybridization reactions were performed in flame sealed 40 µl glass capillaries in 10 µl volumes containing 10 mM Hepes-HCl (pH 7.5), 0.6 M NaCl, 2 mM EDTA, 3000 dpm of globin cDNA (2.59×10^7 dpm/µg) and globin mRNA (11.6 µg/ml).

Sealed capillaries were heated for 2 minutes at 100°C and incubated at 70°C for the various times required to obtain the appropriate Cot values. Reactions were stopped by submerging capillaries in a dry ice-ethanol bath.

Individual reactions were assayed for the fraction of cDNA hybridized by the S_1 method essentially as described by Sutton (1971). The ends of capillary tubes were clipped and contents blown into 490 µl of S_1 buffer (1 mM $ZnSO_4$, 0.1 M NaCl, 0.03 M Na acetate, pH 4.5, 25 µg/ml denatured sonicated calf thymus DNA). Samples were split in half and 5 µl of S_1 (4 units/5 µl) were added to one of the halves; both halves were incubated for 60 minutes at 37°C. After incubation, 150 µg of yeast carrier RNA was added to each sample. The samples were then TCA precipitated, filtered on membrane filters and counted in a Toluene-based scintillant. Fraction of cDNA hybridized was determined by dividing the cpm of the digested sample by the cpm of the undigested control.

RESULTS AND DISCUSSION

Reticulocytosis was induced in 100 BALB/c mice as described in Methods. The mice were bled and approximately 36 ml of packed cells were obtained. After lysing the cells in hypotonic buffer, free polysomes were isolated by sedimentation through sucrose and RNA was extracted. The 36 ml of packed cells yielded 1,000 A_{260} units of polysomal RNA.

After ethanol precipitation, the RNA sample was chromatographed on poly(U) Sepharose in order to separate polyadenylated RNA from non-polyadenylated species; the elution profile from the column is shown in Fig. 3. Most of the RNA (98%) does not bind to the column and is recovered in the high salt buffer wash. Sucrose gradient analysis of this RNA (Figure 4) indicates that it is composed primarily of 4, 18, and 28S ribosomal RNA. After a low salt wash, poly(A)-containing RNA was eluted in water at 50°C and ethanol precipitated. This RNA was then pelleted, resuspended, and chromatographed a second time on poly(U) Sepharose. Figure 5 illustrates the elution of RNA from the second column. Approximately 80% of the RNA loaded onto this column was recovered in the water wash.

RNA which eluted in the water fraction of the second poly(U) column was then fractionated by size on 5-20% neutral sucrose gradients. Figure 6 shows the sedimentation profile of RNA centrifuged at 27,000 rpm for 24 hours in a Beckman SW27 rotor. Some 4, 18, and 28S RNA still contaminate the preparation; however, a distinct peak of RNA was detected at 10S. Therefore, fractions 12-15 of this gradient were pooled and ethanol precipitated.

Fig. 3 Poly(U) Sepharose chromatography of reticulocyte polysomal RNA. One thousand A_{260} units of reticulocyte, polysomal RNA were loaded onto a 5 ml Poly(U) Sepharose column in 0.5 M NaCl buffer, washed with 0.5 and 0.1 M NaCl buffer, and eluted in H_2O at 50°C.

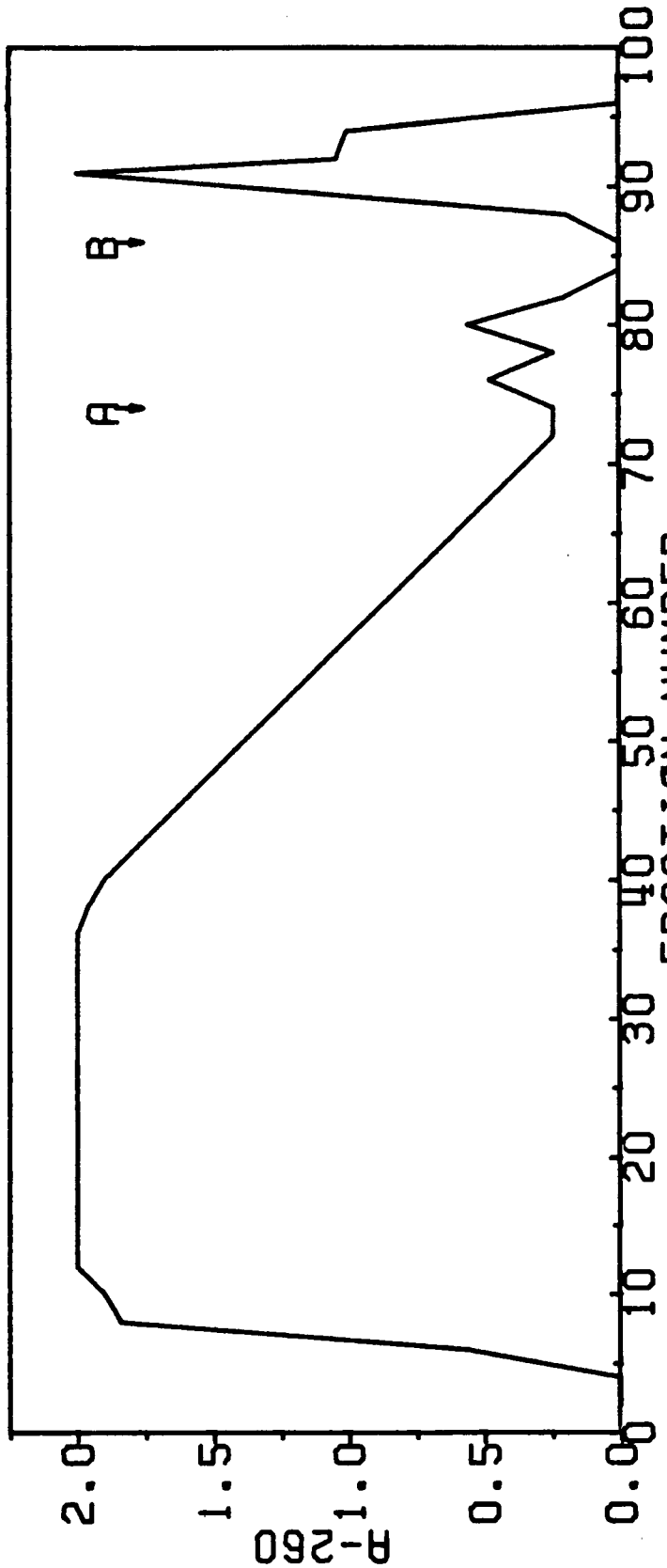
A-280
2.0
1.5
1.0
0.5
0.0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER
A
B

Figure 3

Fig. 4 Sucrose gradient analysis of RNA which does not bind to Poly(U) Sepharose. RNA which eluted from the column in the 0.5 M NaCl wash was concentrated by ethanol precipitation, resuspended and sedimented through a 5-20% sucrose gradient as described in the Methods. Sedimentation is from right to left.

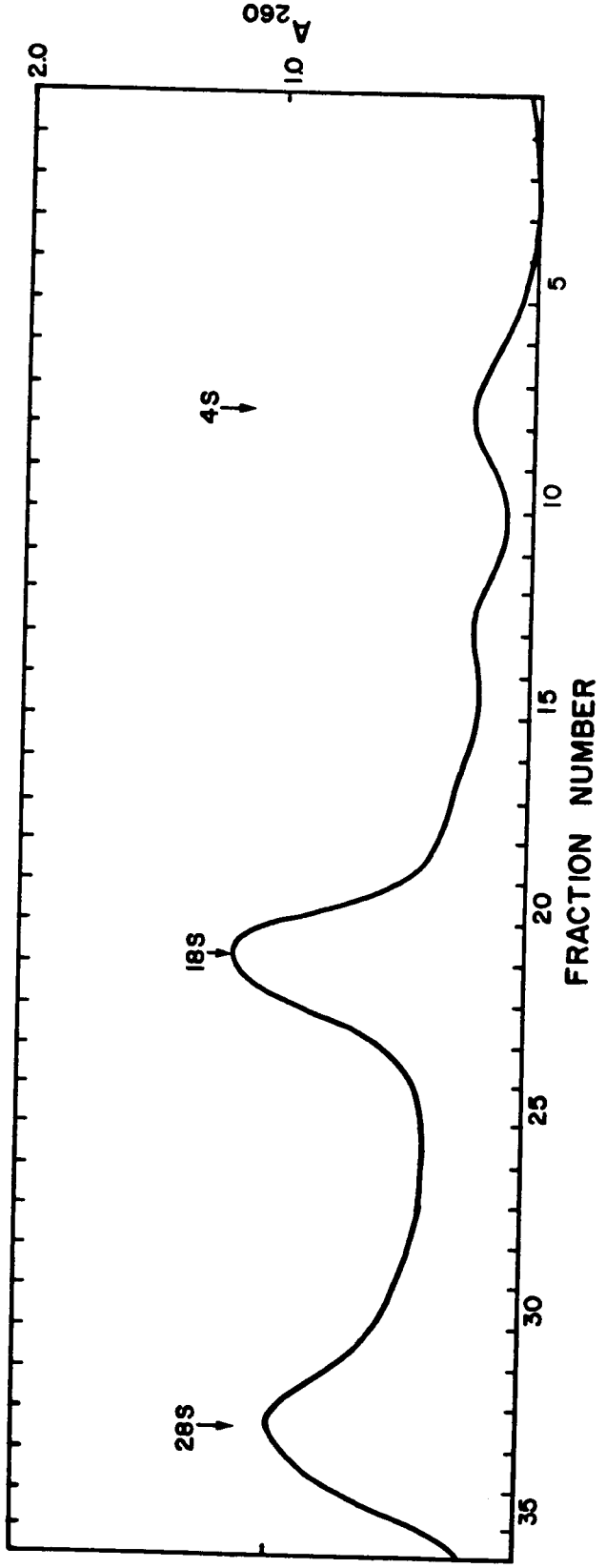

Figure 4

Fig. 5 Poly(U) Sepharose chromatography of reticulocyte RNA bound to first Poly(U) column. RNA which eluted in the H_2O fraction of the first Poly(U) Sepharose column was loaded onto a second column in 0.5 M NaCl buffer. After washing the column with 0.5 M and 0.1 M NaCl buffer, RNA was eluted in H_2O at $50^{o}C$.

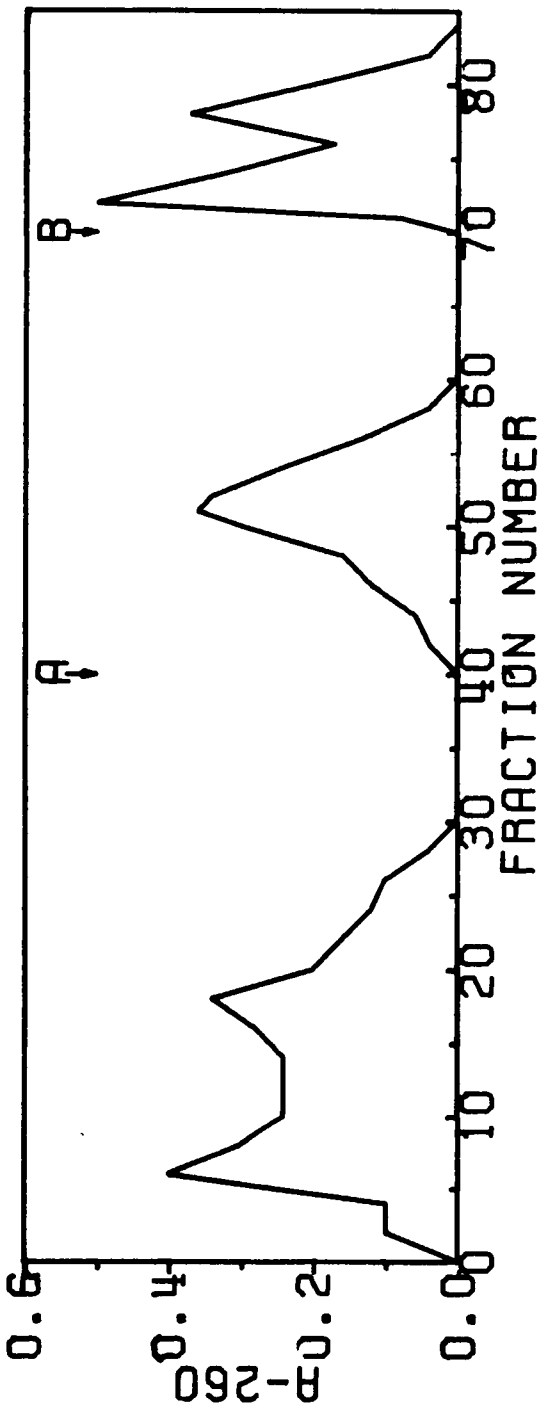

Figure 5

Fig. 6 Sucrose gradient analysis of RNA which bound twice to Poly(U) Sepharose. RNA which was elulted in the H_2O fraction of the second Poly(U) Sepharose column was concentrated by ethanol precipitation, resuspended and sedimented on a 5-20% sucrose gradient as described in the Methods.

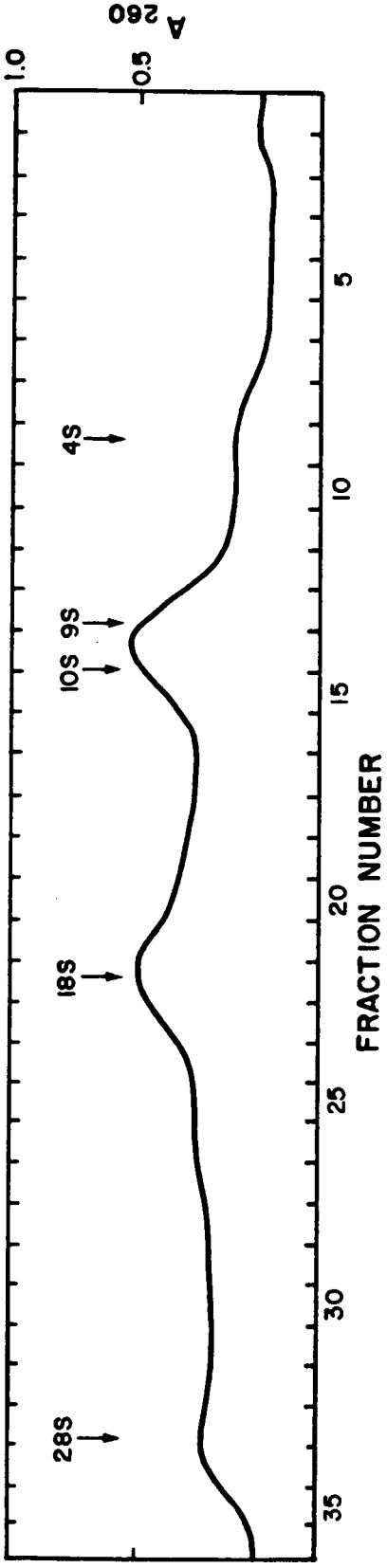

Figure 6

After pelleting, the RNA was resuspended and centrifuged for 14 hours at 41,000 rpm in a second 5-20% sucrose gradient (Fig. 7). The peak fractions (13-15) which again contained 10S RNA were pooled and ethanol precipitated. Approximately 1.0 OD of RNA was recovered in these fractions. This represents 0.1% of polysomal RNA.

In order to determine whether or not the 10S RNA could direct the synthesis of globin protein, an aliquot (3.0 μg) was assayed in the wheat germ free-cell translation system. Half of this reaction was then mixed with mouse hemolysate which contains unlabeled α and β polypeptides and the mixture was chromatographed on a carboxylmethyl cellulose column. The results of this experiment were shown in Figure 8. The elution profile of α and β polypeptide markers was consistent with data of Popp and Baliff and the radioactively labeled product of in vitro translation coeluted with the β polypeptide marker. This result demonstrates that the reticulocyte 10S polyadenylated RNA contains globin β mRNA. However, little α globin peptide was synthesized. Therefore, either α globin mRNA is not present in the 10S preparation or the messenger is present but not translated in the wheat germ system.

The most important test of the purity of a mRNA preparation is the hybridization of this mRNA to cDNA synthesized from it. Therefore, a DNA copy of the 10S mRNA was synthesized and this cDNA was hybridized back to its RNA template (Fig. 9). As indicated in the figure, the reaction is nearly complete by a Cot of 10^{-1}. The best fit curve, which was generated by a

Fig. 7 Sucrose gradient analysis of 8-12S reticulocyte RNA obtained from first sucrose gradient. RNA from the 8-12S region of the first sucrose gradient was pooled, ethanol precipitated, resuspended and sedimented on a second 5-20% sucrose gradient.

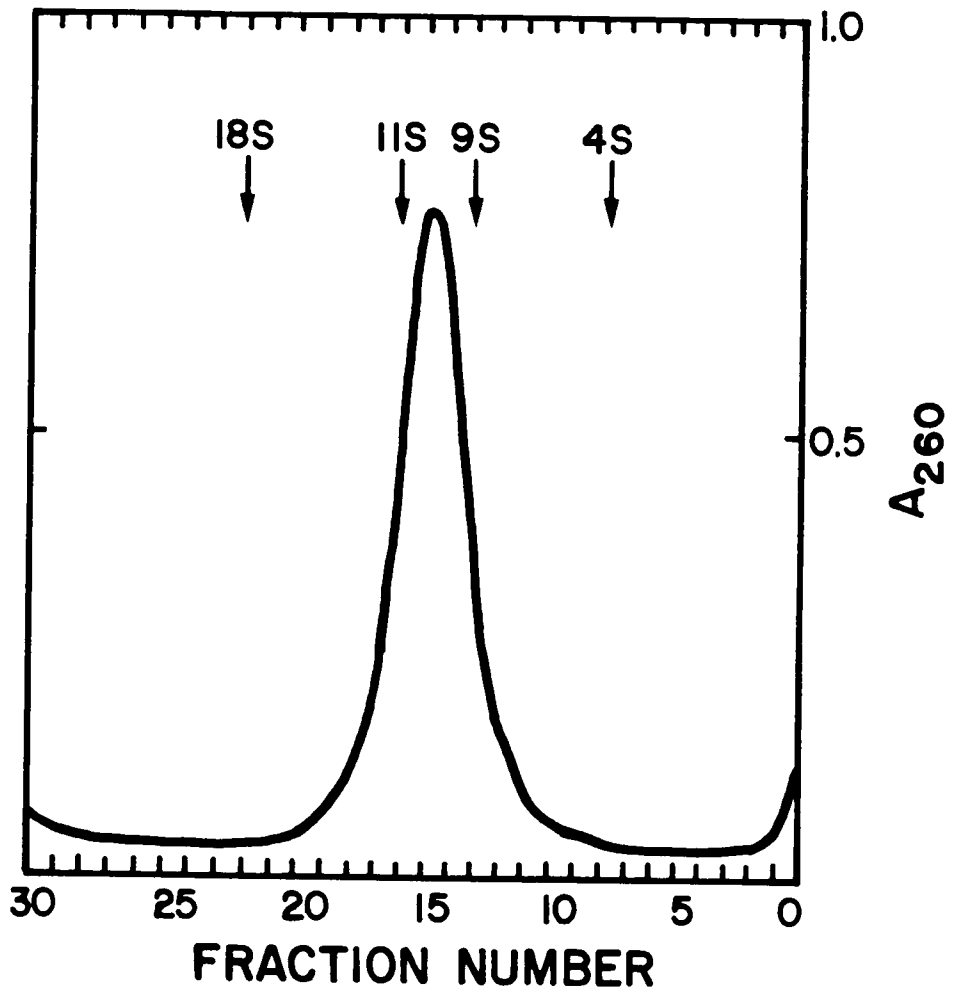

Figure 7

Fig. 8 Carboxymethylcellulose chromatography of proteins synthesized in vitro from reticulocyte 10S mRNA. Peak tubes from the final sucrose gradient were pooled, ethanol precipitated, resuspended and translated in the wheat germ system. The identity of products synthesized in vitro were determined by chromatography of denatured proteins as described in the Methods.

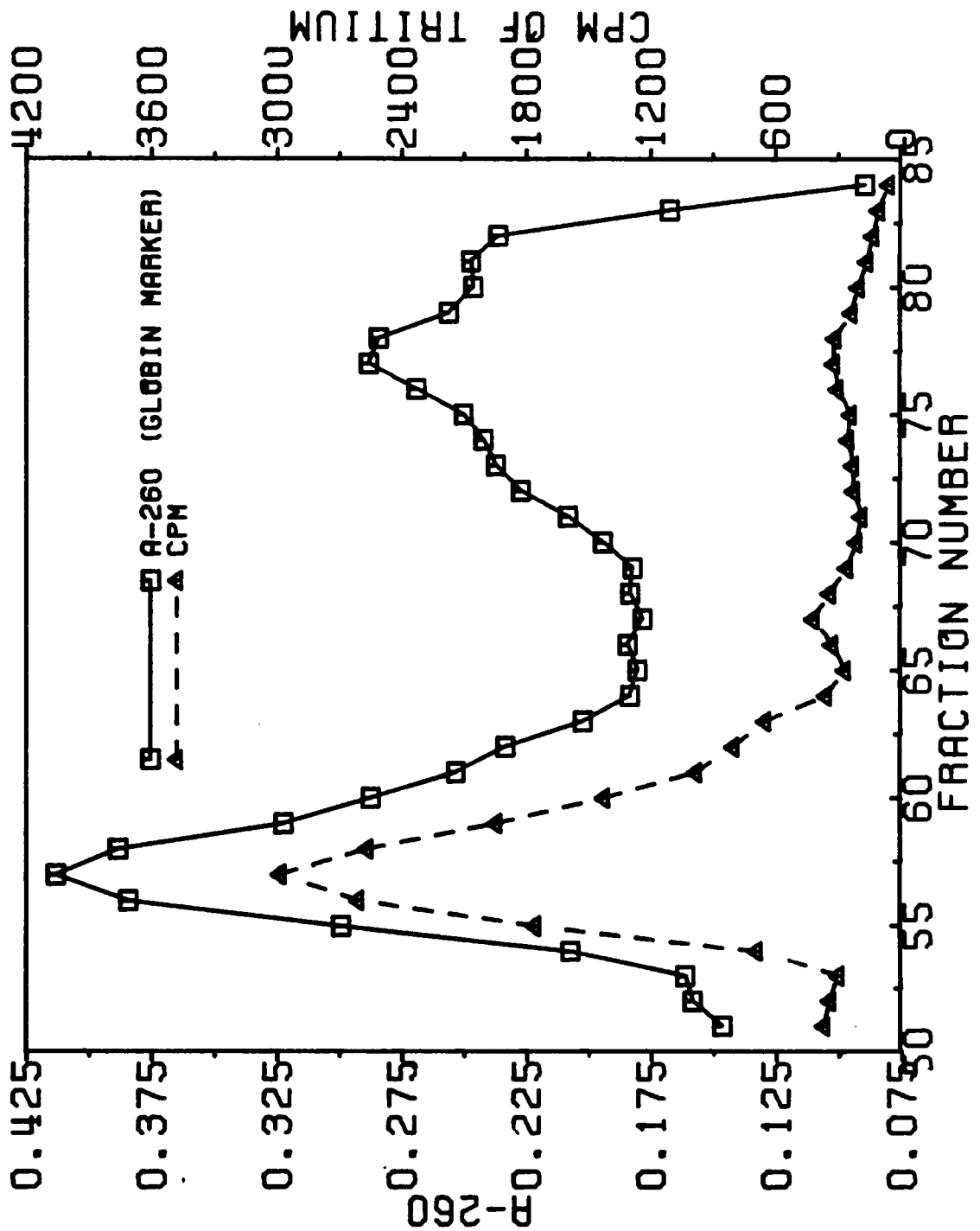
A-260 (GLOBIN MARKER)
CPM
A-260
CPM OF TRITIUM
FRACTION NUMBER
0.425
0.375
0.325
0.275
0.225
0.175
0.125
0.075
4200
3600
3000
2400
1800
1200
600
0
50
55
60
65
70
75
80
85

Figure 8

Fig. 9 Hybridization of globin cDNA to globin mRNA. Globin cDNA synthesized from globin mRNA was hybridized back to its template as described in Methods.

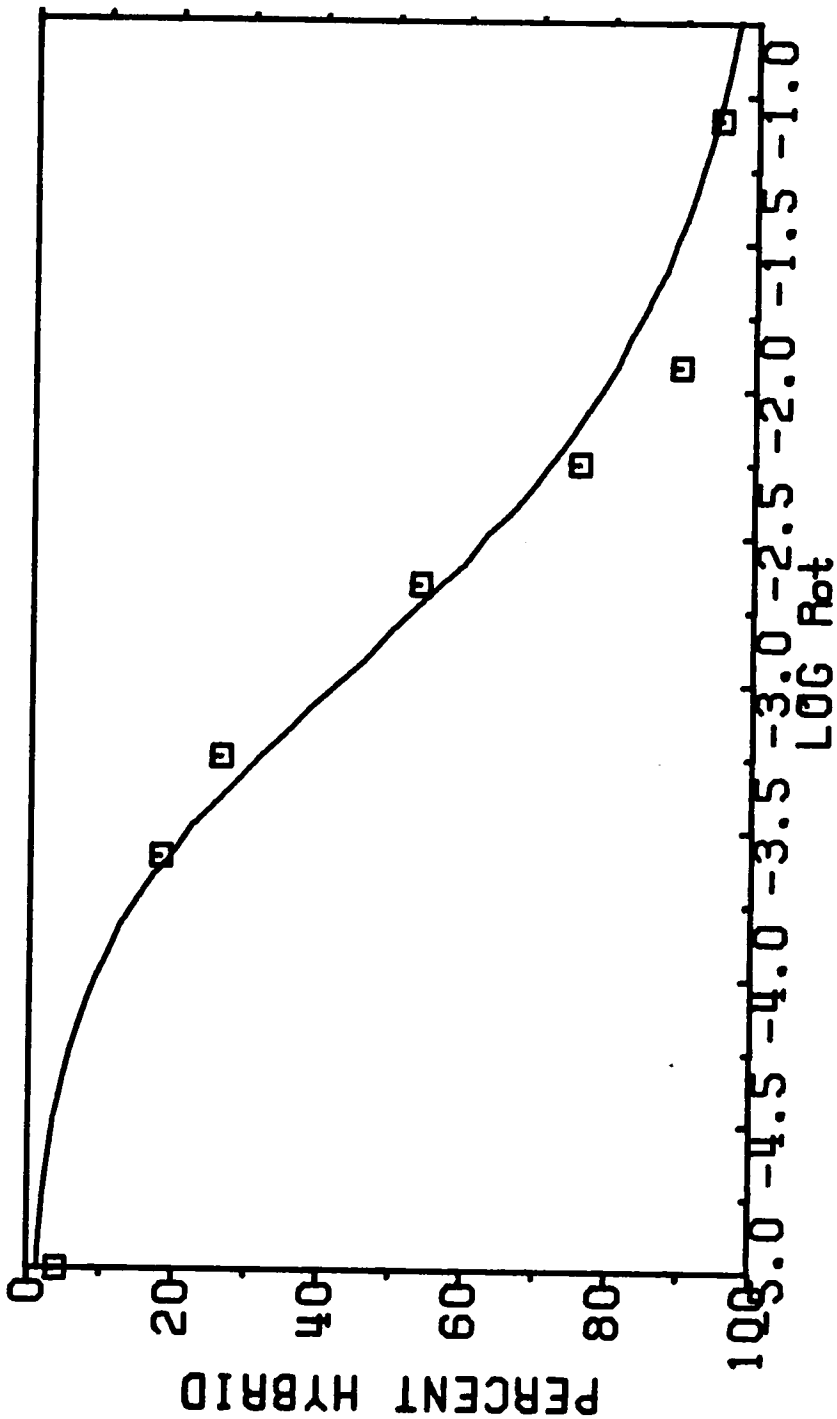

Figure 9

computer program obtained from W.R. Pearson (Pearson, et al. 1977), yielded a $Cot_{1/2}$ of 4.5×10^{-4}. This $Cot_{1/2}$ value corresponds to a complexity of approximately 675 nucleotides (Britten and Davidson, 1968) which is in close agreement with the complexity of mouse β globin mRNA (623 nucleotides) known from the sequencing data of Konkel, et al. (1979).

CHAPTER II

ISOLATION OF WHEAT GERM POLYMERASE II

INTRODUCTION

Large quantities of RNA polymerase are required for in vitro chromatin transcription studies and for many years only E. coli RNA polymerase could be obtained in high enough yields to make these experiments feasible. However, the transcription of chromatin with a procaryotic polymerase can provide only a limited understanding of the mechanisms involved in the regulation of eucaryotic gene expression. Therefore, the isolation of large quantities of polymerase II from a eucaryotic organism has been attempted by many groups.

In 1975, Jendrisak and Burgess reported the isolation of RNA polymerase II from wheat germ. In this procedure several milligrams of purified enzyme could be rapidly obtained from one kilogram of starting material. Chapter II describes the isolation of this enzyme in our laboratory.

METHODS

All of the methods were exactly as described by Jendrisak and Burgess (1975) except the RNA polymerase assay was as follows: a 5 µl aliquot of the appropriate fraction was added to a reaction mixture containing 200 µl of denatured calf thymus DNA (250 µg/ml) and 50 µl of a 5x mix containing 50 mM Tris-HCl pH 7.9, 5 m$\underline{M}$ $MnCl_2$, 100 m$\underline{M}$ $MgCl_2$, 250 m$\underline{M}$ $(NH_4)_2SO_4$, 2m$\underline{M}$ GTP, 2 m$\underline{M}$ CTP, 2 m$\underline{M}$ ATP, 6×10^{-4} m$\underline{M}$ cold UTP, 20 µCi/ml ^{3}H-UTP

(14 Ci/mmole) and 250 µgms/ml BSA. ($MgCl_2$ and $MnCl_2$ were added to the 5x mix on the day of use in order to avoid the precipitation of these salts during freezing and thawing.) The complete assay mixture was incubated for 15 min at 25°C and RNA was precipitated by the addition of 250 µl of 10% TCA containing 25 mM $Na_4P_2O_7$. After 10 min at 4°C, the precipitates were collected on membrane filters, washed with 5% TCA and 70% ethanol, dried and counted in a Toluene based scintillation fluid.

RESULTS AND DISCUSSION

One kilogram of raw wheat germ was ground for one minue at top speed in a one-gallon Waring blender in 4.0 liters of TED (0.050 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 1.0 mM dithiothreitol) + 0.075 M $(NH_4)SO_4$. After adding an additional liter of the same buffer, the homogenate was centrifuged for 15 min at 10,000 x g and the resulting supernatant was filtered through one layer of miracloth. This crude extract contained 7,975 units of pol II activity in a total volume of 4.65 liters (Table 1).

Polymin P was then added (0.075 vol. of 10%) and the precipitated protein was pelleted. After several washes, the pellet was resuspended in 2.0 liters of TED + 0.2 M $(NH_4)SO_4$ and assayed for pol II activity; the Polymin P eluate contained 8,500 units of activity in 1.98 liters of solution (Table I). The increased activity observed after Polymin P precipitation was also observed by Jendrisak and Burgess (1975) and apparently results from the loss of an inhibitor of pol II activity.

Table 1. Summary of Yield of Wheat Germ RNA polymerase

Fraction Description	Volume (ml)	Activity (units)	Yield (%)
Crude extract	4,650	7,979	100
Polymin P eluate	1,985	8,500	107
$(NH_4)_2SO_4$	220	5,590	70
DEAE-cellulose peak	8.20	1,977	25
$(NH_4)_2SO_4$	13.2	1,191	15
Phosphocellulose peak	7.50	1,162	14.5

The enzyme was then precipitated by the addition of 20 grams of solid $(NH_4)_2SO_4$ per 100 ml of solution and pelleted by centrifugation. After resuspending the pellet in TEDG (0.050 m$\underline{M}$ Tris-HCl pH 7.9, 0.1 m$\underline{M}$ EDTA, 1.0 m$\underline{M}$ dithiothreitol, 25% ethylene glycol) + 0.1% Brij 35, this fraction was assayed for polymerase activity. Approximately 5,500 units were recovered in a volume of 220 ml (Table 1).

The polymerase was applied to a DEAE-cellulose column equilibrated in TEDG + 0.15 $\underline{M}$ $(NH_4)_2SO_4$. After washing the column with this buffer, bound protein was eluted with 0.25 $\underline{M}$ $(NH_4)_2SO_4$ in TEDG and each fraction was assayed for polymerase activity (Fig. 10). Peak fractions were pooled and contained 1,977 units of enzyme in 82 ml of solution (Table 1).

RNA polymerase was then precipitated by the addition of 30 grams of $(NH_4)_2SO_4$ per 100 ml of solution and pelleted by centrifugation. The resulting pellet was dissolved in TEDG and reassayed for polymerase activity. Approximately 1,200 units were recovered. The enzyme was then applied to a phosphocellulose column equilibrated with TEDG + 0.075 M $(NH_4)_2SO_4$. After washing the column with this buffer, bound protein was eluted with TEDG + 0.15 M $(NH_4)_2SO_4$, and each fraction was assayed for polymerase activity (Fig. 11). The peak fractions were pooled and dialyzed against one liter of 50 mM Tris-HCl pH 7.9, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol. The final yield of RNA polymerase was 1,162 units in a volume of 7.5 ml.

Fig. 10 DEAE-cellulose chromatography of wheat germ polymerase II. After separation from nucleic acids by Polymin P precipitation, a fraction of the wheat germ proteins were precipitated with ammonium persulfate, resuspended in 0.15 M ammonium sulfate buffer, bound to DEAE-cellulose, and eluted with a 0.15 to 0.25 M ammonium sulfate buffer. Individual fractionationswere assayed for polymerase activity as described in the Methods.

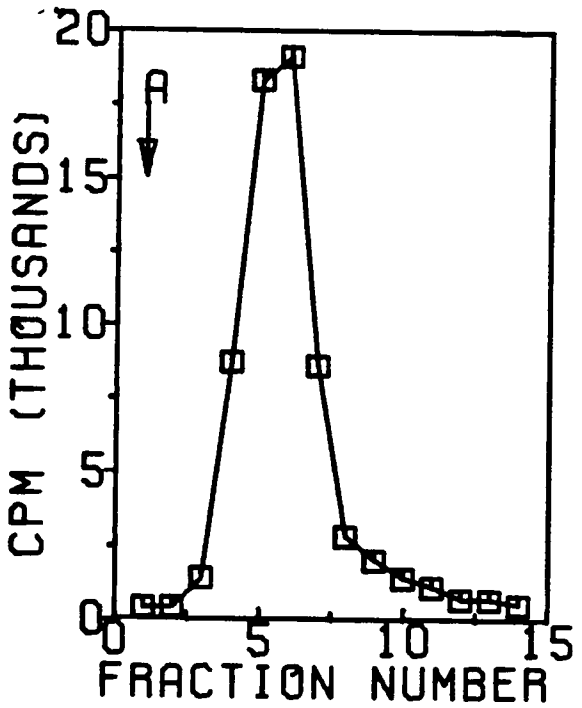

Figure 10

Fig. 11 Phosphocellulose chromatography of wheat germ polymerase II. DEAE-cellulose fractions which contained peak activities were pooled and precipitated with ammonium sulfate. After resuspension in 0.075 M ammonium sulfate buffer, proteins were loaded onto a phosphocellulose column and eluted with 0.15 M ammonium sulfate buffer.

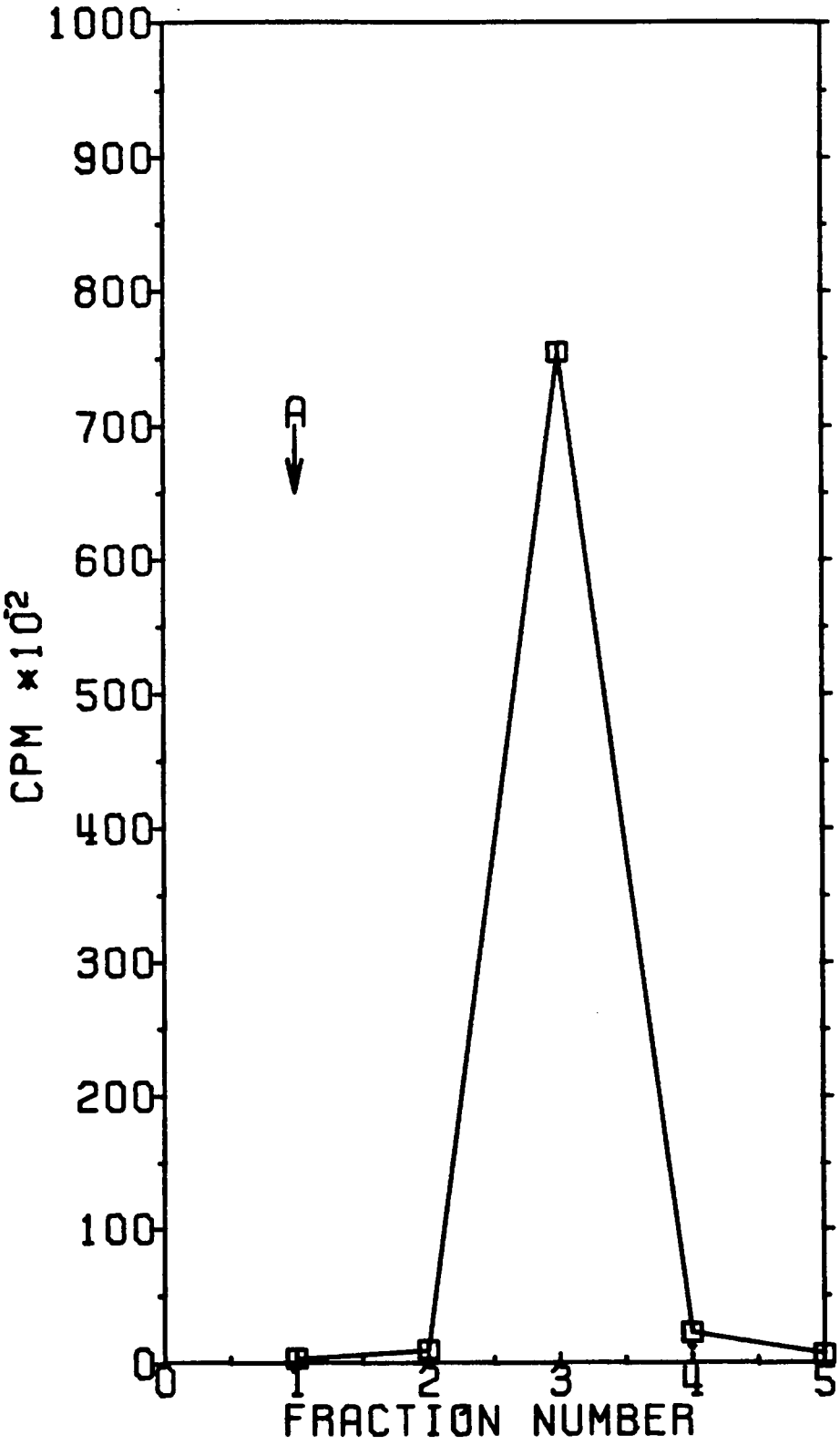
1000
900
800
700
600
500
400
300
200
100
0
CPM *10²
A
0
1
2
3
4
5
FRACTION NUMBER

Figure 11

Specific activity was subsequently calculated after measuring the optical density of the enzyme at 280 nm and assuming an extinction coefficient of 7.7 (Jendrisak, et al. 1976). The value of 267 units per milligram of protein agreed well with the value (250 U/mg) obtained by Jendrisak and Burgess (1975).

CHAPTER III

ISOLATION AND CHARACTERIZATION OF MYELOMA LIGHT CHAIN mRNA

INTRODUCTION

The isolation of myeloma light chain mRNA is much more difficult than the isolation of globin mRNA. Unlike reticulocytes in which 90% of the mRNA is globin mRNA, less than 40% of the mRNA population of myeloma cells is light chain RNA. Also, myeloma cells apparently contain hundreds of mRNAs which are approximately the same size as light chain RNA (see Results). Therefore, fractionation of myeloma mRNA on the basis of size is difficult.

The procedure for isolation of light chain RNA is similar to the isolation of globin RNA with the exception that membrane-bound polysomes are selected instead of free polysomes. Light chain mRNA, like most mRNAs which encode proteins to be excreted from the cell, are translated on the endoplasmic reticulum. Membrane-bound and free polysomes are separated on discontinuous sucrose gradients and RNA is extracted. Polyadenylated RNA is then isolated on poly(A) Sepharose and sedimented in sucrose gradients.

METHODS

Myeloma Tumors

MOPC-31C cells, originally isolated by M. Porter, were obtained from Dr. W.A. Yang at the Oak Ridge National Laboratories. The cells were maintained *in vivo* in BALB/c mice by serial transplantation of subcutaneous tumors.

Isolation of Membrane-Bound Polysomes

MOPC-31C tumors were removed into ice cold homogenization buffer (50 mM Tris-HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 10^{-4} M cycloheximide, 0.1% diethyl pyrocarbonate) and thoroughly homogenized in 4 volumes of this buffer in a Wheaton 40 ml homogenizer with a loose fitting (B) pestle. Cellular debris was pelleted by centrifugation for 20 minutes at 20,000 x g and the super-natant was filtered through one layer of myracloth. Fifteen ml aliquots of the sample were loaded onto discontinuous sucrose gradients composed of 7 ml of 1.5 M sucrose and 15 ml of 1.75 M sucrose in homogenization buffer minus diethyl pyrocarbonate and centrifuged for 1 hour at 25,000 rpm in a Beckman SW27 rotor (Honjo, et al. 1976). The whole 1.5 M sucrose layer was removed and retained as the source of membrane-bound polysomes.

RNA Extraction

Polysomes in 1.5 M sucrose were diluted to 20 ODS/ml and made 0.1 M Tris-HCl, pH 9.0, 1.0% sodium dodecyl sulfate (SDS) and 50 µg/ml Proteinase K. After 30 minutes of digestion at 4°C, RNA was phenol extracted as described in Part I, Chapter I.

Isolation of Polyadenylated RNA

Poly(A)-containing RNA was isolated by Poly(U) Sepharose chromatography as described in Part II, Chapter I.

Sucrose Gradient Sedimentation

Sucrose gradient sedimentation was performed as described in Part II, Chapter I.

Translation of Myeloma mRNA

Myeloma mRNA was translated in the wheat germ cell-free system as described in Part II, Chapter I, except that the final concentration of myeloma RNA was 15.1 µg/ml.

Analysis of Products Synthesized in Wheat Germ System

Ninety-five microliters of the 100 µl translation reaction was made 10 mM EDTA and digested for 30 minutes with 40 µg of RNAse A. The mixture was then made 1% SDS and 1% mercaptoethanol and dialyzed for 24 hours against 2 one liter changes of 0.06 M Tris, pH 6.7, 1% SDS, and 1% mercaptoethanol. One hundred microliters of the sample (~30,000 cpm) were then mixed with an equal volume of 2X application buffer (0.12 M Tris pH 6.7, 2% SDS, 40% glycerol, 1.0% mercaptoethanol and 0.004% bromophenol blue), heated for 2 minutes at 100°C and electrophoresed on 13% SDS, polyacrylamide gels exactly as described by Maizel (1971). A 3% stacking gel (Laemmli, 1970) was used.

Synthesis of cDNA

cDNA was synthesized as described in Part II, Chapter I.

Preparation of Labeled MOPC-31C Heavy and Light Polypeptides

Labeled heavy and light polypeptides were prepared as described by Laskov and Scharff (1970). Solid tumors were fragmented with scissors and a single cell suspension in Eagles medium (containing 1/20th of the normal amino acids) was prepared by several gentle passages of minced tissue through a 20 ml syringe without a needle. The cells were washed two times in amino acid depleted media and resuspended in 10 ml of the same medium at 5 x 10^6 cells/ml. Two and one-half ml of neutralized ^{14}C-amino acid mix (New England Nuclear) was added and the cells were incubated for 3 hours at

37°C. Following the incubation, the cells were pelleted and the supernatant was made 0.01% bovine serum albumin. Proteins were subsequently precipitated by the addition of an equal volume of 10% TCA. Precipitated protein was pelleted, washed twice in acetone, dissolved in 1.0% SDS and stored at -20°C.

RESULTS AND DISCUSSION

Myeloma tumors (35 gm) were removed from 100 BALB/c mice and homogenized. Following homogenization, membrane-bound polysomes were isolated on discontinuous sucrose gradients and 825 ODS of RNA were extracted from the polysomal preparation. Polyadenylated RNA was then selected by two rounds of chromatography on Poly(U) Sepharose. Figures 12 and 13 illustrate the elution of myeloma RNA from the columns. Approximately 90% of the RNA eluted in the 0.5 and 0.1 M salt wash of the first Poly(U) column; this RNA was identified as 4S, 18S, and 28S ribosomal RNA by sedimentation on 5-20% sucrose gradients (Fig. 14).

RNA which eluted in the H_2O fraction of the second Poly(U) column constituted approximately 10% of the total polysomal RNA preparations. This RNA was still contaminated with ribosomal RNA as indicated by the sucrose gradient profile depicted in Figure 15, but did contain other RNA species which sedimented between 4 and 18S. Since a mRNA of approximately 12S was predicted by the amino acid sequence of light chain protein, fractions 13 through 17 were pooled and ethanol precipitated. Fractions 18 through 21 were also pooled since a small peak of RNA at 15S was detected.

Fig. 12 Poly(U) Sepharose chromatography of membrane-bound, polysomal RNA from mouse myeloma tumors. Eight hundred and twenty-five A_{260} units of membrane-bound, polysomal RNA from mouse myeloma tumors were loaded onto a 5 ml Poly(U) Sepharose column in 0.5 M NaCl buffer, washed with 0.1 M NaCl buffer (A) and eluted in H_2O at 50°C (B).

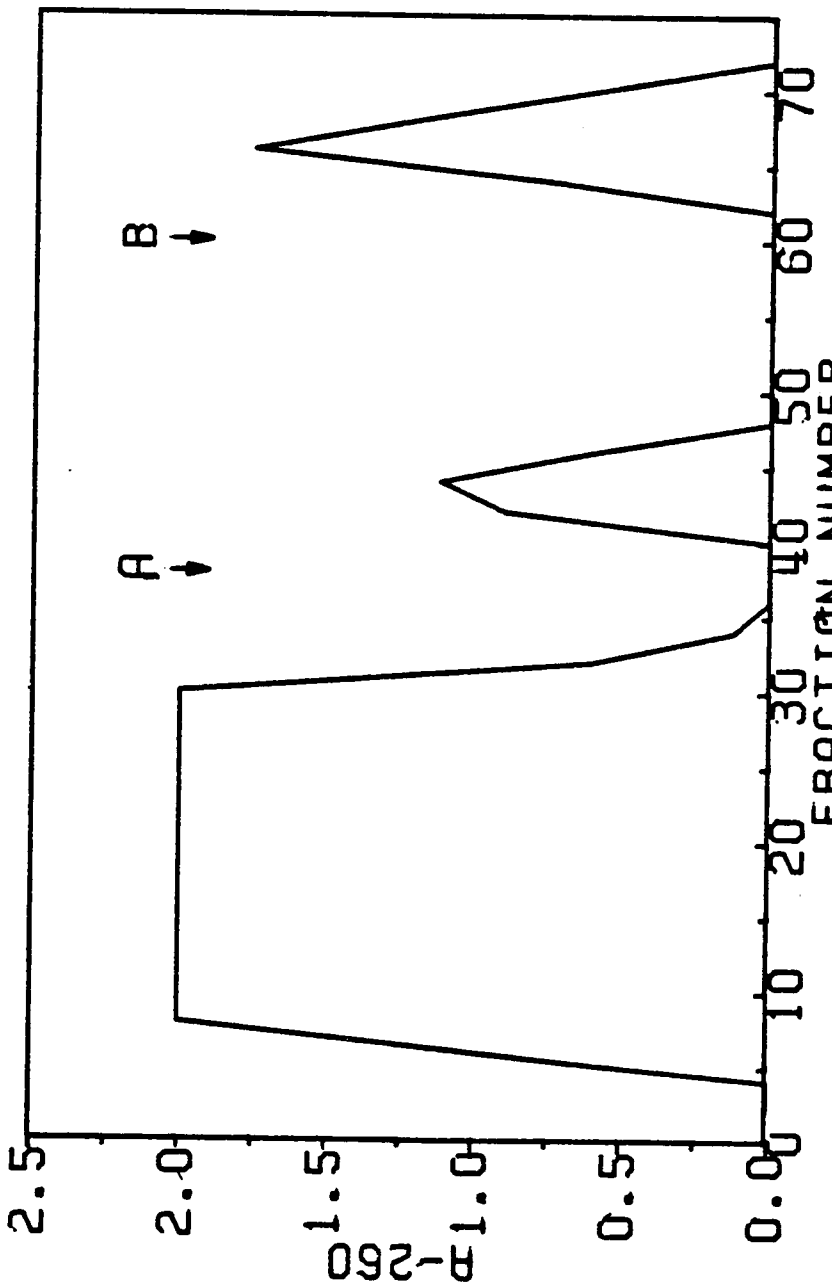

Figure 12

Fig. 13 Poly(U) Sepharose chromatography of myeloma RNA which bound to first Poly(U) colulmn. RNA which eluted in the H_2O fraction of the first Poly(U) Sepharose column was loaded onto a second column in 0.5 M NaCl buffer. After washing the column and 0.1 M NaCl buffer (A), RNA was eluted in H_2O at $50^{o}C$ (B).

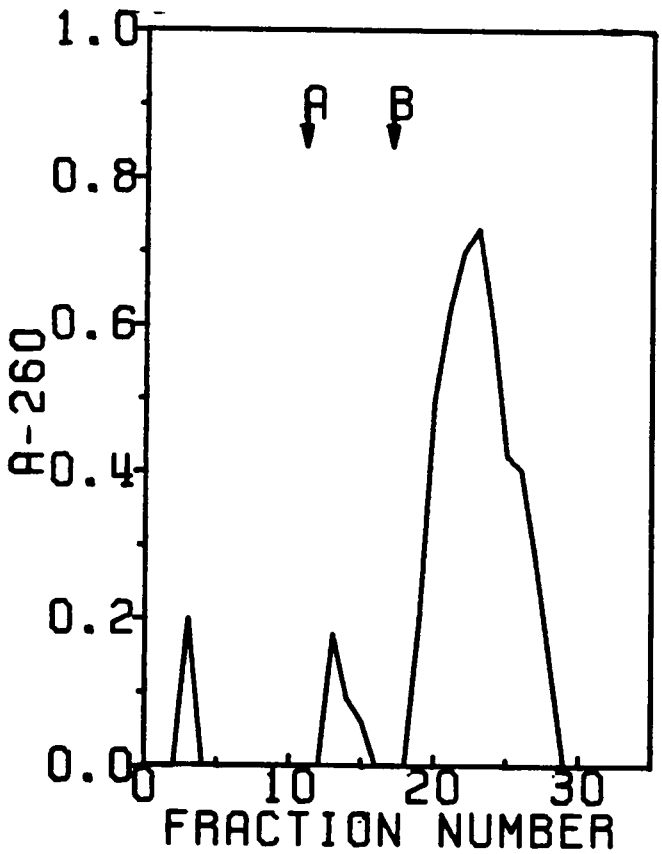

Figure 13

Fig. 14 Sucrose gradient analysis of RNA which does not bind to Poly(U) Sepharose. RNA which eluted from the column in the 0.5 M NaCl wash was concentrated by ethanol precipitation, resuspended and sedimented through a 5-20% sucrose gradient as described in the Methods. Sedimentation is from right to left.

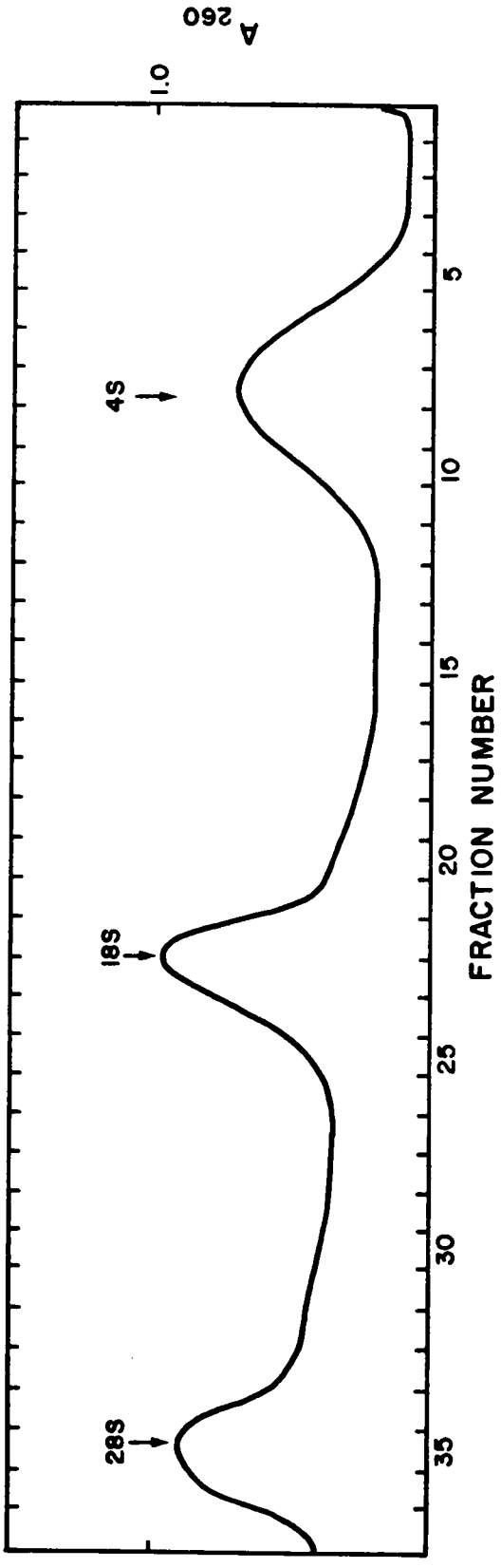

Figure 14

Fig. 15 Sucrose gradient analysis of myeloma RNA which bound twice to Poly(U) Sepharose. RNA which was eluted in the H_2O fraction of the second Poly(U) Sepharose colulmn was concentrated by ethanol precipitation, resuspended and sedimented on a 5-20% sucrose gradient as described in the Methods.

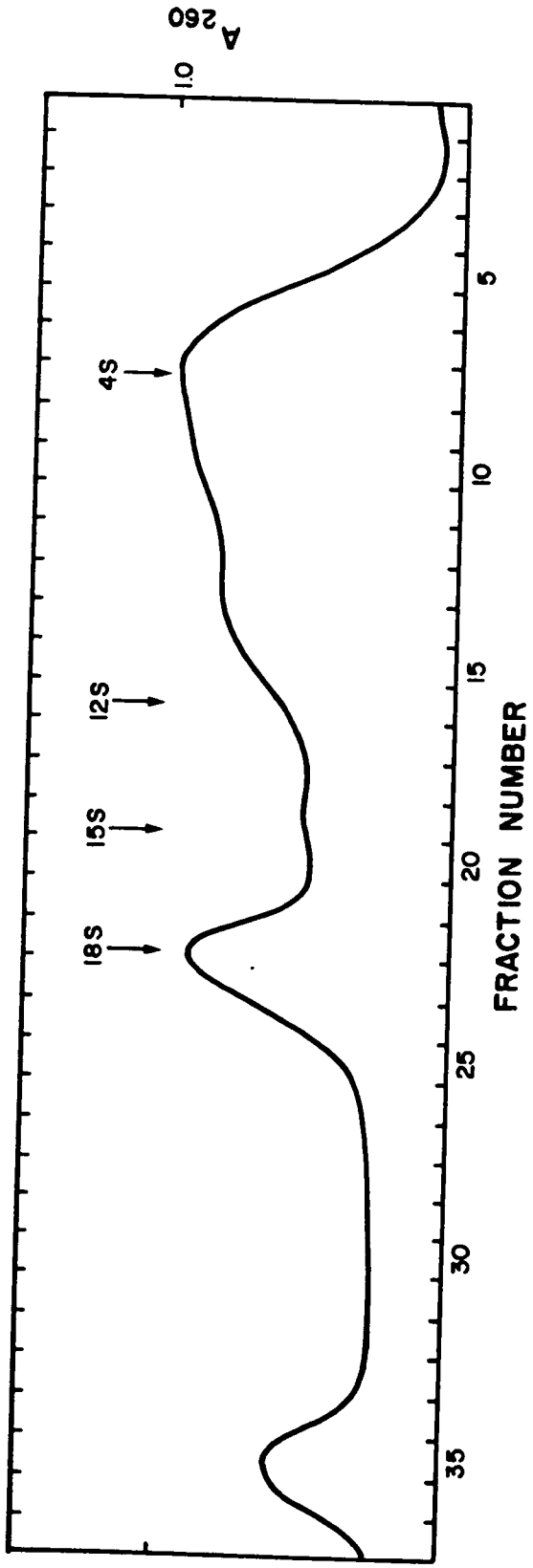

Figure 15

Figures 16 and 17 illustrate the profiles obtained when pooled fractions from the first sucrose gradients were sedimented again on 5-20% gradients. Fractions 8 and 9 of Figure 16 and fractions 6-8 of Figure 17 were pooled and sedimented a third time through 5-20% sucrose gradients. As indicated in Figures 18 and 19, relatively pure populations of 12 and 15S RNA were obtained by pooling fractions 11-13 of Figure 16 and fractions 13-15 of Figure 17, respectively. Approximately 1.8 ODS of 12S RNA and 0.75 ODS of 15S RNA were recovered.

Both 12 and 15S RNAs were subsequently translated in wheat germ cell-free system and the products of in vitro translation were analyzed on 13% SDS, polyacrylamide gels. Figure 20 illustrates the profile of labeled proteins synthesized from 12S. Although the 15S RNA did not direct the synthesis of a discrete protein, a prominent protein of approximately 26,000 daltons was synthesized from the 12S RNA. This protein, which is about 2,000 daltons larger than the mature myeloma light chain protein (Fig. 21), corresponds to light chain precursor. Burstein, et al. (1976) have described several κ light chain proteins which are initially synthesized as precursors; these polypeptides are 20-22 amino acids larger than the mature protein. The additional amino acids are cleaved at the cell membrane immediately before the protein is excreted from the cell.

Having demonstrated that the 12S RNA codes for a protein which corresponds to light chain precursor, a cDNA copy was synthesized and hybridized back to its template. The results of this experiment are illustrated in Figure 22. Surprisingly, only 35% of the cDNA was hybridized by a Cot of 0.6. If the RNA preparation were pure light chain mRNA

Fig. 16 Sucrose gradient analysis of 10-13S myeloma RNA obtained from the first sucrose gradient. RNA from the 12S region of the first sucrose gradient was pooled, ethanol precipitated, resuspended and sedimented on a second 5-20% sucrose gradient.

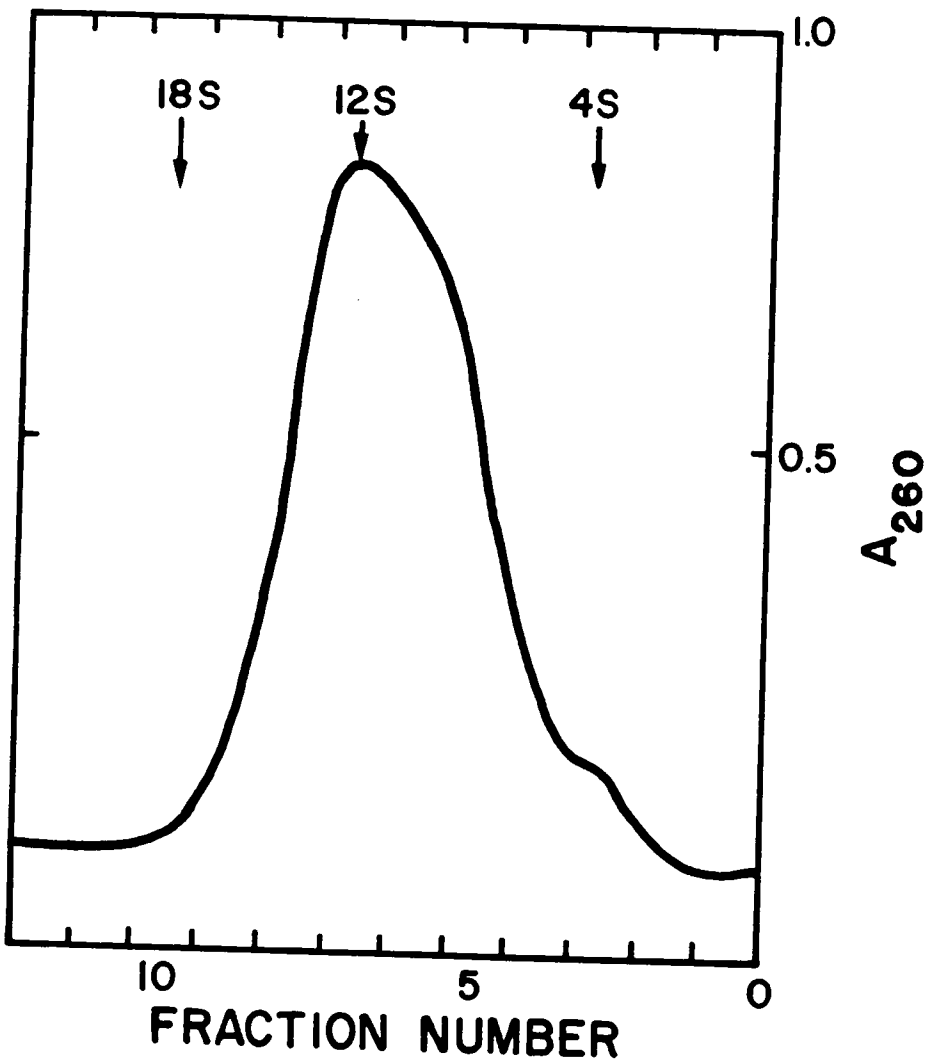

Figure 16

Fig. 17 Sucrose gradient analysis of 14-17S myeloma RNA obtained from the first sucrose gradient. RNA from the 15S region of the first sucrose gradient was pooled, ethanol precipitated, resuspended and sedimented on a second 5-20% sucrose gradient.

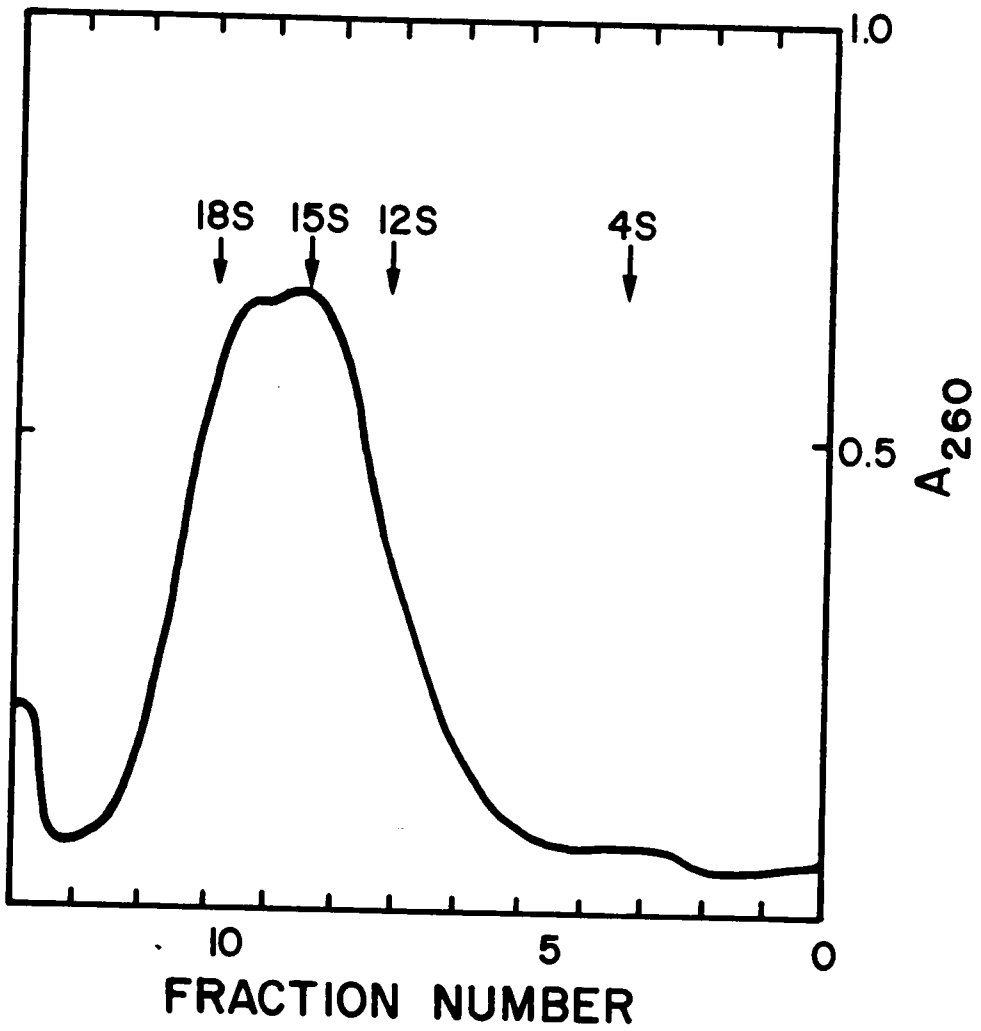

Figure 17

Fig. 18 Sucrose gradient analysis of 12S RNA of Figure 5. RNA in fractions 11-13 of the sucrose gradient depicted in Fig. 5 was pooled, ethanol precipitated, resuspended and sedimented on a third 5-20% sucrose gradient.

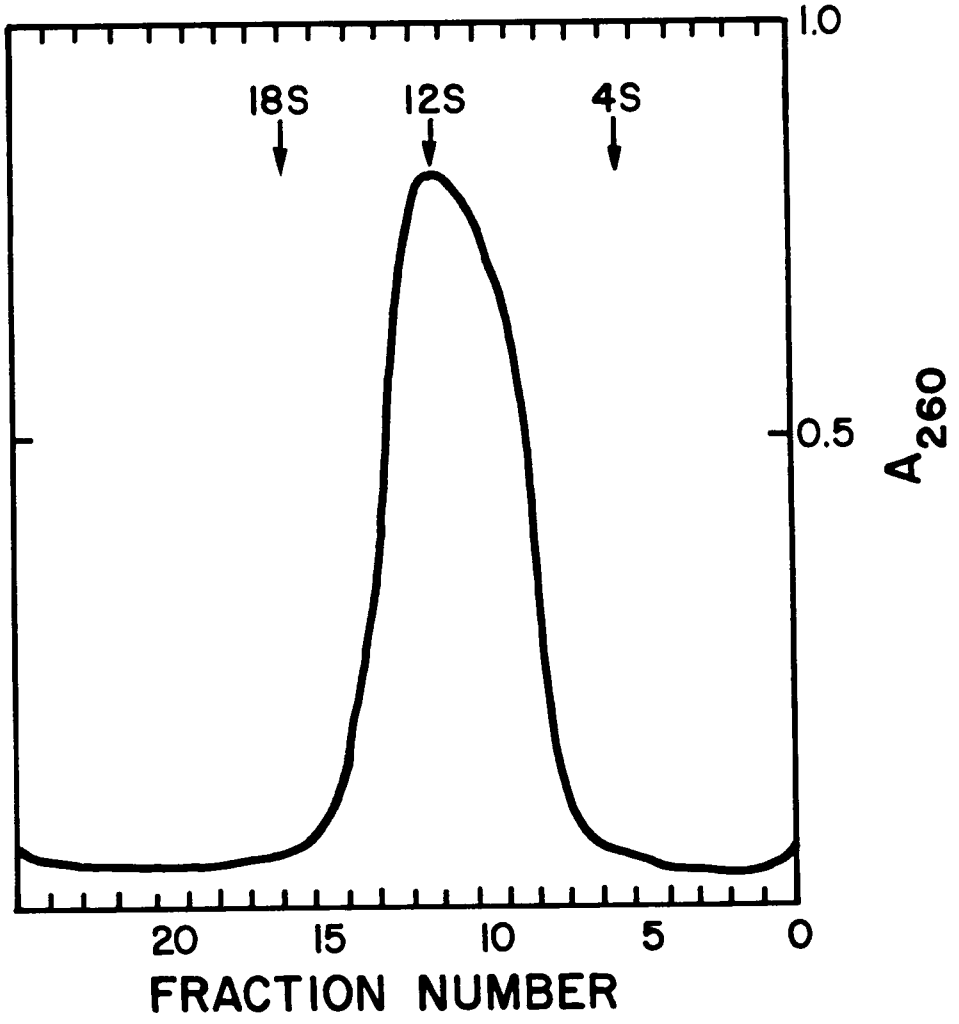

Figure 18

Fig. 19 Sucrose gradient analysis of 15S RNA of Fig. 6. RNA in fractions 13-15 of the sucrose gradient depicted in Fig. 6 was pooled, ethanol precipitated, resuspended and sedimented on a third 5-20% sucrose gradient.

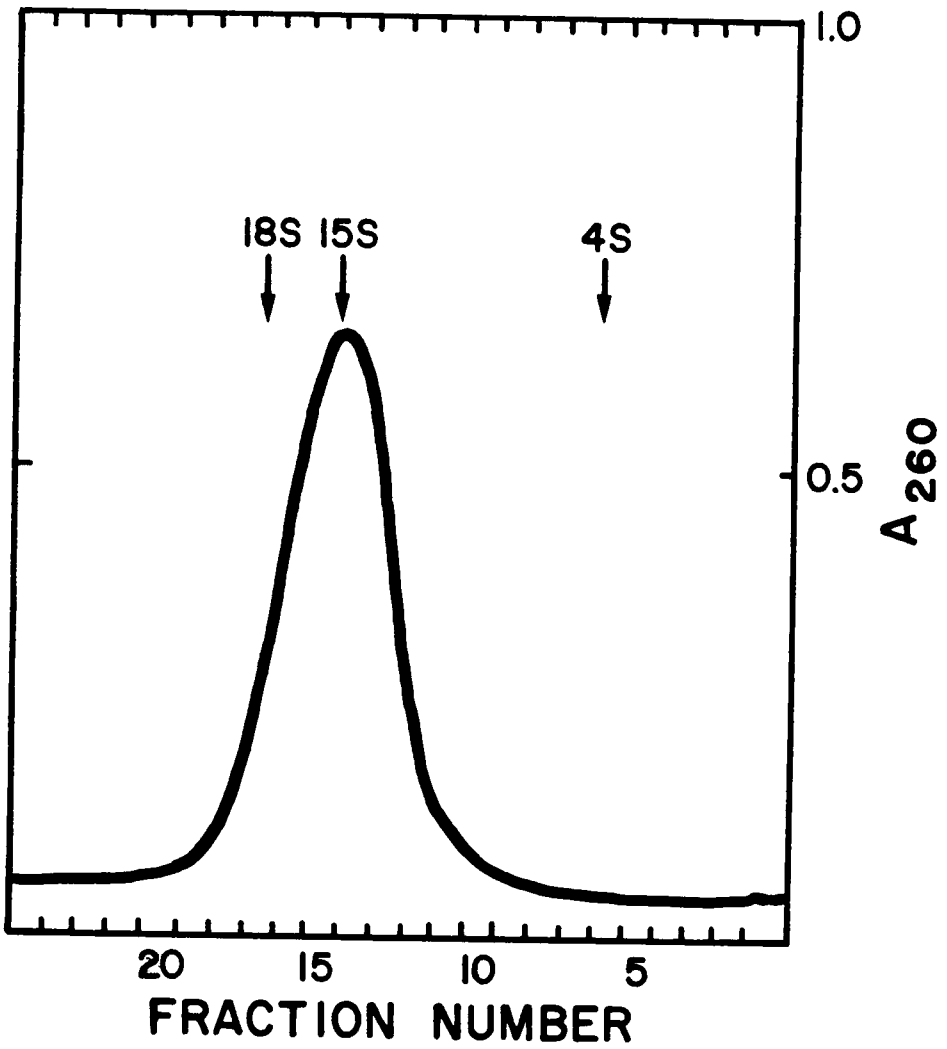

Figure 19

Fig. 20 SDS-polyacrylamide gel electrophoresis of proteins synthesized in vitro from myeloma 12S RNA. Twelve S RNA from the final sucrose gradient was pooled, ethanol precipitated, resuspended and translated in the wheat germ cell-free system. The identity of products synthesized in vitro was determined by electrophoresis of denatured proteins as described in the Methods.

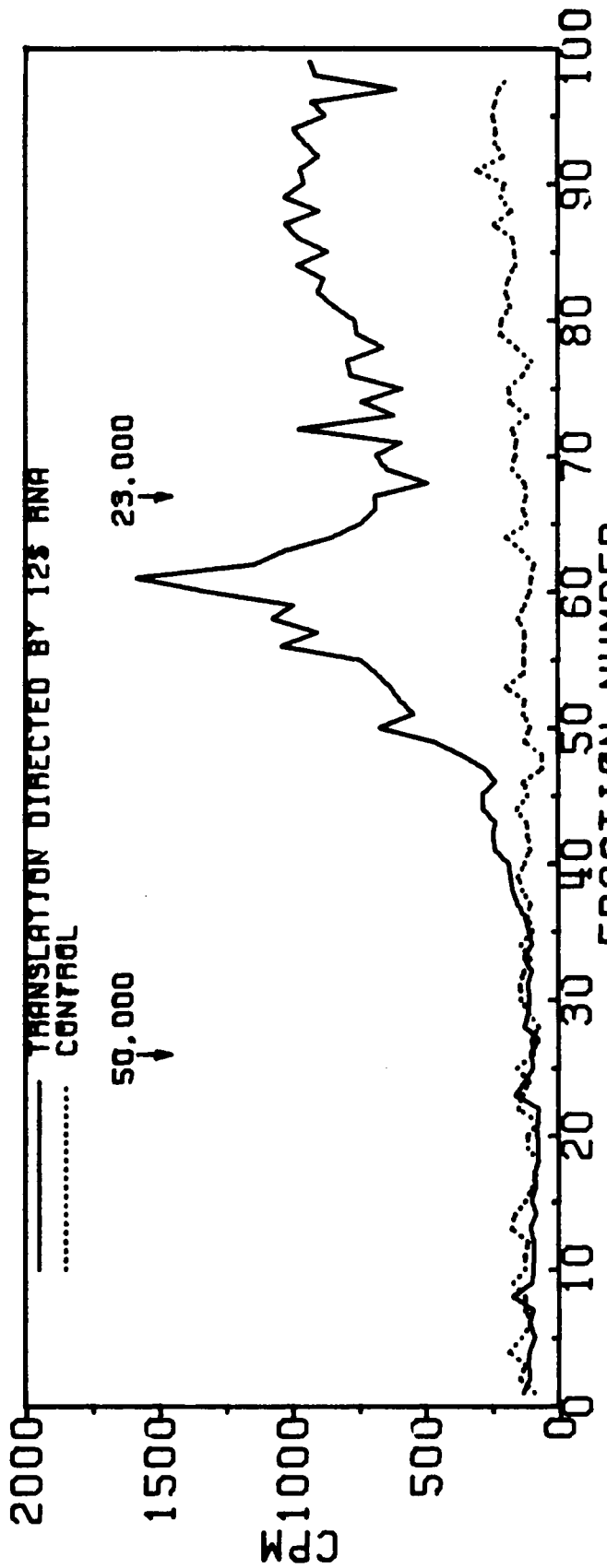
TRANSLATION DIRECTED BY 12S RNA
CONTROL
23.000
50.000
CPM
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 20

Fig. 21 SDS-polyacrylamide gel electrophoresis of myeloma light and heavy chain markers. Myeloma light and heavy chain proteins synthesized in vivo were separated by electrophoresis on SDS-polyacrylamide gels.

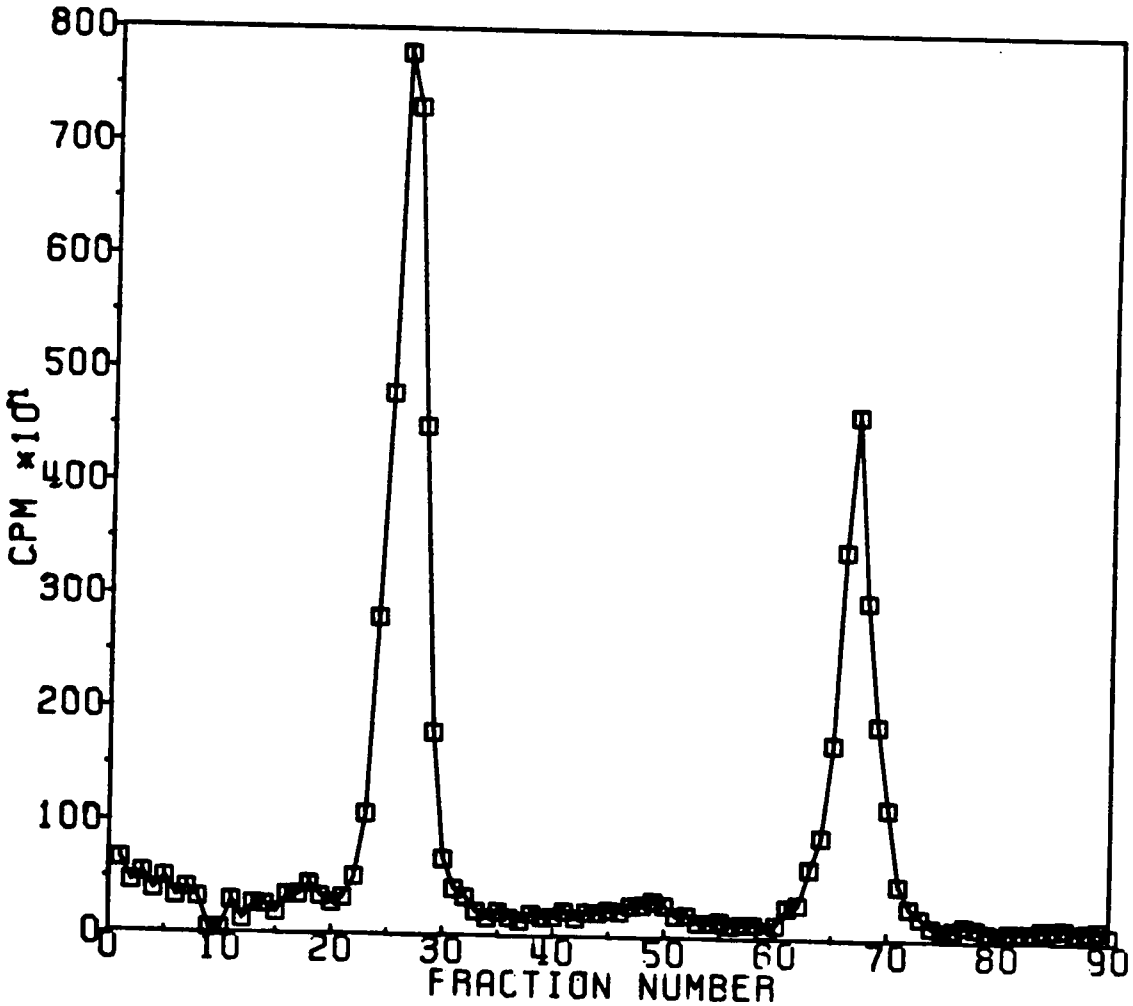
800
700
600
500
400
300
200
100
0
CPM x10
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 21

Fig. 22 Hybridization of myeloma cDNA to myeloma mRNA. Myeloma light chain cDNA synthesized from myeloma mRNA was hybridized back to its template as described in the Methods.

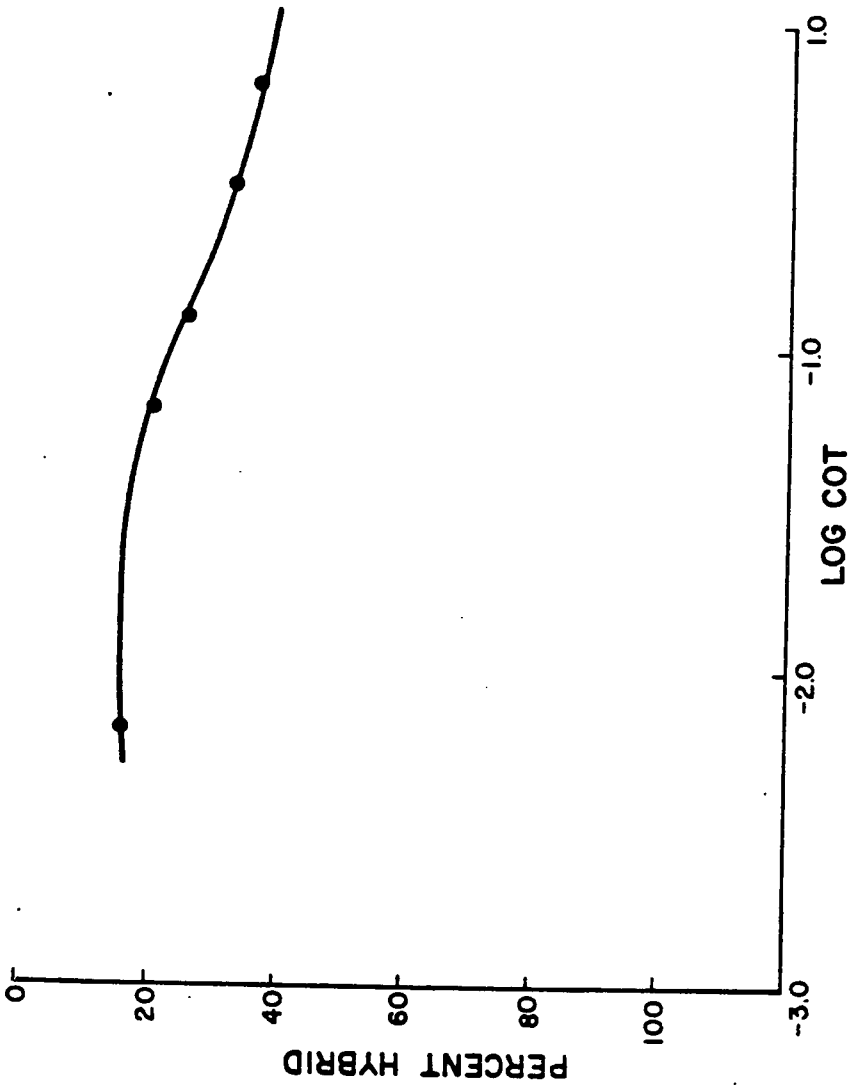

Figure 22

(approximately 1300 nucleotides in length) the reaction should be completed by a Cot of 0.1. This result indicates that the 12S RNA preparation contains hundreds of different RNAs and is, therefore, unsuitable as a template for the synthesis of light chain-specific cDNA.

One explanation for the in vitro synthesis of only one major polypeptide from an RNA preparation which contains hundred of different mRNAs is that only the light chain message is efficiently translated in the wheat germ cell-free system. Therefore, it is possible that this property of light chain RNA could be used in a purification scheme.

Consequently, membrane-bound polysomes were prepared from a new batch of tumors and polyadenylated RNA was isolated as described above. The preparation was then sedimented on 5-20% sucrose gradients for 5 hours longer than previous gradients in order to obtain better separation of individual RNA species. Aliquots from ten fractions between 4 and 18S were then assayed in the cell-free translation system. Figure 23 illustrates the results of this experiment. As indicated in the figure, 12S RNA was at least two times more efficient than the other RNAs in directing the synthesis of protein in vitro. This one fraction, which contained 20 µg of RNA, was ethanol precipitated and subsequently run on a second gradient. During the sedimentation of this RNA, the centrifuge failed, the tubes collapsed and all of the sample was lost.

Although the results depicted in Fig. 23 suggested that a relatively pure mRNA might be obtained by the sucrose gradient-cell-free translation procedure, another attempt at isolating the myeloma light chain message was

Fig. 23 Cell-free translation of sucrose gradient fractions of myeloma mRNA. RNA which bound twice to Poly(U) Sepharose was sedimented on a 5-20% sucrose gradient. Aliquots of individual fractions were translated in the wheat germ translation system.

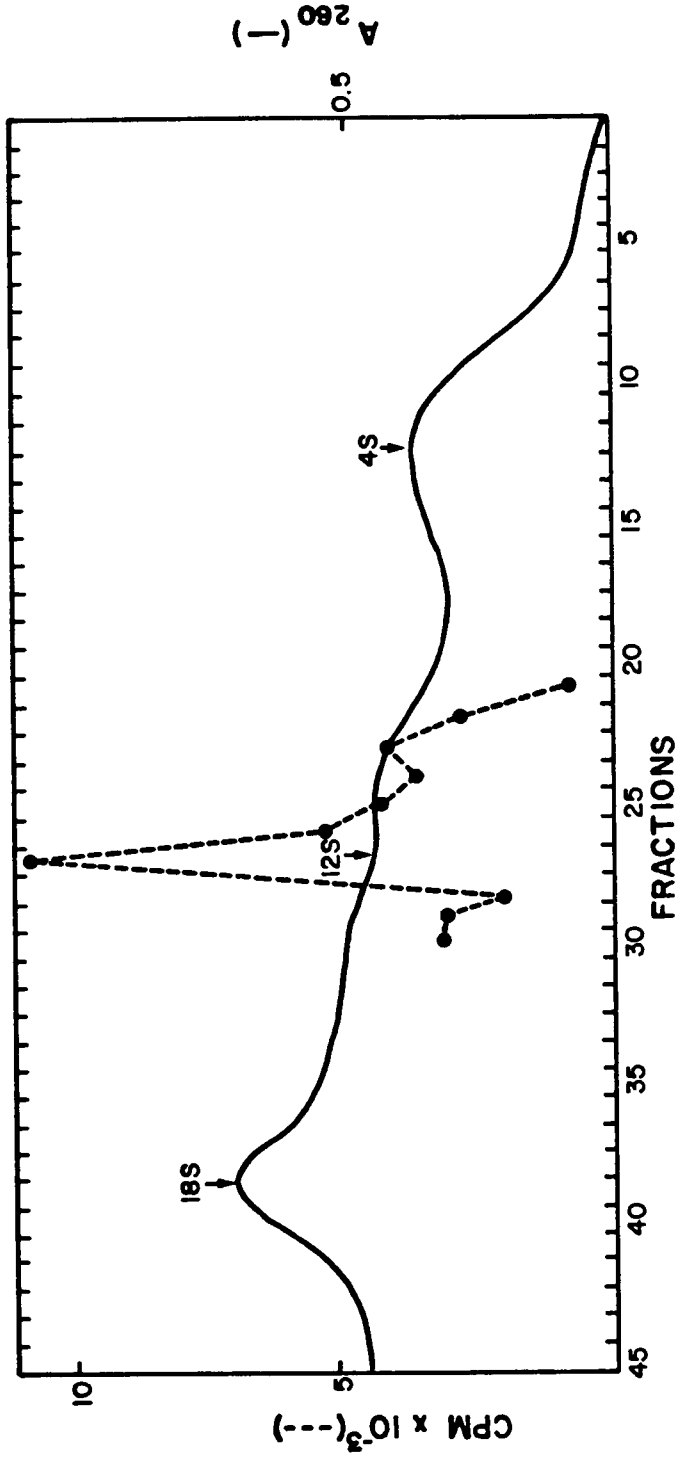
A_{260} (—)
0.5
4S
12S
18S
FRACTIONS
CPM x 10^{-3} (---)
10
5
5
10
15
20
25
30
35
40
45

Figure 23

postponed when the cell line was lost. New cells were obtained; however, by this time an even more serious problem which involved the project as a whole had arisen. Zasloff and Felsenfeld published data (Zasloff and Felsenfeld, 1977; Zasloff and Felsenfeld, 1978) which demonstrated that artifactual results are obtained in in vitro transcription experiments with E. coli polymerase. Artifacts result from the propensity of E. coli polymerase to use endogenous RNA instead of DNA as a template in these reactions. Although the transcription experiments proposed in Part II, Introduction were to be performed with wheat germ polymerase, preliminary experiments indicated that this polymerase might also use RNA as a template. Obviously, without the in vitro chromatin transcription assay, the project as diagrammed was impossible.

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VITA

Tim Mendler Townes was born in Birmingham, Alabama on May 19, 1951. His family moved to Knoxville, Tennessee in 1955 and he attended elementary schools in that city. He graduated from Bearden High School in 1969 and then graduated from the University of Tennessee in June, 1973 with a Bachelor of Science degree in Zoology. The next two and one-half years were spent in study for a Master's degree in Zoology and this degree was awarded in December, 1975.

He then entered the Microbiology Department at the same university and studied for a Doctor of Philosophy degree, which was awarded in August, 1980. In January, 1980, he began postdoctoral training in the laboratory of Dr. Jerry Lingrel at The University of Cincinnati.

He is married to the former Jeannette B. Gilchrist of Woodbridge, Virginia.