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I am submitting herewith a dissertation written by Dennis Roop entitled "A Comparison of Methods for Fractionating Chromatin into Transcriptionally Active and Inactive Components." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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A COMPARISON OF METHODS FOR FRACTIONATING CHROMATIN INTO TRANSCRIPTIONALLY ACTIVE AND INACTIVE COMPONENTS

A Dissertation

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ABSTRACT

Three methods for fractionating chromatin into transcriptionally active and inactive components (chromatography on ECTHAM cellulose, differential sedimentation on sucrose gradients and DNase II digestion followed by differential precipitation with $MgCl_2$) are described in Chapter I. The components obtained by fractionating chromatin with these procedures are characterized by criteria routinely employed (template activity assayed with E. coli RNA polymerase, thermal stability and localization of labeled nascent RNA). Subsequent chapters describe more stringent assays for evaluating chromatin fractionation methods and the preparation of reagents necessary for performing these assays.

The isolation and translation of mouse globin mRNA are described in Chapter II. The synthesis of DNA complementary to globin mRNA is also described in this chapter. Chapter III describes an attempt to purify immunoglobulin light chain mRNA from myeloma cells (MOPC-31C). The translation of the light chain mRNA in the wheat germ and Krebs ascites cell-free systems and the synthesis of light chain cDNA are also discussed in this chapter. The susceptibility of cDNA labeled with deoxy [8-^{3}H] guanosine 5'-triphosphate to tritium exchange under conditions used for nucleic acid hybridization studies and methods to avoid this problem are discussed in Chapter IV.

Mouse fetal liver chromatin, which is active in the transcription of globin genes, was fractionated by ECTHAM-cellulose chromatography,

differential sedimentation on sucrose gradients and digestion with DNase II. The distribution of globin genes in DNA extracted from these fractions was determined by hybridization analysis with globin cDNA (Chapter V). Components obtained by fractionating mouse fetal liver chromatin by ECTHAM-cellulose chromatography and DNase II digestion were transcribed by wheat germ RNA polymerase II. The presence of globin mRNA sequences in these transcripts was determined by hybridization analysis with globin cDNA (Chapter VI).

The results obtained in the gene distribution experiments reveal no difference in globin gene content in fractions obtained by the methods employed. The results obtained by assaying fractions for the ability to support the synthesis of globin genes with wheat germ RNA polymerase II, however, indicate that fractionation into active and inactive components has occurred and that chromatography on ECTHAM cellulose is the method of choice for fractionating chromatin. These results are summarized in Chapter VII.

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LIST OF ABBREVIATIONS

ATP = Adenosine Triphosphate

CTP = Cytidine Triphosphate

GTP = Guanosine Triphosphate

UTP = Uridine Triphosphate

dATP = Deoxyadenosine Triphosphate

dCTP Deoxycytidine Triphosphate

dGTP = Deosyguanosine Triphosphate

dTTP = Deosythymidine Triphosphate

AMV = Avain Myeloblastosis Virus

cDNA = Complementary DNA

mRNA = Messenger RNA

SDS = Sodium Dodecyl Sulfate

BBOT = 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene

PPO = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

TCA = Trichloroacetic Acid

BMV = Brome Mosaic Virus

T_m = Median Thermal Dissociation Temperature

EDTA = Ethylenediaminetetraacetate Disodium Salt

BSA = Bovine Serum Albumin

CMC = Carboxymethyl Cellulose

poly(U) = Poly (Uridylic Acid)

PMSF = Phenylmethylsulfonylfluoride

HgUTP = Mecurated Uridine Triphosphate

DNase I = Deoxyribonuclease I

DNase II = Deoxyribonuclease II

E. coli = *Escherichia coli*

CHAPTER I

METHODS FOR CHROMATIN FRACTIONATION

Introduction

Several methods have been described for fractionating euchromatin ("transcriptionally active") and heterochromatin ("transcriptionally inactive"). The first step in all of these procedures consists of fragmenting the chromatin. This is usually accomplished by sonication, mechanical shear or enzyme digestion. All the steps following fragmentation are based on the assumption that the more diffuse euchromatin components have sufficiently different physicochemical properties from the more condensed heterochromatic material to permit separation by physical means.

The first attempt to fractionate chromatin used differential centrifugation (Frenster et al., 1963). Similar methods have been employed by others (Chalkley and Jensen, 1968; Duerksen and McCarthy, 1971; Murphy et al., 1973; Berkowitz and Doty, 1975). Other methods include chromatography on ECTHAM cellulose (Reeck et al., 1972; Howk et al., 1975), DNase II digestion followed by differential precipitation (Gottesfeld et al., 1974), gel-exclusion chromatography (Janowski et al., 1972), and density gradient centrifugation (Rickwood et al., 1974; Willetts and Wells, 1976).

Three of these methods, chromatography on ECTHAM cellulose, differential sedimentation on sucrose gradients, and DNase II digestion

have been chosen for comparison. These procedures have been evaluated using criteria routinely employed by others (template activity assayed with E. coli RNA polymerase, thermal stability and localization of labeled nascent RNA) and by more stringent criteria discussed in the following chapters.

Materials and Methods

Animals. Male BALB/c (Cum) mice, 14 wk-old, were obtained from Cumberland View Farms, Clinton, TN.

Myelomas. Myeloma cell line MOPC-31C, originally established by M. Potter, was obtained from W. K. Yang, Oak Ridge National Laboratory. The line was maintained by serial transplantation as a subcutaneous solid tumor in 14 wk-old mice.

Reagents. The following reagents were used: DNase II (Worthington HDAC); [3_H] UTP 16 Ci/mmol (Schwarz/Mann); Escherichia coli RNA polymerase (Miles); RNase-free sucrose (Schwarz-Mann); [3_H] uridine 27.8 Ci/mmol (New England Nuclear).

Isolation of Nuclei

Nuclei were isolated by a modification of the procedure described by Hymer and Kuff (1964). Details are given in Appendix A.

Preparation of Chromatin

Chromatin was prepared from nuclei essentially as described by Shaw and Huang (1970). Details are given in Appendix A.

Sonication of Chromatin

This procedure is described in Appendix A.

Fractionation of Chromatin by ECTHAM-cellulose Chromatography

Sonicated chromatin was fractionated on ECTHAM cellulose as described by Reeck et al. (1972). Details are given in Appendix A.

Fractionation of Chromatin on Sucrose Gradients

Sonicated chromatin was fractionated on 0.17-1.7M sucrose gradients as described by Murphy et al. (1973). Details are given in Appendix A.

Fractionation of Chromatin with DNase II

Chromatin was fractionated by DNase II digestion followed by differential precipitation with $MgCl_2$ as described by Gottesfeld et al. (1974). Details are given in Appendix A.

Assay of Template Activity of Chromatin with E. coli RNA Polymerase

This procedure is described in Appendix A.

Thermal Analysis of Chromatin

This procedure is described in Appendix A.

Labeling of Nascent RNA

This procedure is described in Appendix A.

Results

ECTHAM-cellulose Fractionation

The elution profile of sonicated adult liver chromatin from ECTHAM cellulose is shown in Fig. 1-1. As the basic solution (0.01M Tris - 0.01M NaCl) titrates the binding groups on the column, chromatin is gradually eluted. The weakest bound particles are eluted in the fractions where the pH rises. When fractions across the profile are assayed for template capacity with E. coli RNA polymerase, the late eluting fractions are observed to have the highest template activity. This is consistent with the report of Simpson (1974a).

The following fractions were pooled from this column: 71-89 (designated early), 90-111 (designated middle) and 112-130 (designated late). The template activity of these pooled fractions is shown in Table I-1. The thermal stability of these fractions was also examined. The early fraction has the highest Tm and the late fraction the lowest. This is in agreement with data reported by Reeck et al. (1972).

The elution profile from ECTHAM cellulose of myeloma chromatin which was prepared from cells briefly labeled with [$^{3}_{H}$] uridine is shown in Fig. 1-2. The labeled nascent RNA elutes in the same position as the late eluting fractions. This is consistent with the finding of Simpson (1974b).

Sucrose Gradient Fractionation

Sonicated myeloma chromatin (labeled with [$^{3}_{H}$] uridine), was fractionated on 0.17M-1.7M sucrose gradients (Fig. 1-3). The labeled

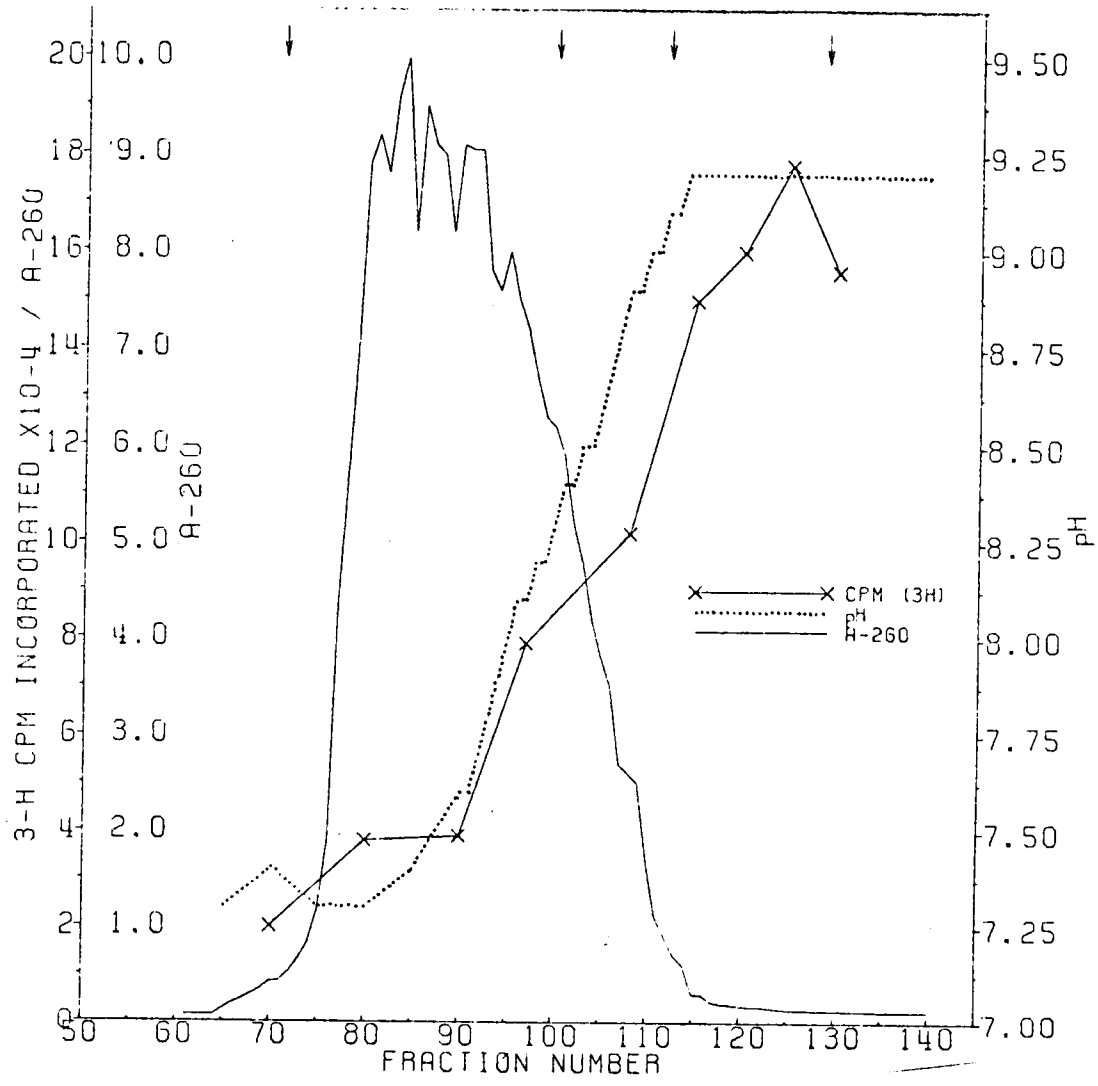


Fig. 1-1. ECTHAM-cellulose fractionation of adult mouse liver chromatin (assayed for template activity with E. coli RNA polymerase).

Table I-1. Characteristics of fractionated chromatin

Chromatin Source	Template Activity of Chromatin (^{3}H UMP cpm incorporated X $10^{-3}/A_{260}$)[a]	T_m Value of Chromatin[b]
ECTHAM Early	26	77.5
" Middle	95	75.3
" Late	156	72.9
Sucrose A	188	73.0
" B	130	75.6
" C	45	77.0
DNase II PI	70	77.9
" PII	103	76.3
" SII	174	70.6
Chromatin (unfractionated)	37	77.2
Chromatin (unfractionated, sonicated)	77	76.1
Calf Thymus DNA	411	-

[a]Assayed with E. coli RNA polymerase as described in Appendix A.

[b]T_m values were determined as described in Appendix A.

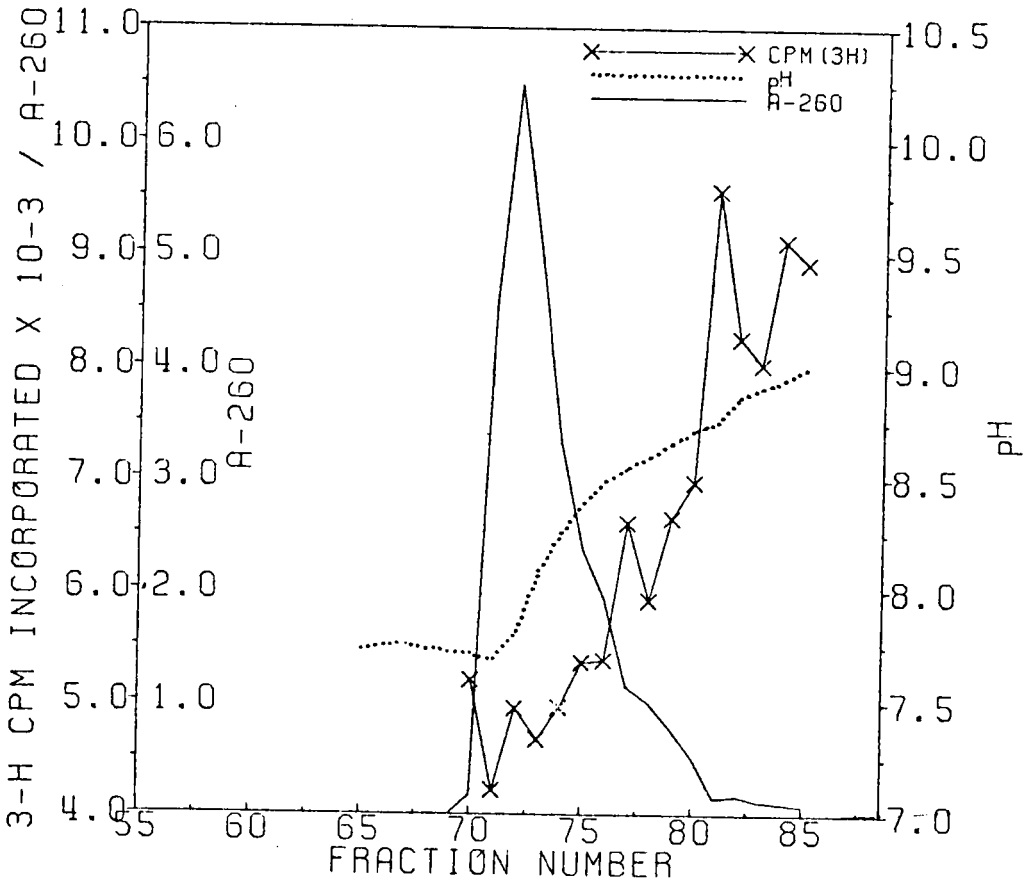


Fig. 1-2. Distribution of labeled nascent RNA associated with myeloma chromatin fractionated on ECTHAM cellulose.

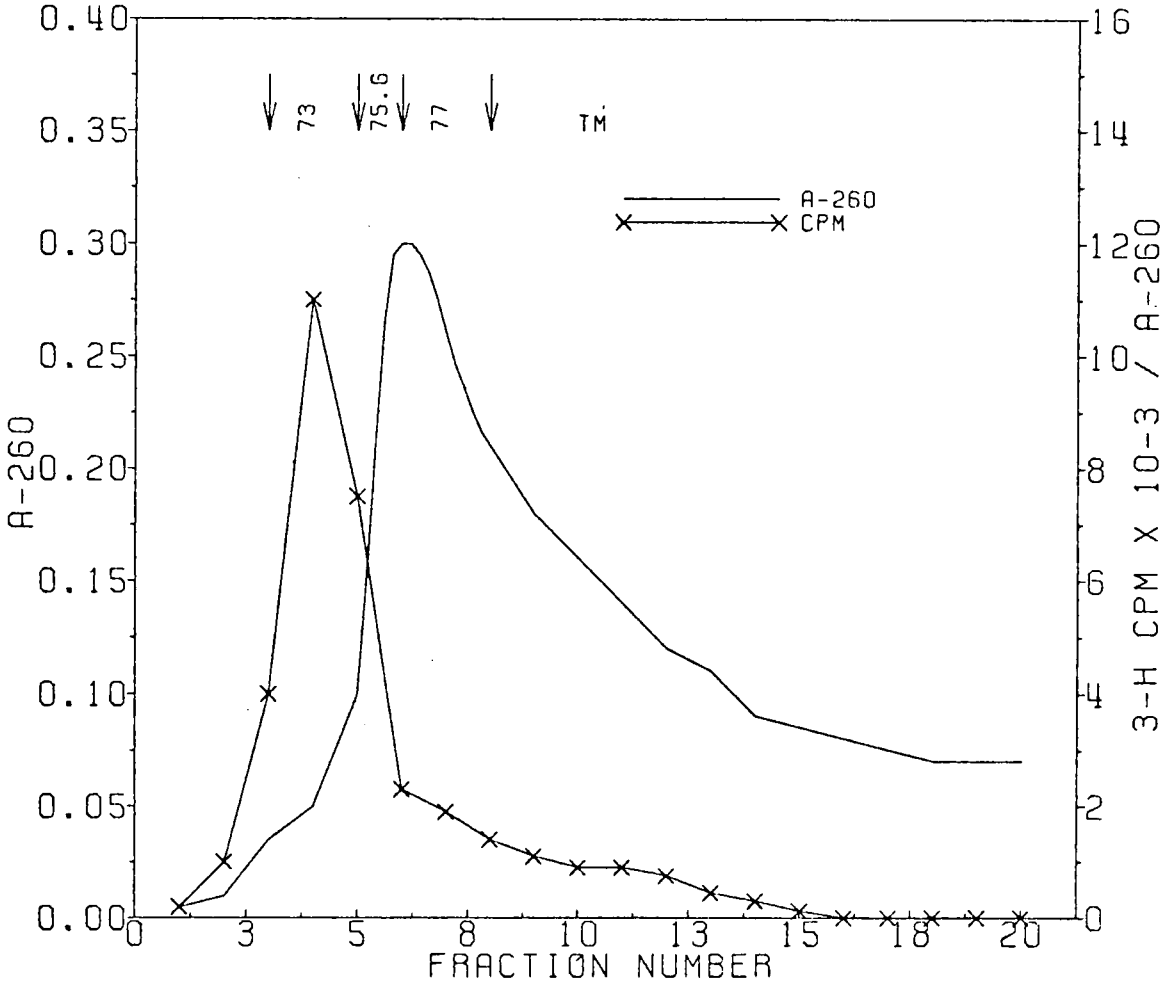


Fig. 1-3. Distribution of labeled nascent RNA associated with myeloma chromatin fractionated on 0.17 - 1.7M sucrose gradients. Sedimentation was from left to right and centrifugation was as described in Materials and Methods. T_m values are given for the indicated fractions.

nascent RNA sediments to the same position in the gradient as the slowly sedimenting fractions of chromatin. This is consistent with other published reports (Murphy et al., 1973; Berkowitz and Doty, 1975). The thermal stability of the following fractions was examined: A (4 and 5), B (6), and C (7 and 8). The Tm values for these fractions are shown in Fig. 1-3 and Table I-1. The slowly sedimenting fractions have the lowest Tm. Similar results have been reported by others (Chalkley and Jensen, 1968; Duerksen and McCarthy, 1971; Berkowitz and Doty, 1975). Chromatin from myeloma cells, which were not incubated with [$^{3}_{H}$] uridine, was fractionated in a similar manner for determination of the template activity with E. coli RNA polymerase. The slowly sedimenting fraction has the highest template activity (Table I-1). This is consistent with the reports of Murphy et al. (1973) and Berkowitz and Doty (1975).

DNase II Fractionation

DNase II is an endonuclease which catalyzes the hydrolysis of deoxyribonucleotide linkages, yielding products ending in 3'-phosphates (Laskowski, 1959). This enzyme differs from DNase I in respect to the substrate linkage hydrolyzed, the pH optimum and susceptibility to magnesium ion inhibition above 1 $+10^{-3}$ M (Benardi and Griffe, 1964). The scheme used in fractionating chromatin with DNase II is shown in Fig. 1-4. The association of labeled nascent RNA with DNase II fractions, obtained from treatment of myeloma chromating (labeled with $^{3}_{H}$ uridine), is shown in Table I-2. The SII (active) fraction contains approximately 46% of the total radioactivity. Bonner et al. (1973)

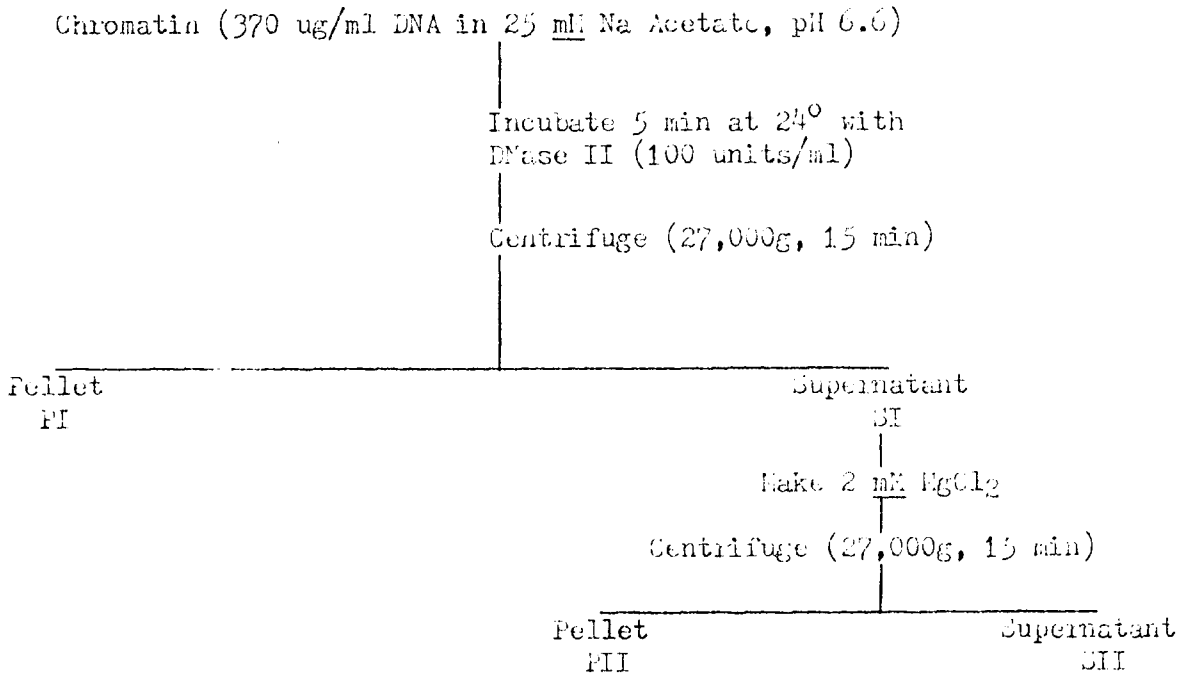


Fig. 1-4. Scheme for fractionating chromatin with DNase II.

Table I-2. Association of labeled nascent RNA with DNase II fractionated chromatin.

Fraction	Percent of total chromatin present in fraction[a]	Percent of total radio-activity in fraction	^{3}H CPM X $10^{-3}/A_{260}$
PI	76.3	51.5	3
PII	11.1	2.9	1.2
SII	12.6	45.6	16.2

[a]Absorbance at 260 nm was measured in 0.9 N NaOH.

report finding 70% of the total radioactivity in the SII fraction. The template activity and thermal stability of DNase II fractions are shown in Table I-1 (p. 6). The SII fraction has the highest template activity when assayed with E. coli RNA polymerase; this is consistent with findings of Bonner at al. (1973). The Tm of the SII fraction is substantially lower than that observed for the PI fraction or unfractionated chromatin. This appears to be partially due to digestion by DNase II since the percent hyperchromicity is low for this fraction, and DNA extracted from this chromatin has a low Tm and percent hyperchromicity compared to DNA from unfractionated chromatin (data shown in Chapter V, p. 69).

The following observation was made during experiments with the DNase II fractionation procedure. Under the conditions used for DNase II digestion, a substantial amount of chromatin is digested in the absence of DNase II (Table I-3). It was of interest to compare the SII fractions obtained with and without DNase II digestion. SII fractions obtained under both conditions were placed on 5-20% sucrose gradients, containing 0.01M Tris (pH 8.0), and centrifuged in a SW27.1 rotor for 13 hr at 27,000 rpm (Fig. 1-5). Profiles observed for both fractions are essentially identical. The profile of the SII fraction obtained by DNase II digestion is very similar to that observed by Gottesfeld et al. (1975). The digestion observed in the absence of DNase II, presumably due to endogenous nuclease in the chromatin, can be substantially reduced by dialyzing the chromatin against 1mM EDTA (Table I-3). The effect of the endogenous nuclease during the DNase II fractionation procedure is

Table I-3. The effect of incubating chromatin with and without DNase II

Treatment	Fraction	Percent of total chromatin present in fraction
Chromatin (dialyzed vs. 25mM Na Acetate, pH 6.6) incubated with DNase II	PI	74.1
	PII	15.2
	SII	10.7
Chromatin (dialyzed vs. 25mM Na Acetate, pH 6.6) incubated without DNase II	PI	75.4
	PII	17.5
	SII	7.1
Chromatin (dialyzed vs. 5mM Tris, pH 7.0) incubated with DNase II	PI	70.3
	PII	18.6
	SII	11.1
Chromatin (dialyzed vs. 5mM Tris, pH 7.0) incubated without DNase II	PI	76.5
	PII	16.3
	SII	7.2
Chromatin (dialyzed vs. 5mM Tris, pH 7.0; 1mM EDTA) incubated without DNase II	PI	98.2
	PII	.5
	SII	1.3

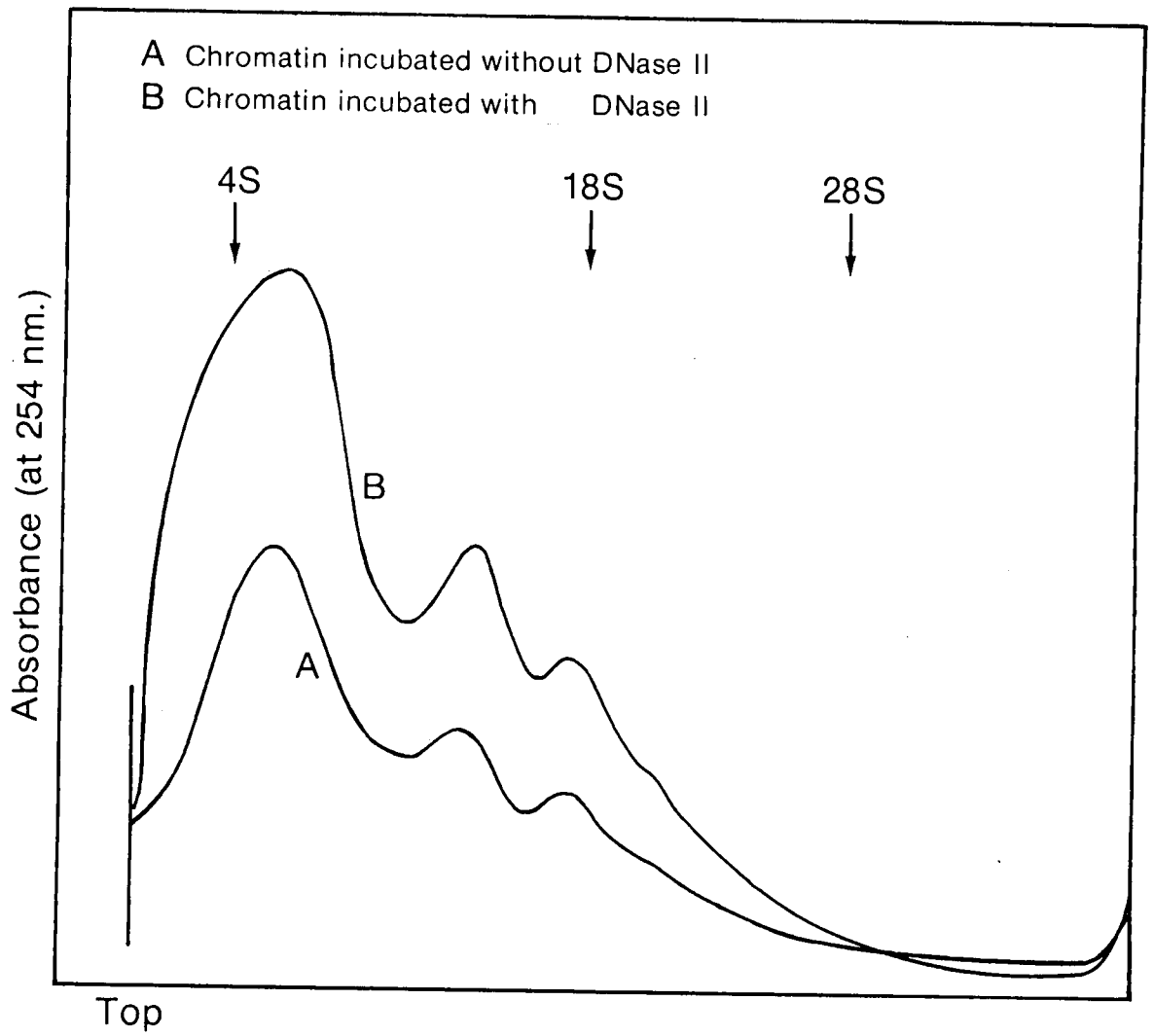


Fig. 1-5. Sucrose gradient analysis of SII chromatin obtained by incubation in the presence (B) or absence (A) of DNase II. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

not known. Recently, Paul and Duerksen (1976) have described a fractionation procedure which is based on a Mg/Ca-dependent autodigestion of chromatin.

Discussion

When chromatin fractions obtained by ECTHAM-cellulose chromatography, differential sedimentation or DNase II digestion are judged by the following criteria: template activity assayed with E. coli RNA polymerase, thermal stability and association of labeled nascent RNA, fractions are observed which appear to be transcriptionally active components of chromatin. Although the physical characteristics of increased template activity and lower thermal stability are consistent with this chromatin being transcriptionally active in vivo, similar results would be obtained if the chromatin was damaged. The criterion of localization of labeled nascent RNA with the transcriptionally active component has recently been questioned (Seidman and Cole, 1977), since the RNA may bind to sites generated during the fractionation process.

It was of interest to design experiments that would provide a more stringent analysis of procedures used to fractionate chromatin. The following approach was taken. Chromatin actively synthesizing globin mRNA was obtained from mouse fetal livers and fractionated by methods discussed. One experiment consisted of determining the distribution of globin genes in the various fractions using globin cDNA. The other involved transcribing fractions with wheat germ RNA polymerase II and assaying these transcripts for the presence of globin mRNA sequences.

Initially, the use of the immunoglobin light chain genes as an inactive control was planned. The preparation of the reagents necessary to perform these experiments and the performance of the experiments are described in the following chapters.

CHAPTER II

THE ISOLATION OF GLOBIN mRNA AND THE SYNTHESIS OF GLOBIN cDNA

Introduction

Membrane-free polysomes were isolated from mice in which reticulocytosis had been induced by treatment with phenylhydrazine. RNA was extracted from the polysomes, and the poly(A)-containing fraction was isolated by chromatography on poly(U)-Sepharose. The poly(A)-containing RNA was fractionated on successive sucrose gradients and a component with a sedimentation coefficient of 9-10S was obtained. The ability of this RNA to direct the synthesis of mouse globin in a cell-free protein synthesizing system was determined. This RNA was then used as a template for the synthesis of cDNA with AMV reverse transcriptase. The size of the cDNA was determined by sucrose gradient sedimentation. The specificity of the cDNA was assayed by hybridization to mouse globin mRNA, mouse fetal liver DNA and mouse myeloma RNA.

Materials and Methods

Animals. Male BALB/c (Cum) mice, 14 wk-old, were obtained from Cumberland View Farms, Clinton, TN.

Reagents. The following reagents were used: phenylhydrazine hydrochloride (Sigma); poly(U)-Sepharose (Pharmacia); RNase-free sucrose (Schwarz-Mann); S1 nuclease (Sigma); oligo$(dT)_{10}$ (Collaborative Research, Inc.); dATP, dGTP and dTTP (Sigma); [$^{3}_{H}$]-leucine, 60Ci/mmole (New

England Nuclear); [$^{3}_{H}$]dCTP, 20Ci/mmole (Amersham/Searle); AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.); wheat germ (donated by Dixie Portland Flour Mills, Chattanooga, TN); Proteinase K (EM Laboratories, Inc.).

Isolation of Polysomes

Reticulocytosis was induced in BALB/c mice by six daily injections of 0.1 ml of a neutralized aqueous solution of 0.9% phenylhydrazine-HCl (Gorski et al., 1974). On the seventh day the mice were injected with 0.1 ml heparin and blood was collected into ice-cold isotonic medium (0.14M NaCl, 5mM $MgCl_2$, 5mM KCl) containing heparin. The cells were washed three times with isotonic medium and then lysed by the addition of two volumes of hypotonic medium (10mM Tris-HCl, pH 7.4; 2mM $MgCl_2$; 10mM KCl) and vigorous shaking for two min in the cold. The NaCl concentration was then adjusted to 0.15M (Lebleu, 1974). The lysate was centrifuged at 12,000g for 10 min and the supernatant was layered over a 7 ml cushion of 36% sucrose containing 0.1M Tris-HCl, pH 7.4, 15 mM KCl, and 5 mM magnesium acetate and centrifuged at 35,000rpm for 3 hr in a 42.1 rotor (Evans and Lingrel, 1969).

Extraction of RNA

RNA was extracted from polysomes by a modification of the procedure described by Brawerman (1974). Polysomes obtained from 60 ml of blood were suspended in 20 ml of 0.1% SDS containing 50ug/ml of Proteinase K, and held at 0° C for 10 min. The suspension was diluted to a concentration of 20 A_{260} units/ml and made 0.1M Tris=HCl, pH 9.0 and 1.0% SDS. An

equal volume of aqueous phenol (redistilled phenol diluted with distilled H_2O to a concentration of 80% (v/v)) was added and the mixture was stirred vigorously for 10 min. This was followed by centrifugation at 12,000g for 10 min. The aqueous layer was removed and the nonaqueous residue (phenol phase plus gel interphase) was reextracted with 0.5 volume of 0.1M Tris, pH 9.0 and 1% SDS as before. The aqueous phases were pooled and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1), again by stirring and separation by centrifugation as above. This step was repeated two more times. The aqueous phase was then made 2% CH_3COOK (pH 5.5), precipitated by the addition of 2.5 volumes of 95% ethanol and stored at -20C overnight. The RNA was collected by centrifugation at 10,000g for 30 min at -20C. The RNA pellet was washed twice with ethanol - 0.2M NaCl (2:1) to remove residual phenol and dissolved in distilled water for fractionation on poly(U)-Sepharose.

Poly(U)-Sepharose Chromatography

This procedure is described in Appendix A.

Sucrose Gradient Centrifugation

The RNA obtained in the water fraction of the second poly(U)-Sepharose column was fractionated on sucrose gradients as described by Honjo et al. (1974). Details are given in Appendix A.

Wheat Germ Cell-Free Protein Synthesizing System

The preparation of the wheat germ extract was as described by Roberts and Patterson (1973), except that the S-30 fraction was preincubated for 30 min at 30°. Details are given in Appendix A.

Analysis of Cell-Free Products

Fifty ul of the cell-free reaction mixture were added to 250 ul of dialysis buffer (50mM 2-mercaptoethanol, 0.1M $NaPO_4$, pH 7.0) and dialysed for 10-12 hr against two one liter volumes of the same buffer. The sample was added to 1-2 mg of mouse (BALB/c) hemolysate 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).

Synthesis of cDNA

The synthesis of globin cDNA was essentially as described by Kacian and Myers (1976a). Details are given in Appendix A.

Size Determination of cDNA

This procedure is described in Appendix A.

Hybridization of cDNA to Template RNA

Hybridizations were performed in 10 ul volumes in 40 ul glass capillaries (Microcaps), treated as described by Wilson et al. (1975). Each reaction contained 10mM Tris, pH 7.5, 500mM NaCl, 2mM EDTA, 45ng RNA, and 2ng cDNA. The samples were heated at 100° for 2 min and incubated at 70° for various times. Reactions were terminated by placing the capillaries in dry ice-ethanol.

Extraction of DNA

This procedure is described in Appendix A.

Hybridization of cDNA to DNA

This procedure is described in Appendix A.

S1 Nuclease Assay of cDNA-RNA Hybrids

This procedure is described in Appendix A. The TCA precipitable radioactivity was collected on glass fiber filters and treated prior to counting by a modification of the procedure described by Birnboim (1970).

Results

Purification of Mouse Globin Messenger RNA

Approximately 18 ml of packed cells were obtained from 70 mice in which reticulocytosis had been induced. The reticulocyte yield from these animals was 80-90% as judged by staining with cresyl brilliant blue (Lebleu, 1974). RNA (900 A_{260} units) was extracted from the membrane-free polysomes and chromatographed on poly(U)-Sepharose. The elution profile obtained is shown in Fig. 2-1. Most of the RNA does not bind to the column in 0.5M NaCl. The 0.5M NaCl fraction consists of 4S, 18S and 28S RNA as can be seen from sucrose gradient analysis (Fig. 2-2). A small amount of material is observed in the 9-10S region of the gradient. This may be poly(A)-containing RNA which did not bind to the column, or RNA of that size which does not contain poly(A). The RNA eluted in the 0.1M NaCl wash appears to be nearly identical to that in the 0.5M NaCl wash when analyzed on sucrose gradients (data not shown).

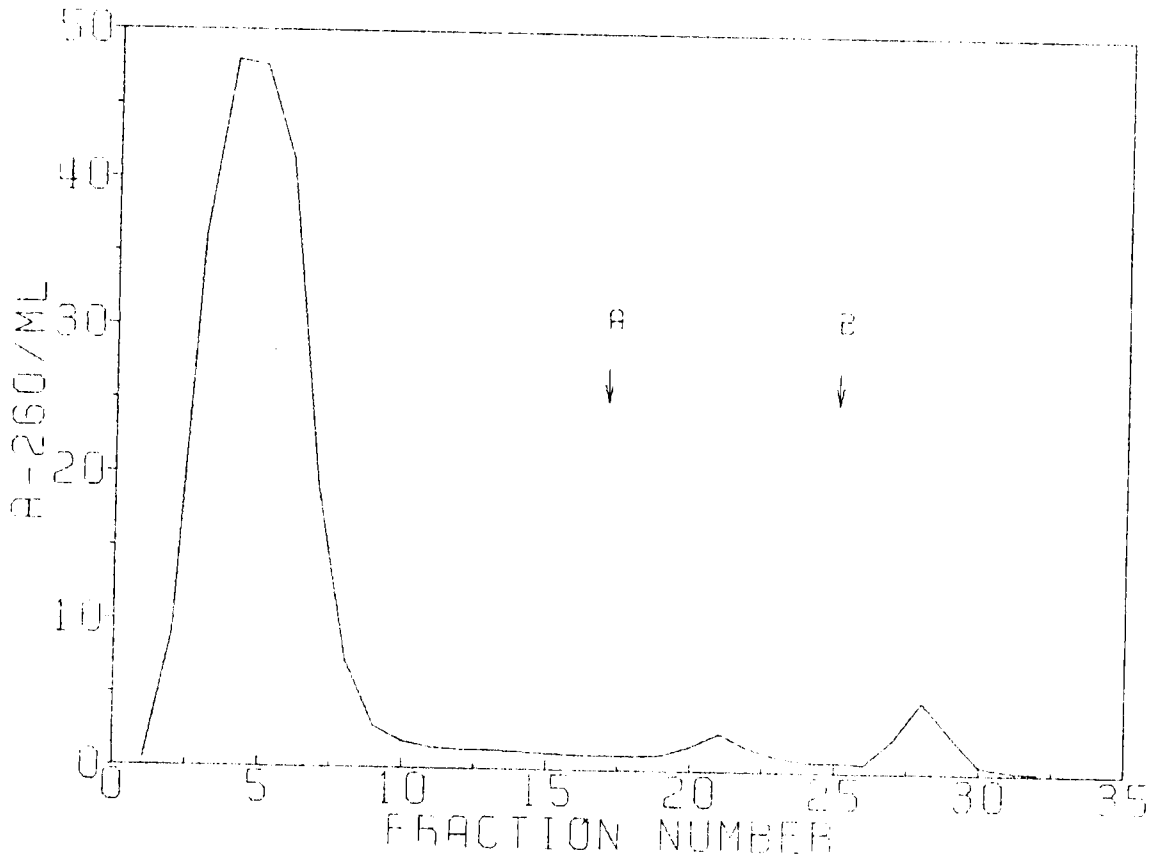


Fig. 2-1. Chromatography of reticulocyte RNA on poly(U)-Sepharose (first column). The RNA was applied in 0.5M NaCl + TS (0.01M Tris, pH 7.5 - 0.5% SDS). The column was washed with 0.1M NaCl + TS (arrow A) and with water at 50° (arrow B).

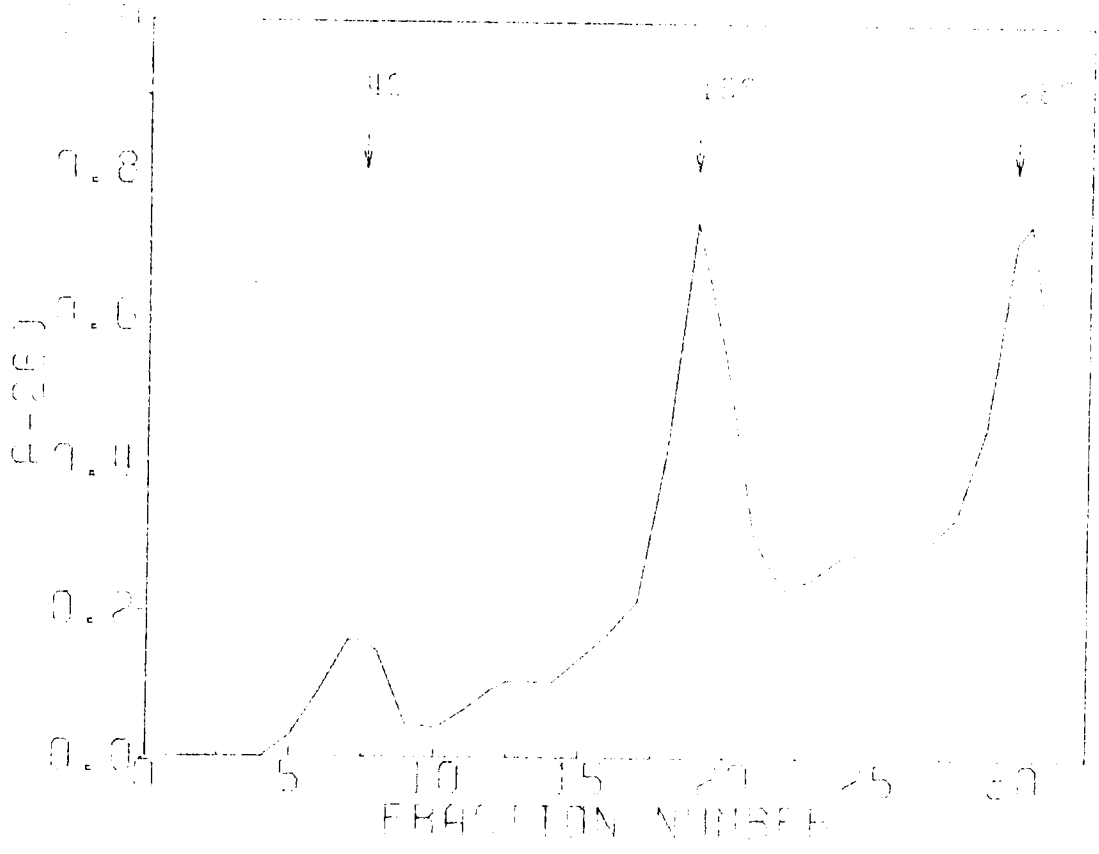


Fig. 2-2. Sucrose gradient analysis of reticulocyte RNA not binding to poly(U)-Sepharose in 0.5M NaCl + TS (first column). Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

The water fraction obtained from the first poly(U)-Sepharose column contained contaminating 18S and 28S RNA and was subjected to poly(U)-Sepharose chromatography again. The elution profile obtained from the second column is shown in Fig. 2-3. The 0.5M NaCl and 0.1M NaCl fractions were analyzed on sucrose gradients to determine whether the 9-10S RNA had failed to bind to the column in the second chromatography step. No demonstrable 9-10S RNA was observed in the 0.5M NaCl fraction (data not shown); however, a small amount of 9-10S RNA was observed in the 0.1M NaCl fraction (Fig. 2-4). No attempt was made to recover this RNA.

The water fraction obtained from the second column was placed on 5-22% sucrose gradients and centrifuged in a SW27 rotor for 24 hr at 27,000 rpm. Examination of the profile of this gradient (Fig. 2-5) reveals that this fraction is still contaiminated with 18S and 28S RNA. Fractions 13-18 (the 9-10S region) were pooled and subjected to fractionation on successive sucrose gradients until a component with a sedimentation coefficient of 9-10S was obtained. A profile of this fraction, which was centrifuged in 5-22% sucrose gradients in a SW41 rotor for 14 hr at 41,000 rpm as shown in Fig. 2-6. Fractions 12-15 were pooled and precipitated with ethanol. Approximately 8 A_{260} units of RNA were recovered; this is 0.9% of the polysomal RNA.

Translation of the Purified RNA in Vitro

The ability of the purified 9-10S RNA to direct the synthesis of mouse globin was assayed in the wheat germ cell-free protein synthesizing

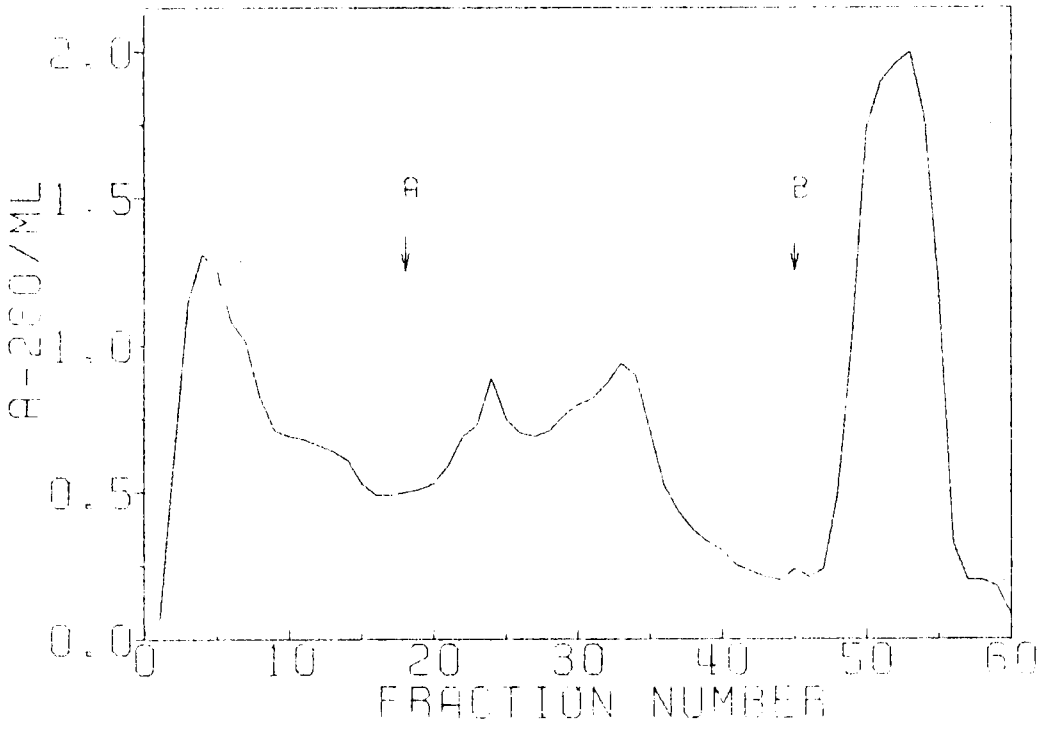


Fig. 2-3. Poly(U)-Sepharose chromatography of reticulocyte RNA obtained in the water fraction from the first poly(U)-Sepharose column. The RNA was applied to the column in 0.5M NaCl + TS and eluted with 0.1M NaCl + TS (arrow A) and water at 50° (arrow B).

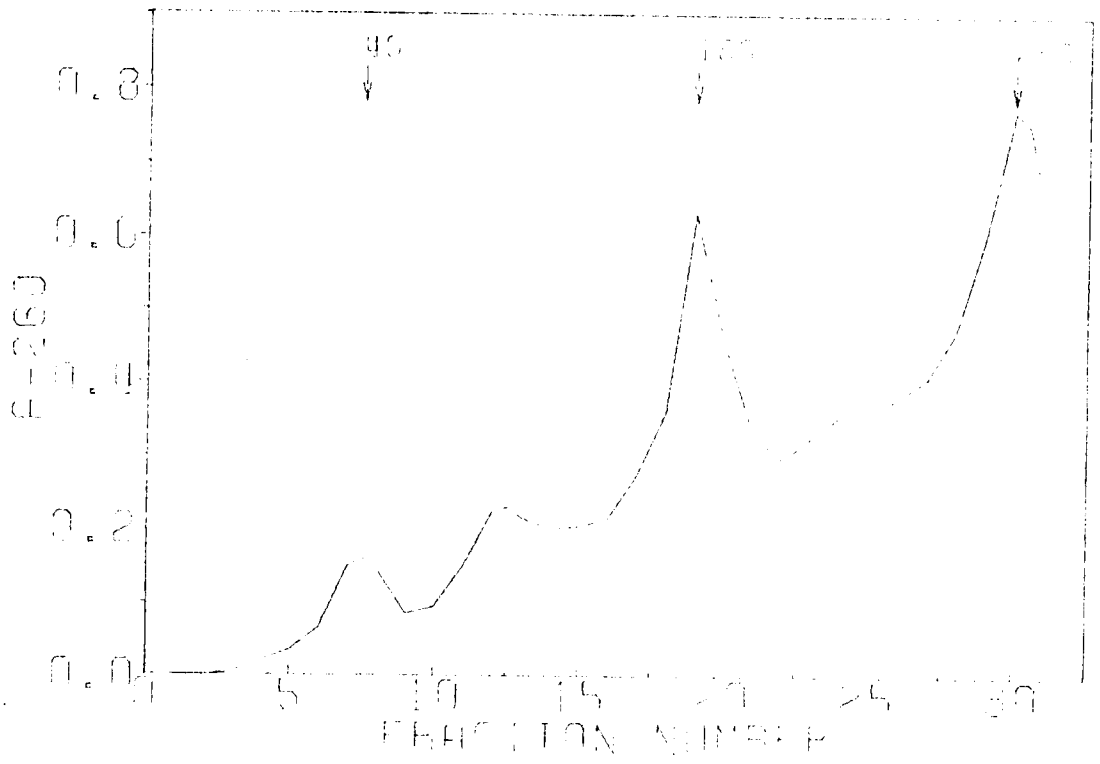


Fig. 2-4. Sucrose gradient analysis of reticulocyte RNA obtained in the 0.1M NaCl + TS wash of the second poly(U)-Sepharose column. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

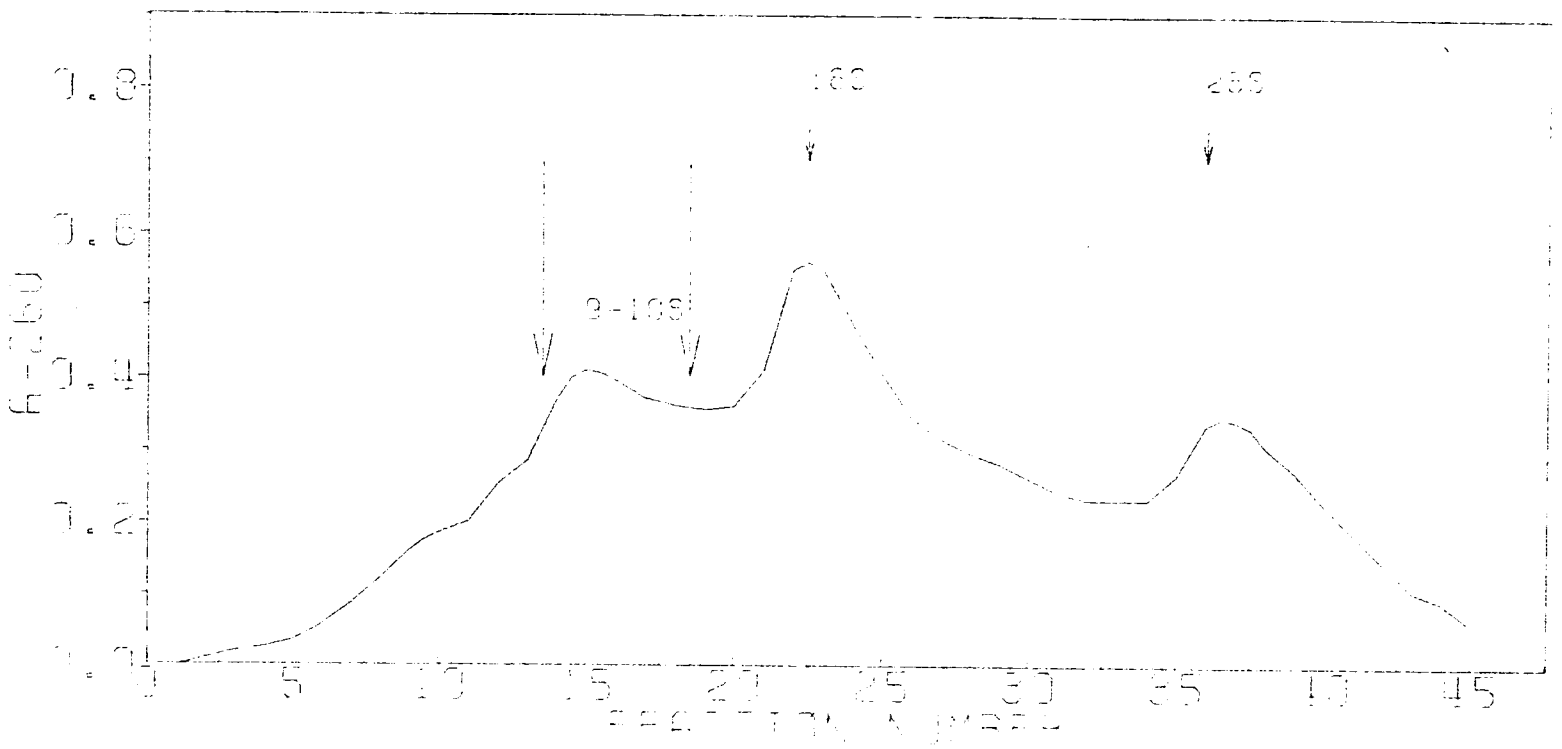


Fig. 2-5. Fractionation of reticulocyte RNA, obtained in the water fraction from the second poly(U)-Sepharose column, on sucrose gradients. Fractions 13-18 (the 9-10S region) were pooled for further fractionation. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

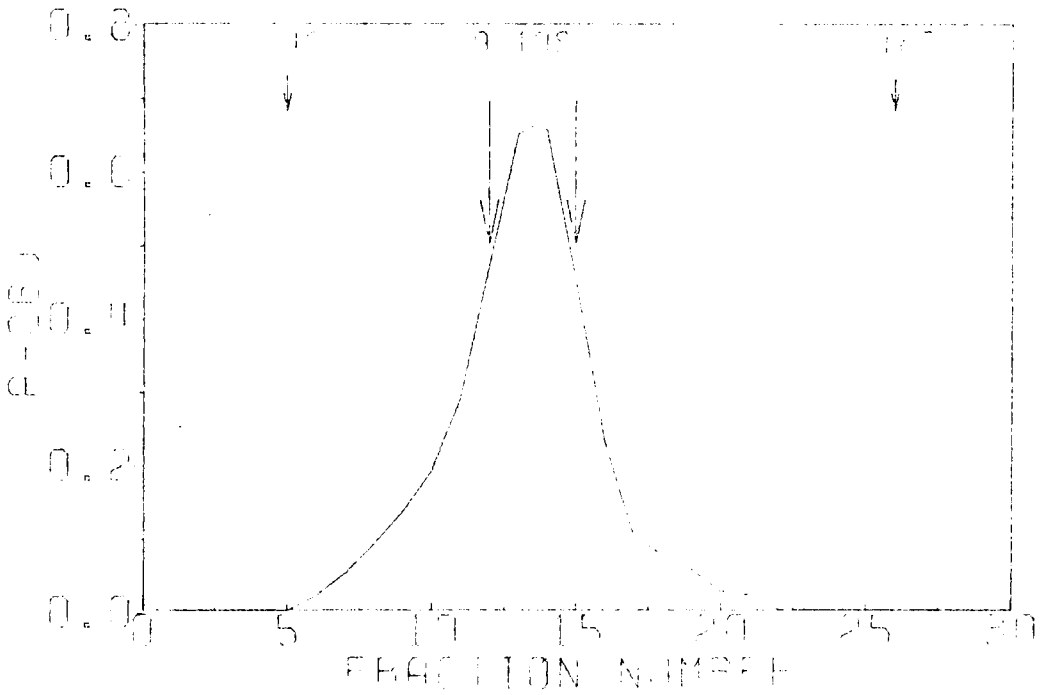


Fig. 2-6. Final sucrose gradient of reticulocyte RNA. Fractions 12-15 (the 9-10S region) were pooled. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

system (Roberts and Patterson, 1973). Under the conditions employed, globin RNA appears to be translated very efficiently in the wheat germ system compared to the BMV RNA standard; 1 ug of globin RNA directs the incorporation of 1.9 pmole of [^{3}H] leucine compared to the incorporation of 0.22 pmole of [^{3}H] leucine for lug of BMV RNA. The labeled cell-free products were added to mouse hemolysate and globin was prepared as described by Clegg et al. (1966). This material was then chromatographed on CMC-23 as described by Popp and Baliff (1973). It can be seen in Fig. 2-7 that this RNA fraction directs the synthesis of both α and β chains. Of the material applied to the column, approximately 90% is eluted in the α and β chain peaks.

Synthesis and Characterization of cDNA

Purified globin mRNA was used as a template for AMV reverse transcriptase to synthesize cDNA (Kacian and Myers, 1976a). The cDNA synthesized has a sedimentation coefficient of 8-9S in neutral sucrose gradients (Fig. 2-8); this corresponds to a complete or nearly complete copy of globin mRNA.

The cDNA was further characterized by hybridization analysis with globin mRNA, mouse DNA and mouse myeloma 15S RNA. The reassociation kinetics observed for cDNA and its template RNA are shown in Fig. 2-9. The values plotted are those that would obtain at 0.18M Na+ (Britten et al., 1974). The $C_o t$ 1/2 value for this curve is 5×10^{-3} and is in close agreement with a value reported by Honjo et al. (1974). The reassociation kinetics of cDNA with fetal liver DNA are shown in Fig. 2-10.

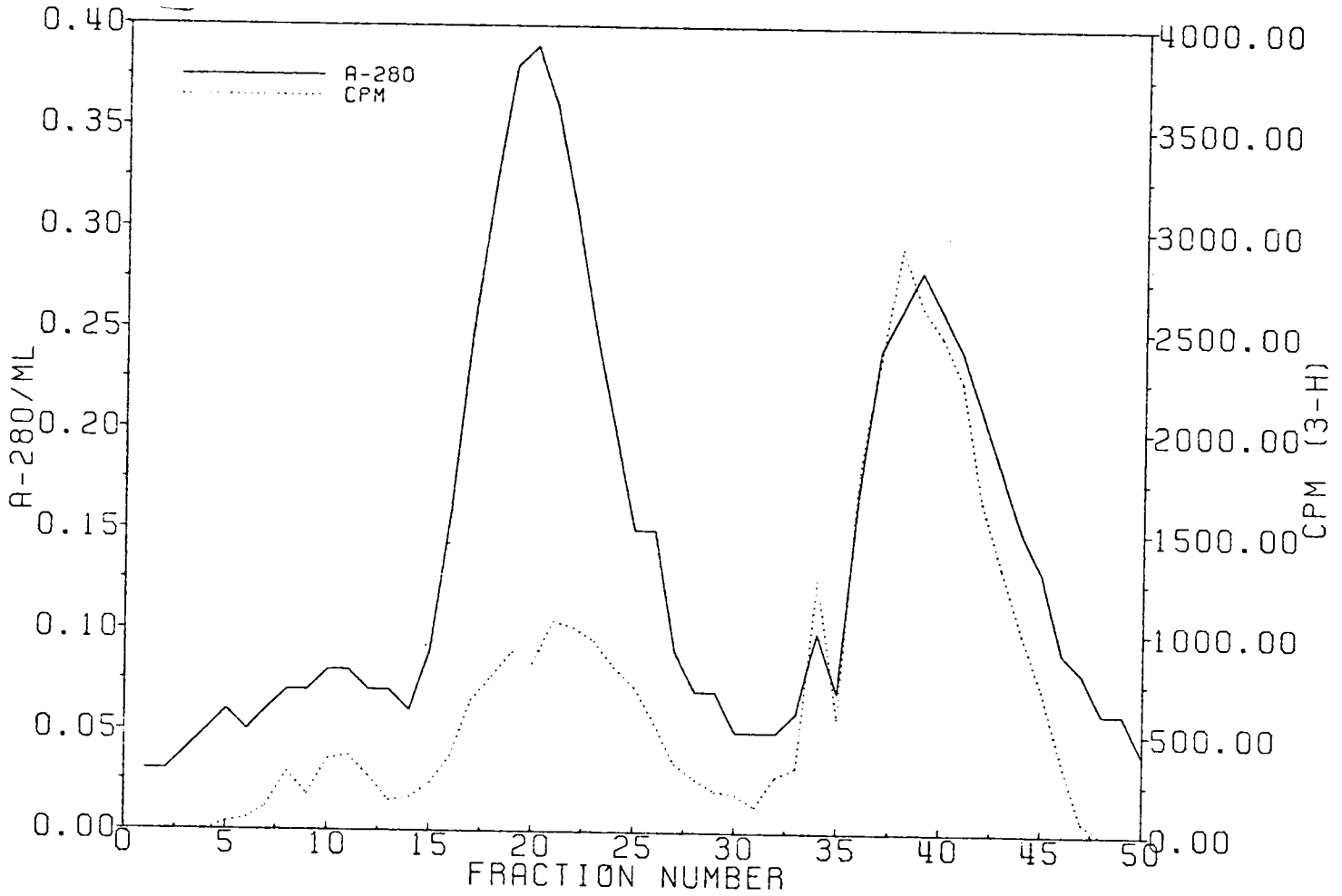


Fig. 2-7. Analysis of reticulocyte RNA cell-free products (synthesized in the wheat germ system) on CM cellulose.

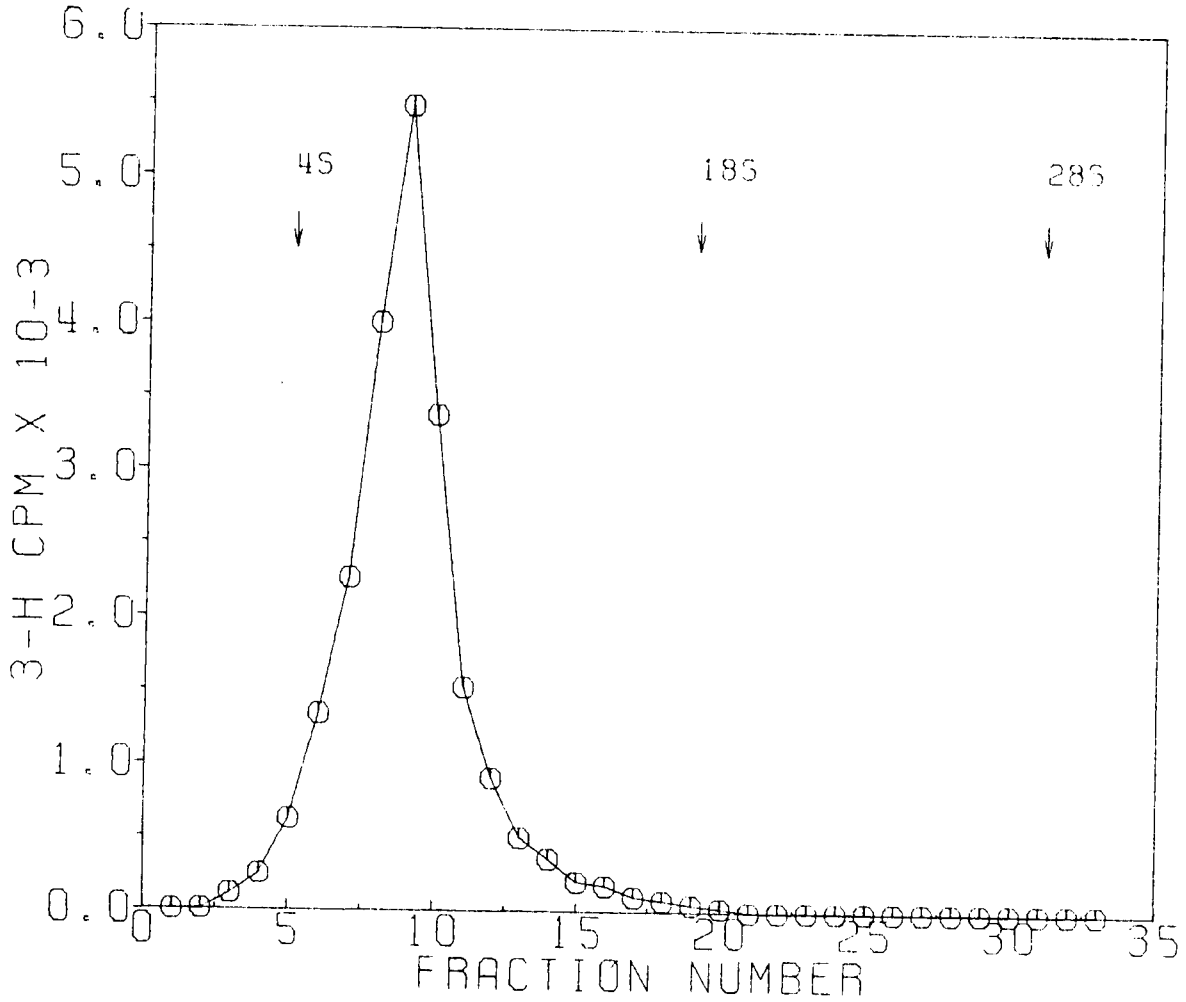


Fig. 2-8. Sucrose gradient analysis of globin cDNA. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

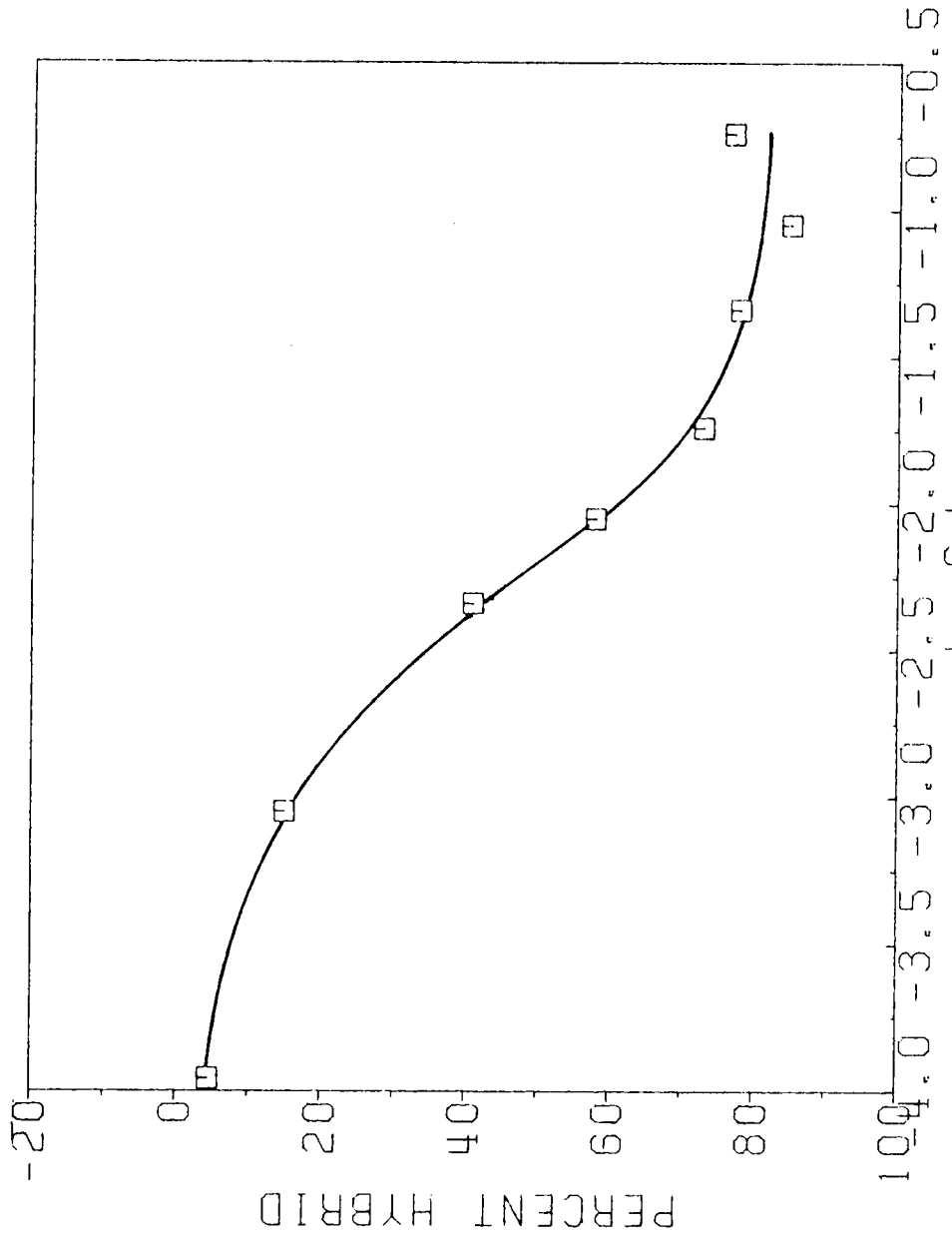


Fig. 2-9. Hybridization kinetics of globin cDNA with its template RNA.

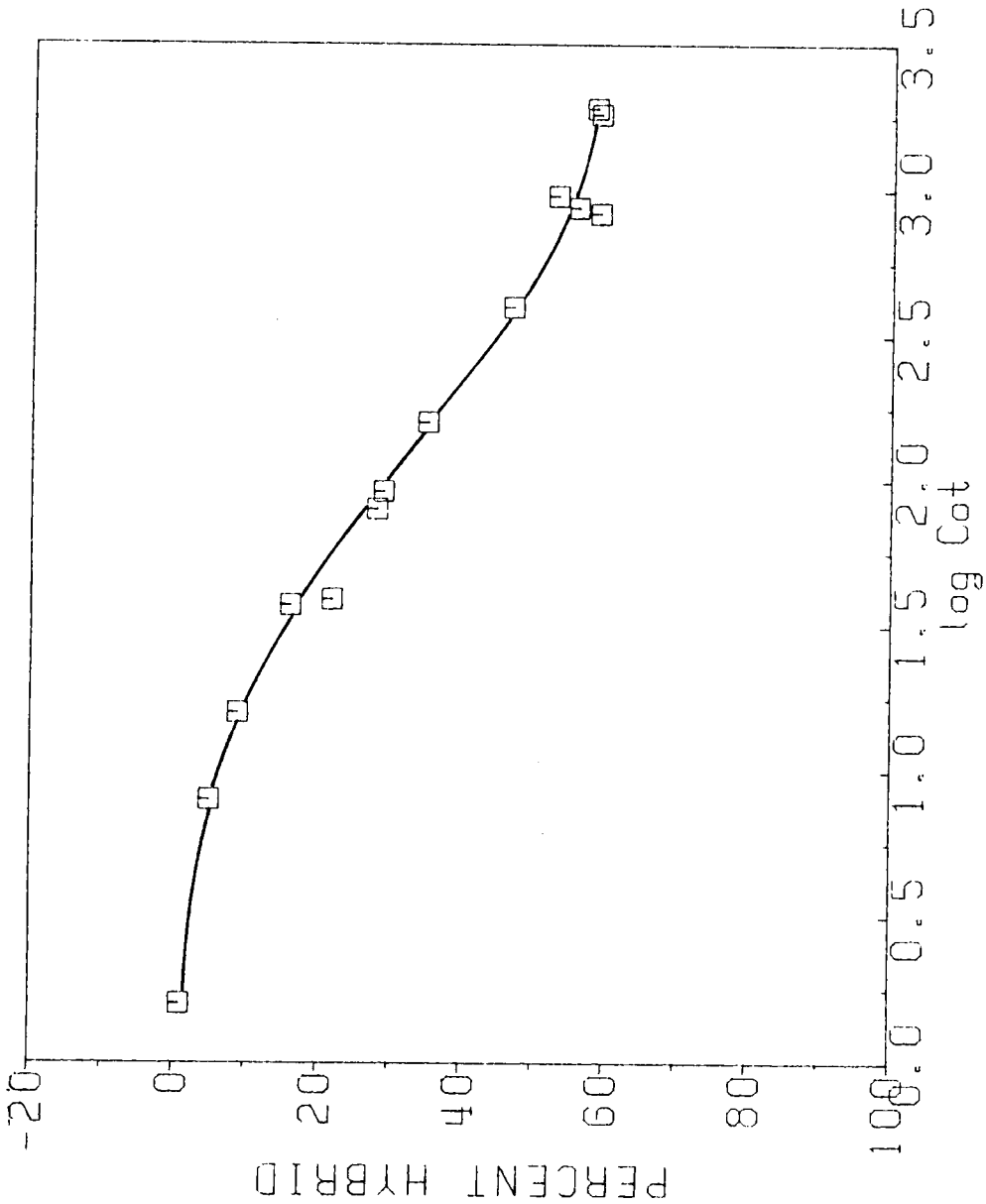


Fig. 2-10. Hybridization kinetics of globin cDNA with fetal liver DNA.

The $C_o t$ 1/2 value for this curve is 100. If this value is corrected for the effect of salt concentration on the rate of reassociation, it is consistent with a value reported by Harrison et al. (1974) for the reassociation of mouse globin cDNA with mouse fetal liver DNA. Harrison et al. (1974) conclude that there are probably only single, but not more than four to five copies each of the α and β globin genes in the mouse germ line. Globin cDNA does not hybridize with 15S RNA isolated from myeloma tumor cells (Table II-1).

Table II-1. Hybridization specificity of mouse globin cDNA

	Globin mRNA	Myeloma 15S RNA
Percent resistance to S1 nuclease	95	2

Reactions were carried out to a $C_o t$ of 9×10^{-1} as described in Materials and Methods except that 10 ul reactions contained 400 ng of RNA.

Discussion

During the development of the procedure for chromatography of poly(U)-Sepharose, it was found that better resolution and higher yields were obtained if samples were heated prior to applying to the column. This probably results from reduced aggregation problems after heating and quick cooling. Samples were also heated and quick cooled prior to fractionation on sucrose gradients; this also improved resolution and yields.

The purified mRNA sediments as a single component with a sedimentation coefficient of 9-10S, as expected for mouse globin mRNA (Williamson et al., 1971). From the results obtained in the wheat germ cell-free protein synthesizing system, the purity is estimated to be at least 90%.

The selection of a procedure for the synthesis of cDNA proved difficult for several reasons. Initially, a problem with a high percentage of the cDNA being resistant to S1 nuclease was encountered. This reaction can be inhibited by actinomycin D. For this purpose, actinomycin D obtained from Calbiochem was not as effective as that obtained from Sigma. It was not determined whether this problem was confined to a particular lot. With the procedure finally selected (Kacian and Myers, 1976a), synthesis of the second strand does not occur, even in the absence of actinomycin D. This appears to be due to the inhibition of RNase H activity by sodium pyrophosphate which is present in the reaction mixture (Kacian and Myers, 1976b). The amount of cDNA resistant to S1 nuclease is routinely 2-3%. Another advantage of the

Kacian procedure is the high yield of cDNA obtained in the absence of actinomycin D; routinely 40% of the RNA is transcribed into cDNA.

The size of the cDNA synthesized in early attempts was short (approximately 4S). It was found that long cDNA could be synthesized if high concentrations of the deoxynucleoside triphosphates were employed (0.5mM) or by using the Kacian procedure. Kacian and Myers (1976a) synthesized complete or nearly complete cDNA for polio RNA (approximately 7500 nucleotides in length). The globin cDNA synthesized appears to be large enough to be complete and the author has recently used this procedure to synthesize long cDNA from ovalbumin mRNA (approximately 1800 nucleotides in length). The length of the cDNA used to determine the presence of globin gene transcripts in RNA transcribed from chromatin can be important. Weiss et al. (1976) have reported that short globin cDNA does not hybridize as well as long globin cDNA to RNA transcribed from chromatin. This is expected since short cDNA, transcribed from the 3' end of template RNA, would not hybridize to incomplete chains of RNA initiated at the 5' end. The concentration of the deoxynucleoside triphosphates in the Kacian procedure is 0.2 mM; this is more economical and facilitates making highly labeled cDNA.

Another problem encountered in the synthesis of cDNA was tritium exchange. This occurred when cDNA, labeled with [^{3}H]dGTP, was incubated at a high temperature for long times. This problem was finally eliminated by using [^{3}H]dCTP as the source of label (this will be discussed in greater detail in Chapter IV).

The results obtained from the hybridization of cDNA with template RNA, fetal liver DNA and myeloma RNA suggest that the globin cDNA is a faithful copy of mouse globin mRNA and is suitable to use in assaying for globin genes or RNA transcripts of globin genes.

CHAPTER III

THE ISOLATION OF MOPC-31C LIGHT CHAIN mRNA AND THE SYNTHESIS OF LIGHT CHAIN cDNA

Introduction

Membrane-bound polysomes were isolated from mouse myeloma tumors (MOPC-31C). RNA was extracted from the polysomes and the poly(A)-containing fraction was isolated by chromatography on poly(U)-Sepharose. The poly(A)-containing RNA was fractionated on successive sucrose gradients and two components with sedimentation coefficients of approximately 12S and 15S were obtained. These RNA fractions were assayed for the ability to direct the synthesis of MOPC-31C light chain in cell-free protein synthesizing systems. The 12S RNA was used as a template for the synthesis of cDNA with AMV reverse transcriptase. The size of the cDNA was determined by sucrose gradient sedimentation. The purity of the cDNA was determined by hybridization to template RNA and to globin mRNA.

Materials and Methods

Animals. Male BALB/c (Cum) mice, 14 wk-old, were obtained from Cumberland View Farms, Clinton, TN.

Myelomas. Myeloma cell line MOPC-31C, originally established by M. Potter, was obtained from W. K. Yang, Oak Ridge National Laboratory.

The line was maintained by serial transplantation as a subcutaneous solid tumor in 14 wk-old mice.

Reagents. The following reagents were used: Poly(U)-Sepharose (Pharmacia); RNase-free sucrose (Schwarz-Mann); S1 nuclease (Sigma); oligo $(dT)_{10}$ Collaborative Research, Inc.); dATP, dGTP and dTTP (Sigma); [^{3}H] leucine, 60 Ci/mmole (New England Nuclear); [^{3}H]dCTP, 20 Ci/m mole (Amersham/Searle); AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.); wheat germ (donated by Dixie Portland Flour Mills, Chattanooga, TN); [^{3}H] and [^{14}C]L-Amino Acid Mixture (New England Nuclear); Proteinase K (EM Laboratories, Inc.).

Isolation of Polysomes

Polysomes were isolated as described by Honjo et al. (1976). MOPC-31C tumors were excised, frozen immediately and stored at -70°. Frozen tumors were placed in 4 volumes of buffer A (50mM Tris HCl, pH 7.8, 25mM KCl, 5mM $MgCl_2$, 10^{-4}M cyclohexamide) containing 0.1% diethyl pyrocarbonate and homogenized in a chilled Waring blender for 30 sec at low speed and then further homogenized with 5 strokes of a loose and 5 of a tight pestle Dounce homogenizer. The homogenate was centrifuged at 20,000g for 20 min. The supernatant was layered on a discontinuous sucrose gradient composed of 7 ml of 1.5M sucrose and 15 ml of 1.75M sucrose, each containing buffer A lacking diethyl pyrocarbonate. After centrifugation at 25,000 rpm (81,500 g) for 60 min in a Beckman SW27 rotor, the polysomes, which had been concentrated in the 1.5M sucrose layer, were harvested.

Extraction of RNA

RNA was extracted from polysomes by a modification of the procedure described by Brawerman (1974). Polysomes obtained from the 1.5M sucrose layer were made 0.1% SDS and 50 ug/ml of Proteinase K were added. After 10 min at 0°C, the polysome suspension was made 0.1M Tris HCl, pH 9.0 and 1.0% SDS. An equal volume of aqueous phenol (redistilled phenol diluted with distilled H_2O to a concentration of 80% (v/v)) was added and the mixture was stirred vigorously for 10 min. This was followed by centrifugation at 12,000 g for 10 min. The aqueous layer was removed and the nonaqueous residue (phenol phase plus gel interphase) was reextracted with 1/2 volume of 0.1M Tris, pH 9.0 and 1% SDS as before. The aqueous phases were pooled and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1); again by stirring and separation by centrifugation as above. This step was repeated two more times. The aqueous phase was then made 2% CH_3 COOK (pH 5.5) and precipitated by the addition of 2.5 volumes of 95% ethanol and stored at -20C overnight. The precipitated RNA was collected by centrifugation at 10,000 g for 30 min at -20C. The RNA pellet was washed twice with ethanol-0.2M NaCl (2:1) to remove residual phenol and dissolved in distilled water for fractionation on poly(U)-Sepharose.

Poly(U)-Sepharose Chromatography

This procedure is described in Appendix A.

Sucrose Gradient Centrifugation

The RNA obtained in the water fraction of the second poly(U)-Sepharose column was fractionated on sucrose gradients as described by Honjo et al. (1974). Details are given in Appendix A.

Preparation of Labeled MOPC-31C Heavy and Light Chains

The procedure of Laskov and Scharff (1970) was used with minor modifications for the preparation and labeling of tumor cell suspensions. MOPC-31C tumor fragments were suspended in 10 ml of amino acid-depleted Eagle's medium (containing 1/20 the normal amounts of the appropriate amino acids) and placed in a 25 ml flask. The suspension was stirred vigorously for 5 min at 37°. Large fragments were allowed to settle and the supernatant was filtered through a 100 mesh stainless steel screen. The cells in the filtrate were washed twice in the amino acid-depleted medium and resuspended in the same medium at a concentration of $5x10^6$ cells/ml. Two and one-half ml of either [^{3}H] or [^{14}C] amino acid mixture (neutralized) was added to 10 ml of cells. The cell suspension was gently shaken on a rotary shaker at 37°C for 3 hr. At this time the cells were pelleted and the secreted proteins, in the supernatant, were precipitated by the addition of 100 ug/ml of carrier BSA and an equal volume of of 10% TCA. The precipitate was washed twice with acetone and dissolved in 1% SDS and stored at -20°C.

Cell-Free Protein Synthesis

The preparation of the wheat germ extract was as described by Roberts and Patterson (1973) except that the S-30 fraction was preincubated for 30 min at 30°C. Details are given in Appendix A.

Translation of myeloma mRNA in the Krebs ascites cell-free system as described by Boime and Aviv (1973) and analysis of the cell-free product was performed by M. P. Stulberg, Oak Ridge National Laboratory.

Analysis of Cell-Free Product

Cell-free reaction mixtures were incubated with 20 ug of RNase A for 20 min at 37°C. The reactions were then made 1% SDS, 0.06M Tris (pH 6.7), 20% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue, and heated for 2 min at 100°C. The cell-free products and standards, which had been treated in a similar manner, were subjected to electrophoresis on 13% polyacrylamide resolving gels (containing a 3% polyacrylamide stacking gel) as described by Laemmli (1970). This procedure is described in detail by Maizel (1971).

Synthesis of cDNA

This procedure is described in Appendix A.

Size Determination of cDNA

This procedure is described in Appendix A.

Hybridization of cDNA to Template RNA

Hybridizations were performed in 10 ul volumes in 40 ul glass capillaries (Microcaps), treated as described by Wilson et al. (1975).

The reaction mixture contained 10mM Tris, pH 7.5, 500mM NaCl, 2mM EDTA, 45 ng RNA, and 2 ng cDNA. The samples were heated at 100°C for 2 min and incubated at 70°C for various times. Reactions were terminated by placing the capillaries in dry ice-ethanol.

S1 Nuclease Assay of cDNA-RNA Hybrids

This procedure is described in Appendix A. The TCA precipitable radioactivity was collected on glass fiber filters and treated prior to counting by a modification of the procedure described by Birnboim (1970).

Results

Purification of MOPC-31C Light Chain RNA

Approximately 600 A_{260} units of RNA were extracted from the membrane-bound polysomes obtained from 25 g of MOPC-31C tumor. This RNA was chromatographed on poly(U)-Sepharose. Most of the polysomal RNA failed to bind to the column in 0.5M NaCl (Fig. 3-1). The 0.5M NaCl and 0.1M NaCl fractions were found to consist of 4S, 18S and 28S RNA by analysis on sucrose gradients (data not shown). The water fraction was passed through poly(u)-Sepharose again (Fig. 3-2). Most of this RNA bound to the column and was eluted with water at 50°C.

The RNA obtained in the water fraction from the second chromatography step was centrifuged in 5-22% sucrose gradients in a SW27 rotor for 24 hr at 27,000 rpm (Fig. 3-3). Most of this RNA sedimented in the 4-16S region of the gradients. A small amount of contaminating 18S and 28S RNA was present. Fractions 8-10 (the 7-10S region) and fractions 11-16

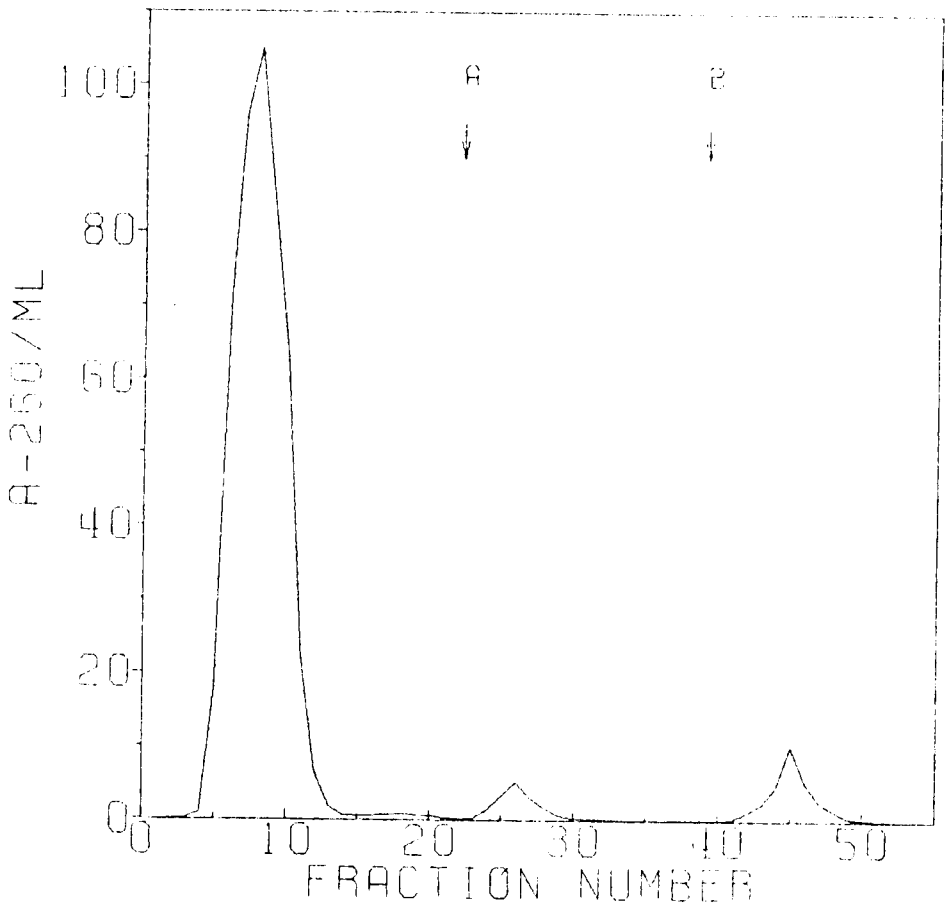


Fig. 3-1. Chromatography of tumor RNA on poly(U)-Sepharose (first column). The RNA was applied in 0.5M NaCl + TS. The column was washed with 0.1M NaCl + TS (arrow A) and with water at 50° (arrow B).

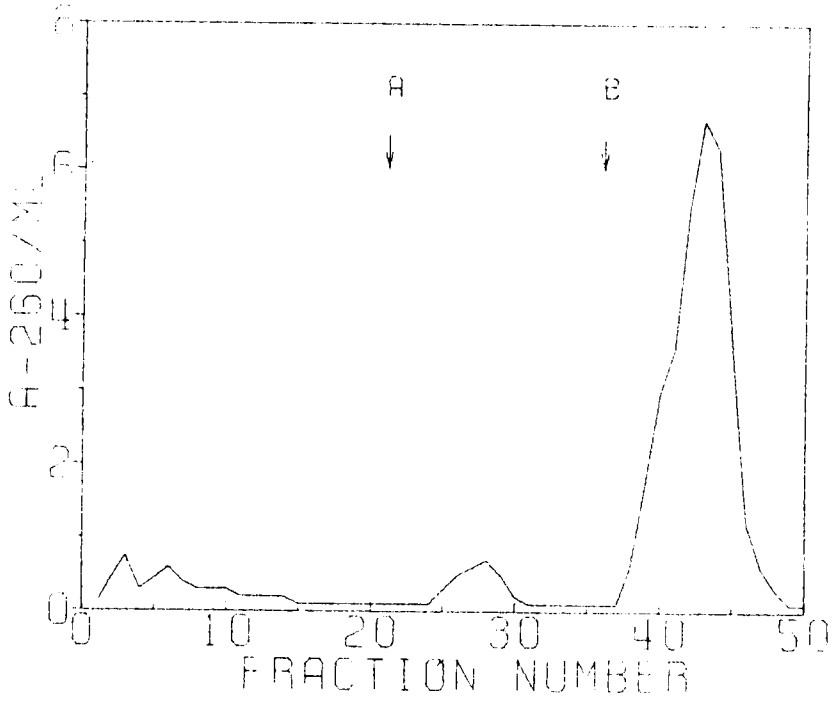


Fig. 3-2. Poly(U)-Sepharose chromatography of tumor RNA obtained in the water fraction from the first poly(U)-Sepharose column. The RNA was applied to the column in 0.5M NaCl + TS and eluted with 0.1M NaCl + TS (arrow A) and water at 50° (arrow B).

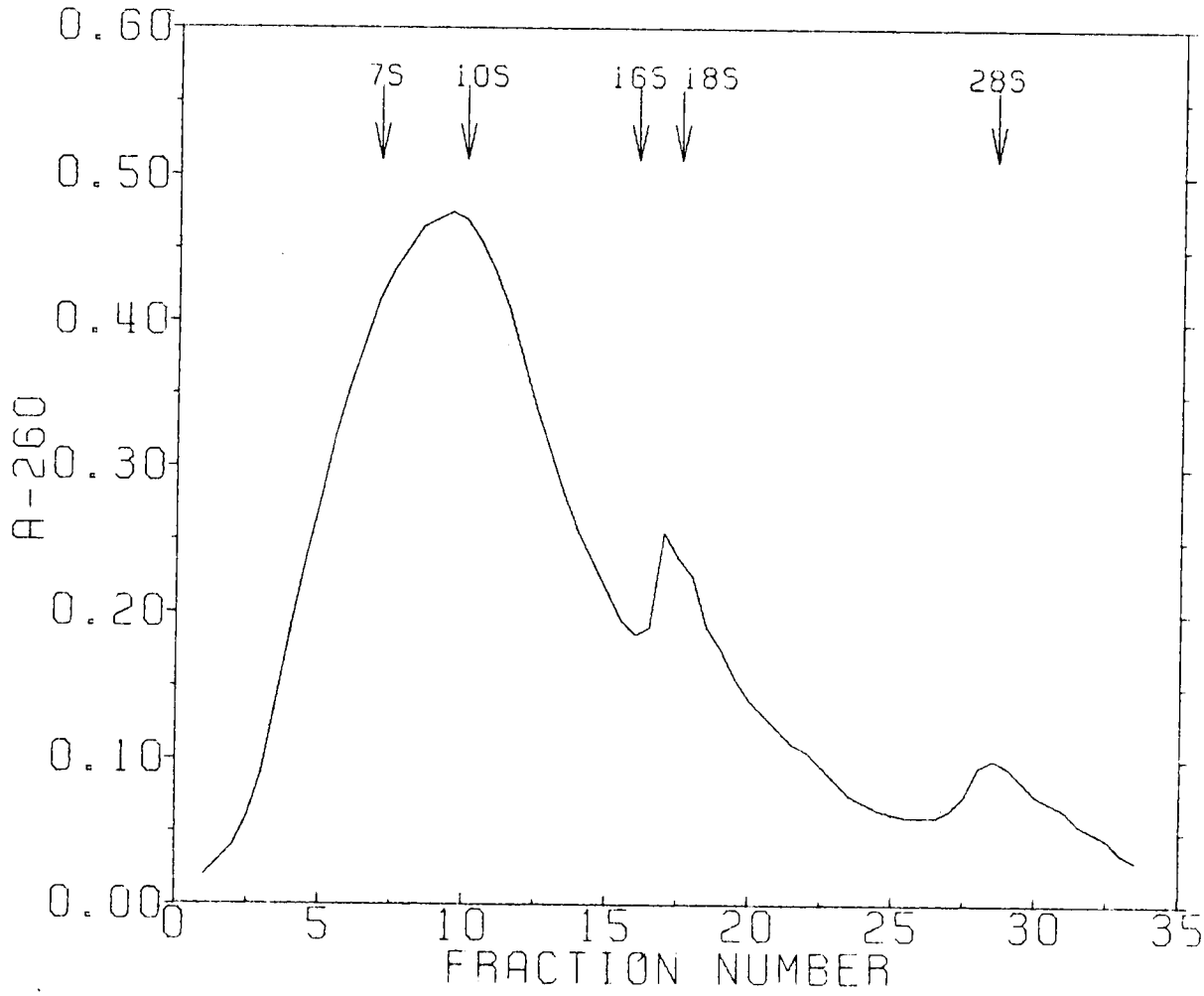


Fig. 3-3. Fractionation of tumor RNA, obtained in the water fraction from the second poly(U)-Sepharose column, on sucrose gradients. Fractions 8-10 (the 7-10S region) and fractions 11-16 (the 10-16S region) were pooled separately for further fractionation. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

(the 10-16S region) were pooled separately for further fractionation on successive sucrose gradients. Two components with sedimentation coefficients of approximately 12S (Fig. 3-4) and 15S (Fig. 3-5) were obtained. Fractions 13-18 of the 12S RNA (Fig.3-4) were pooled and precipitated; 2.2 A_{260} units were recovered (0.37% of the polysomal RNA). Less than 0.06% of the polysomal RNA (0.35 A_{260} units) was recovered in fractions 18-20 of the 15S RNA (Fig. 3-5).

Preparation of Labeled MOPC-31C Heavy and Light Chains

MOPC-31C tumor cells synthesize both heavy (IgF) and light (κ) chains. Labeled heavy and light chains were prepared as described by Laskov and Scharff (1970) and were used as authentic MOPC-31C heavy and light chain markers. The analysis of these markers on 13% SDS-polyacrylamide gels is shown in Fig. 3-6. The heavy chain has a molecular weight of approximately 50,000 (Swan et al., 1972) and the light chain a molecular weight of approximately 23,000 (Burstein et al., 1976).

Translation of the Purified RNA in Vitro

The polypeptides synthesized in response to the 12S RNA in the wheat germ cell-free system were analyzed by SDS-polyacrylamide gel electrophoresis and compared with authentic MOPC-31C heavy and light chains (Fig. 3-7). The major polypeptide synthesized has a molecular weight of approximately 27,000 and probably corresponds to light chain precursor. *In vitro* synthesized precursors with an additional 20-22 amino acids have

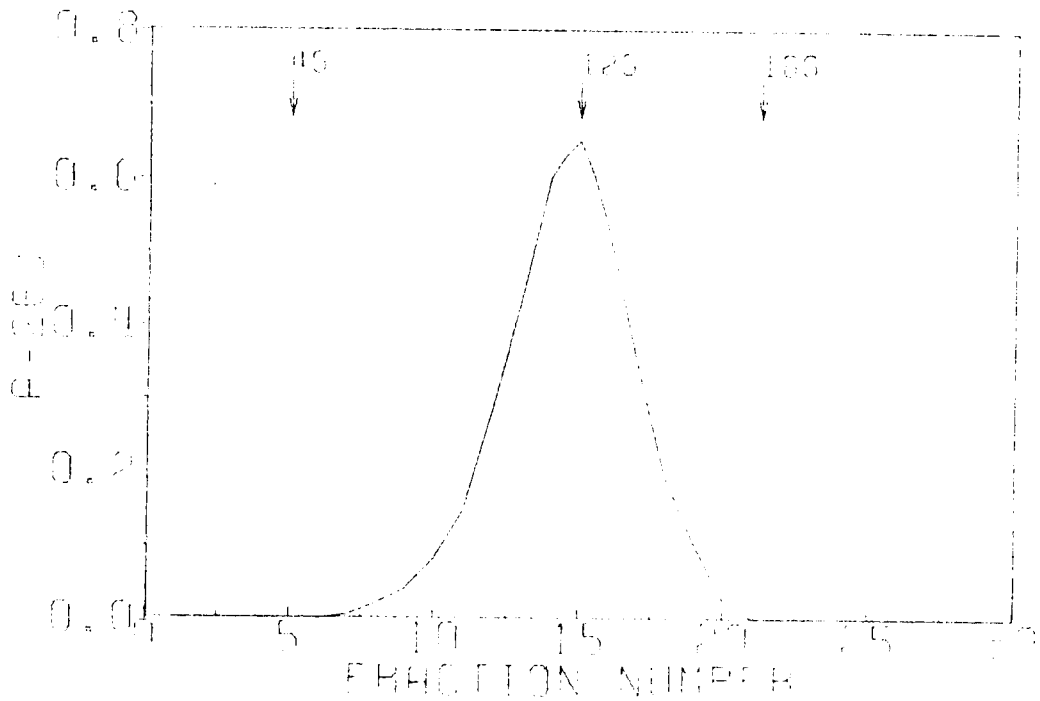


Fig. 3-4. Final sucrose gradient of 12S tumor RNA. Fractions 13-18 were pooled. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

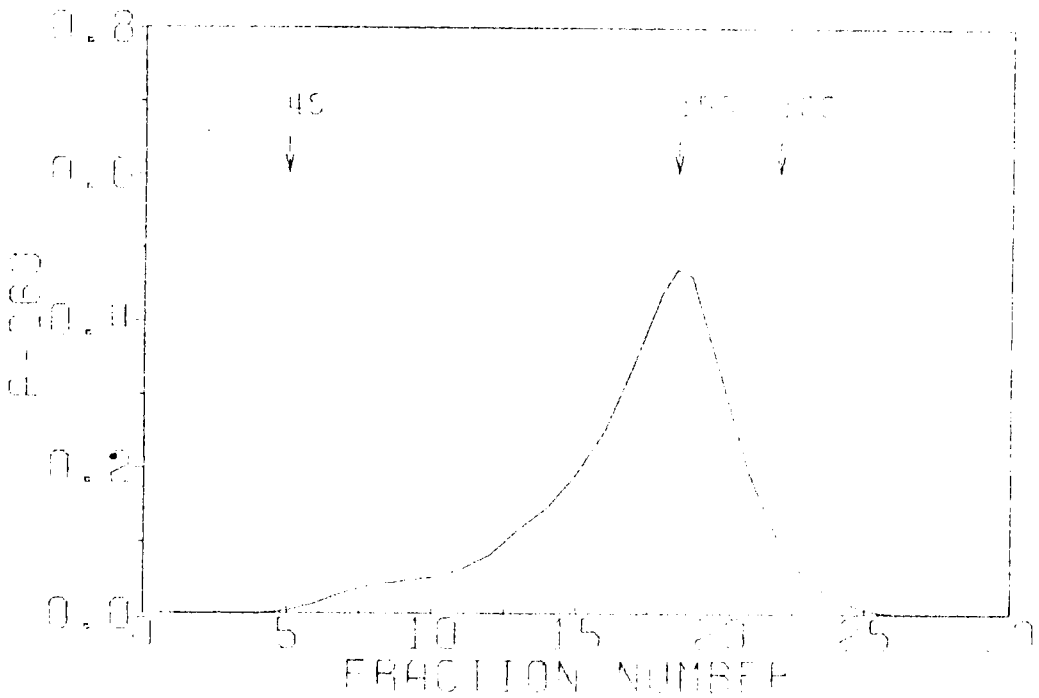


Fig. 3-5. Final sucrose gradient of 15S tumor RNA. Fractions 18-20 were pooled. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

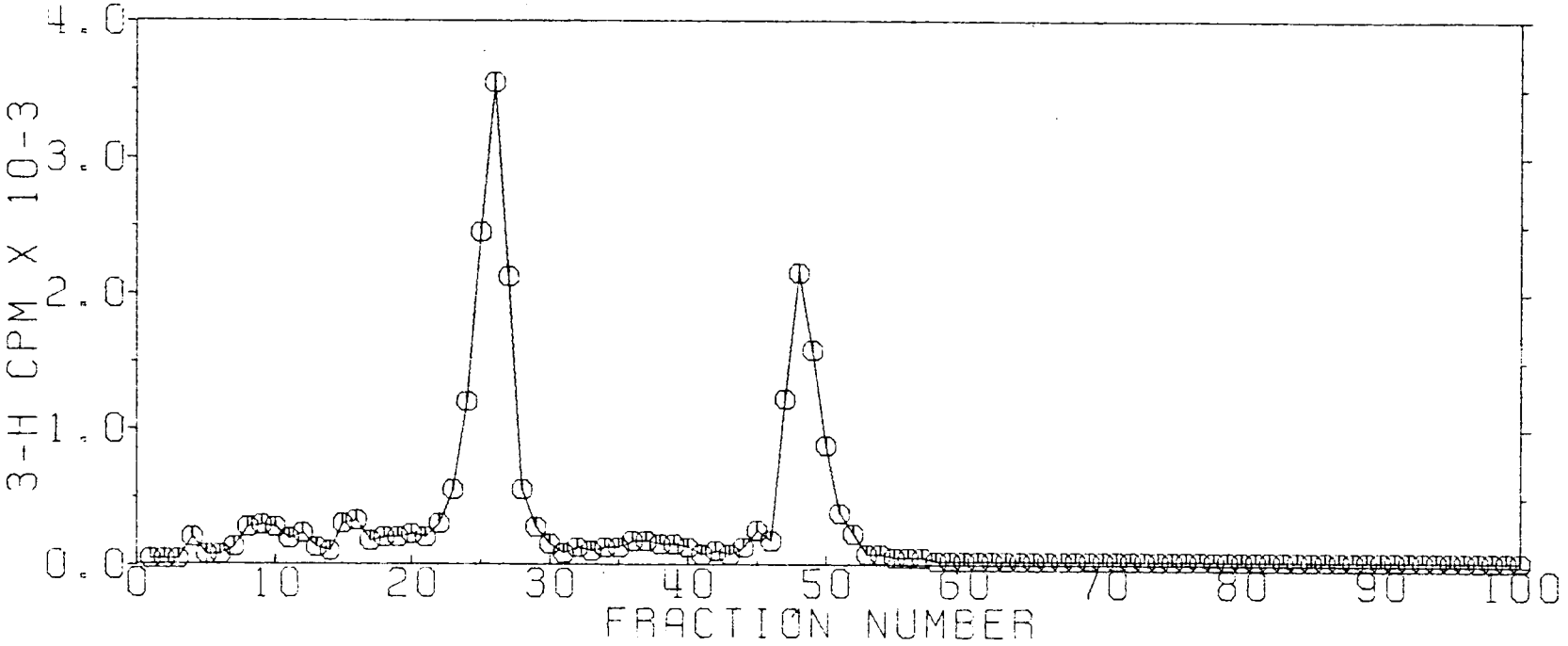


Fig. 3-6. Analysis of MOPC-31C heavy and light chains on SDS-polyacrylamide gels.

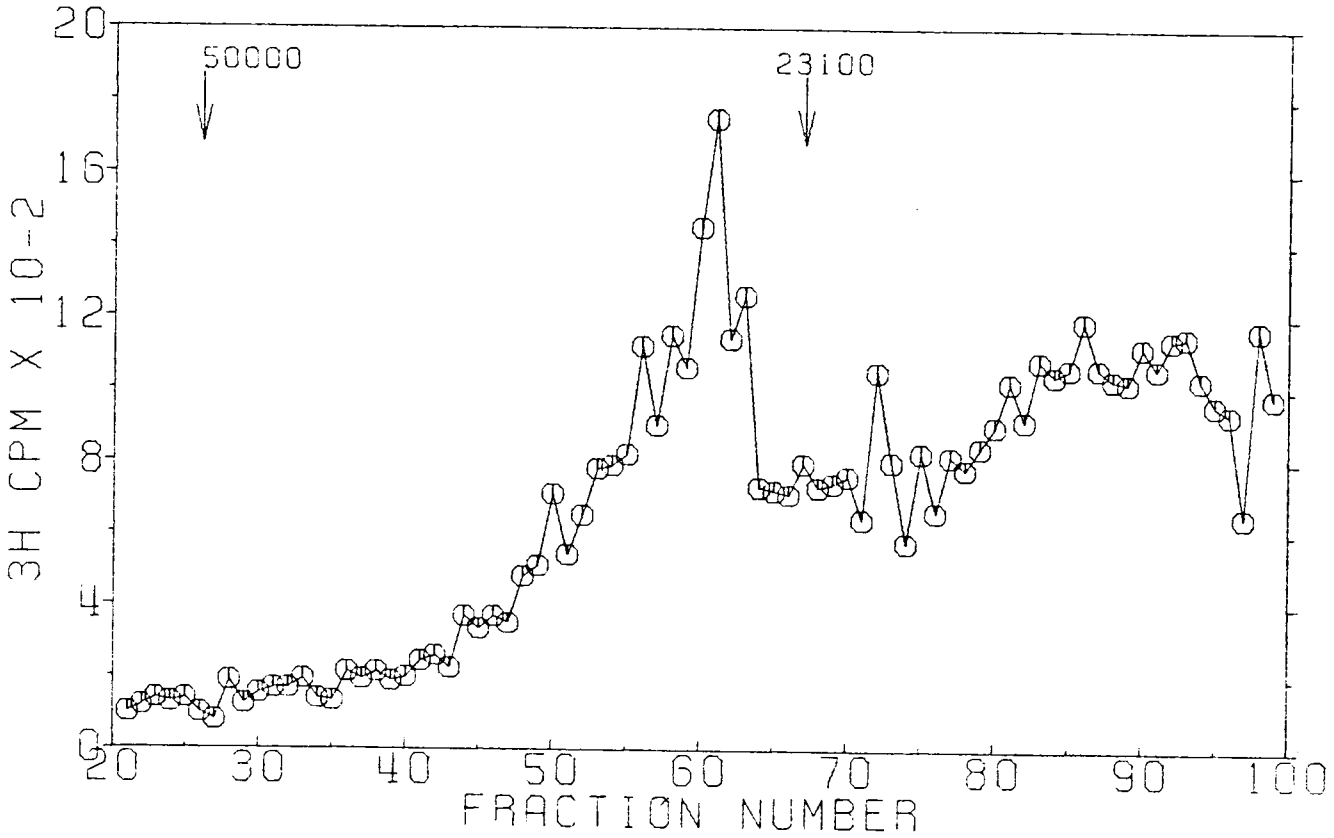


Fig. 3-7. Analysis of 12S tumor RNA cell-free products (synthesized in the wheat germ system) on SDS-polyacrylamide gels.

been described for three κ light chains (Burstein et al. 1976). The 15S RNA did not direct the synthesis of enough cell-free product for further analysis.

The 12S RNA was also translated in the Krebs ascites cell-free system. Initially, a polypeptide with a molecular weight of 27,000 was observed along with some smaller polypeptides. An attempt was made to determine whether the smaller molecular weight products were incomplete light chains or other polypeptides. At the end of the incubation period, the ribosomes were sedimented from the reaction mixture. Both the radioactive products released from the ribosomes and those remaining bound to the ribosomes were analyzed on SDS-polyacrylamide gels (Figs. 3-8 and 3-9). Since the small molecular weight polypeptides are present on both gels, they do not appear to be incomplete light chains. However, this result does not rule out the possibility of degradation of complete light chains after synthesis. The 15S RNA did not direct the synthesis of appreciable product in this system either.

Synthesis and Characterization of cDNA

The 12S RNA was used as template for AMV reverse transcriptase to synthesize cDNA (Kacian and Myers, 1976). The cDNA synthesized has a sedimentation coefficient of 8-9S in neutral sucrose gradients (Fig. 3-10).

The hybridization kinetics of the cDNA with template RNA were examined (Fig. 3-11). The C_0t 1/2 value for this reaction is estimated to be 3. This value can be compared with the value obtained for globin mRNA and its cDNA ($5x10^{-3}$) to estimate the purity of the MOPC-31C light

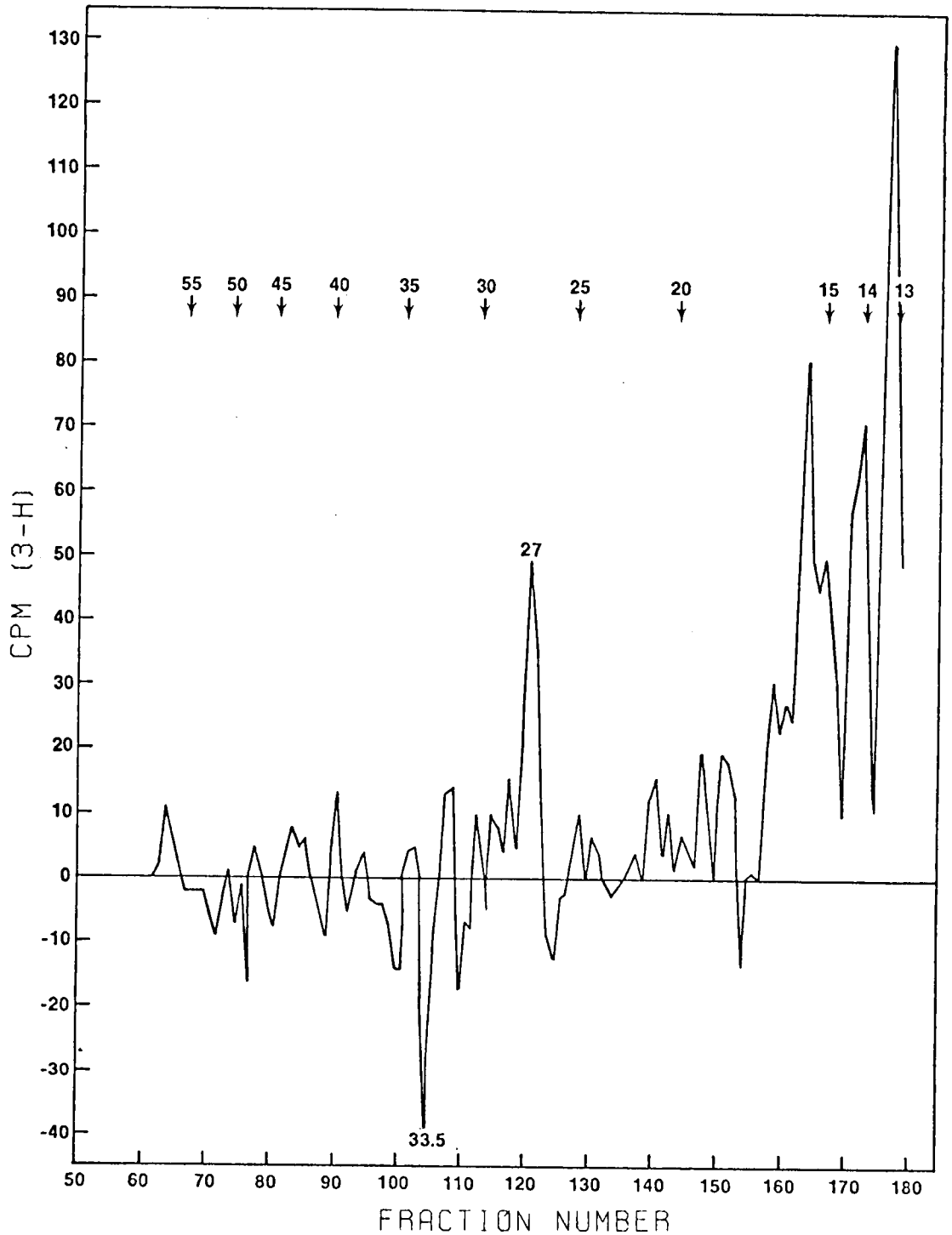


Fig. 3-8. Analysis of 12S tumor RNA cell-free products, released from polysomes, on SDS-polyacrylamide gels (synthesis was in the ascites system). A difference profile for reactions with and without 12S RNA is shown.

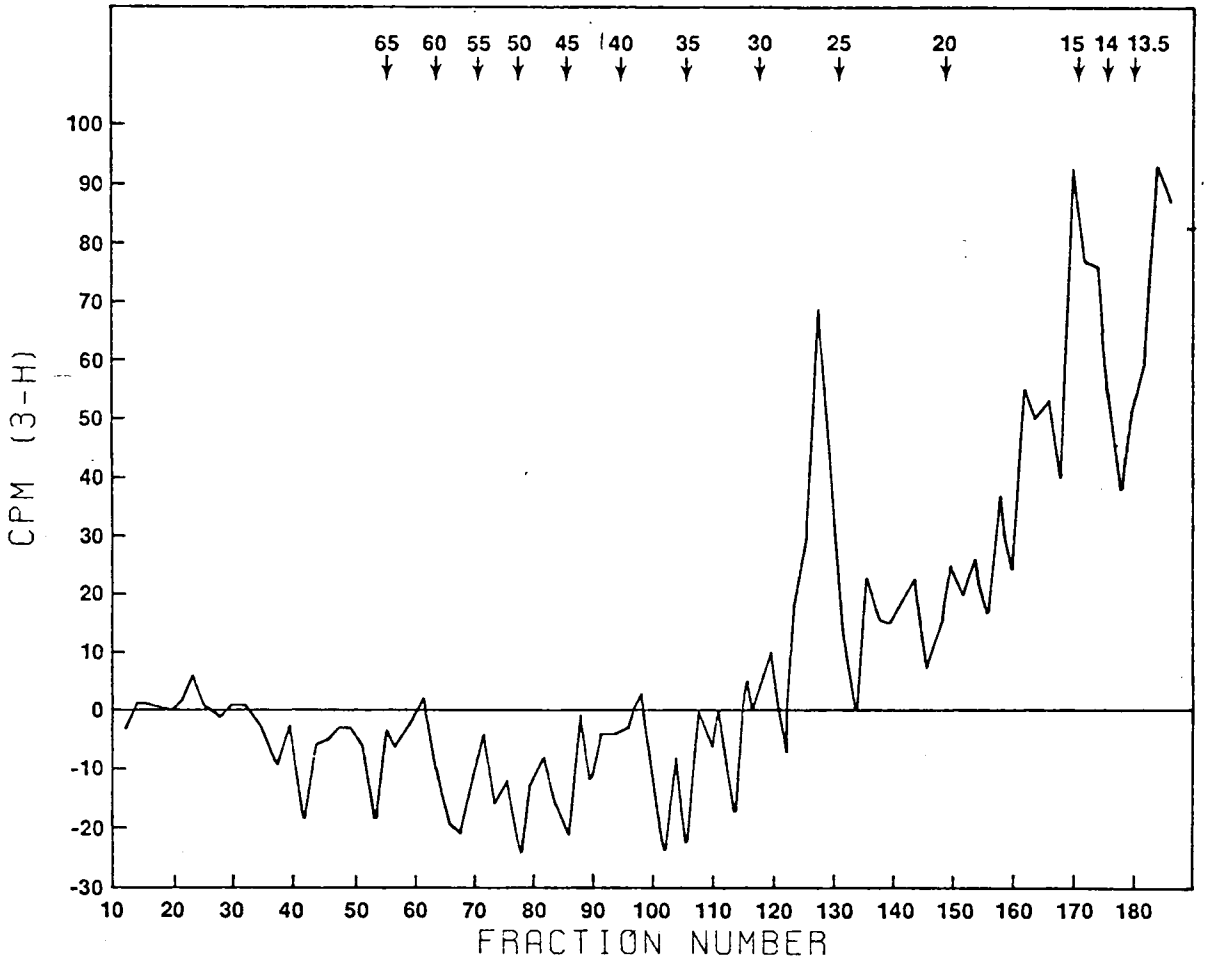


Fig. 3-9. Analysis of 12S tumor RNA cell-free products bound to polysomes on SDS-polyacrylamide gels (snythesis was in the ascites system). A difference profile for reactions with and without 12S RNA is shown.

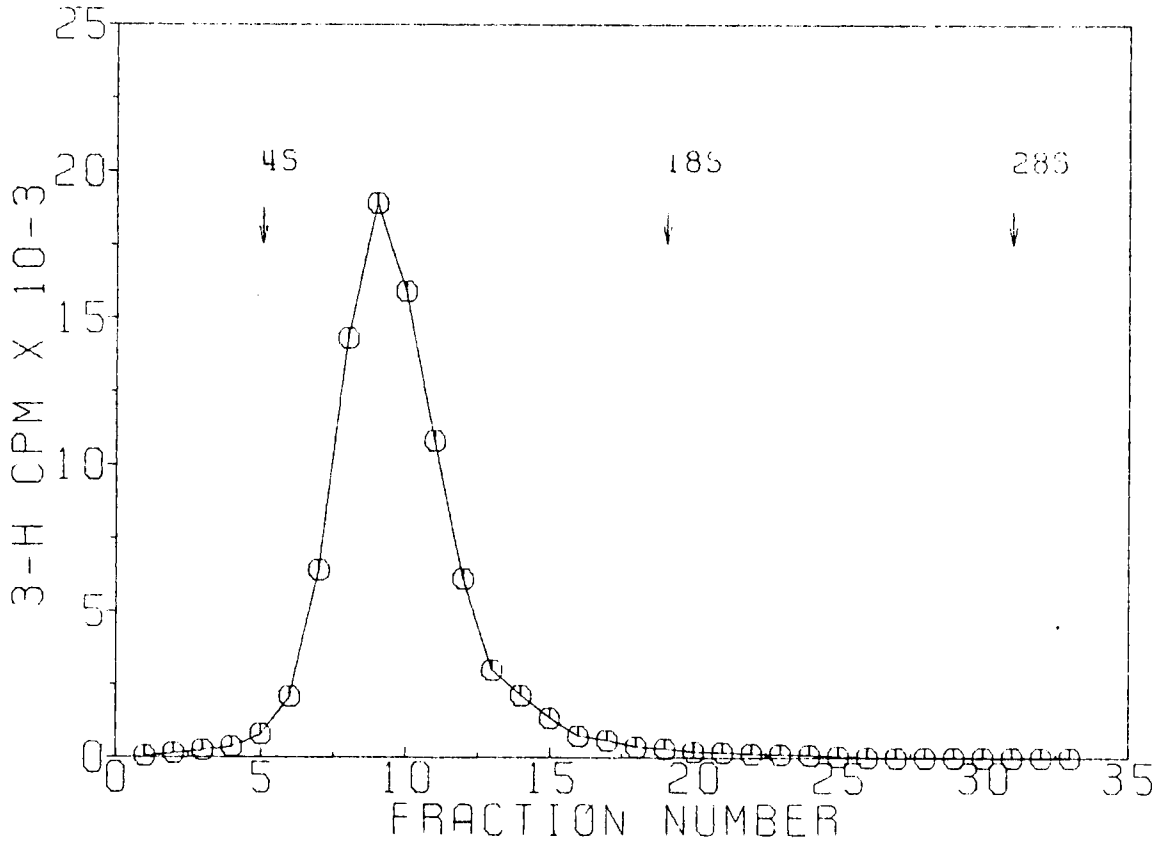


Fig. 3-10. Sucrose gradient analysis of 12S tumor cDNA. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

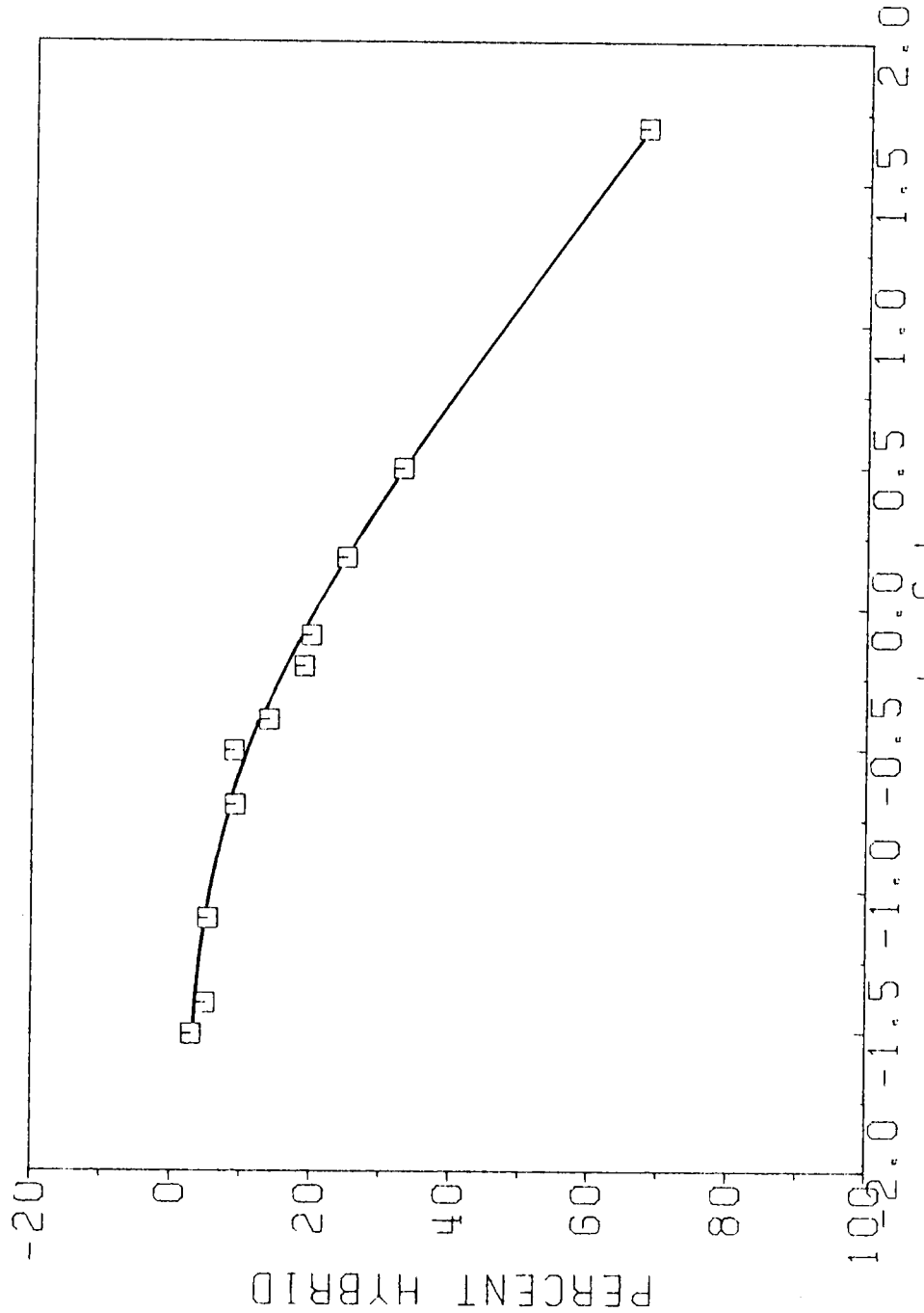


Fig. 3-11. Hybridization kinetics of 12S tumor cDNA with its template RNA.

chain mRNA preparation (Honjo et al., 1974). Since globin mRNA is essentially a 1:1 mixture of α and β globin mRNAs (Orkin et al., 1975) each approximately 600 nucleotides long, the expected C_0t 1/2 value for MOPC-31C light chain (approximately 1,300 nucleotides long) would be $(5x10^{-3})$ X (1300/600+600)=$5.4x10^{-3}$ (Honjo et al., 1974). The calculated purity of the MOPC-31C light chain mRNA is $(5.4x10^{-3}/3)100$ or about 0.2%.

The specificity of the MOPC-31C cDNA was also determined by hybridization to mouse globin mRNA (Table III-1). Thirty-five percent of the MOPC-31C cDNA hybridized to the globin mRNA. Globin cDNA was also hybridized to the MOPC-31C 12S and 15S RNA fractions. Globin cDNA hybridized to the 12S RNA but not the 15S RNA. Appropriate experiments were not performed to determine the percentage of globin mRNA sequences in the MOPC-31C 12S RNA preparation.

Table III-1. Hybridization Specificity of cDNA Preparations

	Source of cDNA	
RNA Source	Globin	MOPC-31C(12S)
globin mRNA	95	35
MOPC-31C(12S)	89	89

Reactions were carried out to a C_0t of $9x10^{-1}$ as described in Materials and Methods except that 10 ul reactions contained 400 ng of RNA. Values shown are percent resistance to S1 nuclease.

Discussion

The purity of the 12S RNA, based on analysis in the cell-free protein synthesizing systems, appears to be greater than that calculated from the hybridization kinetics. This is especially true for the wheat germ system in which a polypeptide with a molecular weight of approximately 27,000 is the major product. The products obtained in the Krebs ascites system also contain a 27,000 molecular weight polypeptide; however, smaller polypeptides are more evident in these products than in those of the wheat germ system. Those polypeptides with molecular weights of 15,000-17,000 could be globin chains. The other RNAs present in the 12S fraction may not be translated well under the conditions employed, or their products may be present at such low concentrations that they are not readily detectable.

It is evident from the broad $C_o t$ range over which light chain cDNA hybridized with its template that some of the other RNAs present in the 12S fraction were transcribed by reverse transcriptase (Fig. 3-11). Since the light chain cDNA was also able to hybridize to some extent with mouse globin mRNA, it was judged unacceptable as a specific probe for the detection of light chain genes or transcripts of these genes. Although it may have been possible to remove cDNA sequences complementary to globin mRNA by hybridization to excess globin mRNA, it would have been difficult to purify the cDNA preparation further.

The purification procedure employed was essentially that used successfully by Honjo et al., (1976), with one exception. They assayed

individual sucrose gradient fractions for the ability to direct the cell-free synthesis of light chains. Future attempts to purify light chain mRNA should include this additional step.

CHAPTER IV

THE SUSCEPTIBILITY OF cDNA LABELED WITH DEOXY[8-^{3}H]GUANOSINE 5'-TRIPHOSPHATE TO TRITIUM EXCHANGE UNDER CONDITIONS USED FOR NUCLEIC ACID HYBRIDIZATION STUDIES

Introduction

Considerable information has been reported on the loss of tritium atoms by exchange from the 8-position of purines in acid or alkaline solutions at various temparatures or in neutral solutions at high temperatures (100° or greater) (for review see Evans et al., 1970). Little information is available concerning the stability of tritiated purines under conditions routinely used for nucleic acid hybridization studies. Harrison et al. (1972) reported that an apparent decrease in the ability of globin cDNA to bind to hydroxyapatite from an initial value of 90% to 60% after 4 weeks of incubation in 0.12M phosphate buffer, pH 6.8 at 60° was due to tritium exchange. It is not clear from this report what labeled precursors were used in the synthesis of the cDNA. If cDNA labeled with ^{3}HdGTP is incubated at 70° for 40 hr in hybridization buffer, 80% of the TCA-precipitable radioactivity is lost. This can be avoided by choosing other labeled precursors for the synthesis of cDNA or by altering the temperature of incubation in the hybridization analysis by the use for formamide.

Materials and Methods

Reagents. The following reagents were used: Chelex 100 (Bio-Rad); dATP, dGTP and dTTP (Sigma); [8-^{3}H]dGTP, 15 Ci/mmol (Amersham/Searle); [5-^{3}H]dCTP, 20 Ci/mmol (Amersham/Searle); [α-^{32}P]dGTP, 150 Ci/mmol (Amersham/Searle); oligo $(dT)_{10}$ (Collaborative Research, Inc.); AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.).

Purification of Globin Messenger RNA

Mouse globin mRNA was prepared as described in Chapter II.

Synthesis of Globin cDNA

The synthesis of globin cDNA using either [^{3}H]dGTP, [^{3}H]dCTP, or [^{32}P]dGTP was essentially as described by Kacian and Myers (1976a). Details are given in Appendix A. The procedure described in Appendix A was modified in some cases to include the passage of the cDNA, after alkaline hydrolysis, through a G-50 Sephadex column containing a layer of Chelex 100 (a chelating resin) in the bottom (Groudine and Weintraub, 1975). This step was included to remove metal ions that may be present in the cDNA preparation, since catalytic degradation of DNA can occur at high temperatures in the presence of metal ions (Britten et al., 1974). Chelex 100 was prepared as described by Willard et al. (1969).

Treatment of DNA Preparations

DNA preparations were placed in 300mM NaCl, 40mM sodium phosphate, pH 6.8 and 2mM EDTA (Buffer A). Two 25 ul aliquots of each mixture were sealed in 40 ul glass capillaries which had been treated as described

by Wilson et al. (1975). After heating at 100° for 2 min, one aliquot was incubated at 70°, or as indicated, and the other aliquot was stored at -20°. At the indicated times each aliquot was precipitated with TCA in the presence of 100 ug of yeast RNA. The percent loss of TCA-precipitable radioactivity was determined using the aliquot stored at -20° as an unincubated control.

Analysis of Treated cDNA Preparations on Sucrose Gradients

cDNA preparations labeled with [^{32}P]dGTP, [^{3}H]dGTP or [^{3}H]dCTP were treated as described above. At the end of 20 hr of incubation, the contents of the capillaries were expelled into 100 ul of buffer A and placed on ice. An aliquot (20 ul) of each mixture was precipitated with TCA and the remainder (100 ul) was placed on 5-20% sucrose gradients containing 10mM Tris (pH 7.5), 100mM KCl and 1mM EDTA. The gradients were centrifuged for 24 hr at 41,000 rpm in a SW41 rotor. The gradients were fractionated into 0.45 ml fractions. An aliquot (0.2ml) of each fraction was counted in 5 ml of Aquasol. The remaining 0.25 ml was precipitated with TCA in the presence of 100 ug of yeast RNA, collected on membrane filters (Millipore, HA) and counted in a toluene-based scintillator containing 0.4% BBOT.

Results

When mouse globin cDNA labeldd with [^{3}H]dGTP was incubated under conditions routinely used for cDNA-RNA or cDNA-DNA hybridization (in buffer A at 70°), 80% of the TCA-precipitable radioactivity was lost after

40 hr of incubation. To insure that this loss did not result from catalytic degradation of the cDNA at high temperatures by metal ions present in the hybridization buffer, the buffer was passed through Chelex 100 (a chelating resin). The cDNA labeled with [^{3}H]dGTP was also passed through a G-50 Sephadex column containing Chelex 100 in the bottom. This cDNA preparation and a preparation of mouse spleen DNA labeled with [^{3}H] thymidine were placed in a Chelex-treated buffer A and incubated as described in Materials and Methods. After 40 hr of incubation, the TCA-precipitable radioactivity was determined (Table IV-1). Again, 80% of TCA-precipitable radioactivity was lost for the cDNA labeled with [^{3}H]dGTP. However, less than 10% of the TCA-precipitable radioactivity was lost for the tritiated mouse spleen DNA.

The loss of TCA-precipitable radioactivity from the cDNA labeled with [^{3}H]dGTP was temperature dependent since it occurred at 70° but not at 25° (Table IV-2). The presence of heterologous DNA in the reaction mixture with the cDNA labeled with [^{3}H]dGTP did not prevent the loss of radioactivity at 70° (Table IV-3).

The following experiment was performed to demonstrate that the loss of TCA-precipitable radioactivity from cDNA labeled with [^{3}H]dGTP at 70° was due to tritium exchange. Globin cDNA labeled with either [^{3}H]dGTP, [^{3}H]dCTP or [^{32}P]dGTP was synthesized. These cDNA preparations were placed in buffer A and incubated as described in Materials and Methods. After 20 hr of incubation, an aliquot of each mixture was precipitated with TCA (Table IV-4). Fifty-five percent of the TCA-precipitable radioactivity was lost for cDNA labeled with [^{3}H]dGTP;

Table IV-1. Incubation of labeled DNAs in Chelex-treated buffer.

Type of labeled DNA	Globin cDNA labeled with [^{3}H]dGTP		Mouse spleen DNA labeled with [^{3}H] thymidine	
Incubation temperature	70°	-20°	70°	-20°
TCA-precipitable cpm	296	1490	756	828
Percent loss of TCA-precipitable radioactivity	80	-	9	-

Reactions were performed as described in Materials and Methods. Incubation was for 40 hr.

Table IV-2. Effect of temperature on loss of TCA-precipitable radioactivity from cDNA labeled with [^{3}H]dGTP.

Incubation temperature	25°	-20°	70°	-20°
TCA-precipitable cpm	1220	1225	730	1215
Percent loss of TCA-precipitable radioactivity	0.4	-	40	-

Reactions were performed as described in Materials and Methods. Incubation was for 12 hr.

Table IV-3. Effect of heterologous DNA on loss of TCA-precipitable radioactivity from cDNA labeled with [^{3}H]dGTP

Incubation temperature	70°	-20°	70°	-20°
Streptococcus faecalis DNA (1mg/ml)	+	+	-	-
TCA-precipitable cpm	316	1204	369	1213
Percent loss of TCA-precipitable radioactivity	74	-	70	-

Reactions were performed as described in Materials and Methods. Incubation was for 30 hr.

Table IV-4. The stability of cDNA labeled with [^{3}H]dGTP, [^{3}H]dCTP or [^{32}P]dGTP at 70°.

Source of label	[^{3}H]dGTP		[^{3}H]dCTP		[^{32}P]dGTP	
Incubation temperature	70°	-20°	70°	-20°	70°	-20°
TCA-precipitable cpm	1386	3082	1056	1089	3045	2934
Percent loss of TCA-precipitable radioactivity	55	-	3	-	0	-

Reactions were performed as described in Materials and Methods. Incubation was for 20 hr.

however, essentially no loss occurred for cDNA labeled with [^{3}H]dCTP or [^{32}P]dGTP. An aliquot of each mixture was also placed on 5-20% sucrose gradients; centrifugation was as described in Materials and Methods. The gradients were fractionated and total radioactivity was determined for part of each fraction and TCA-precipitable radioactivity for the remainder.

The majority of the radioactivity of the cDNA labeled with [^{3}H]dGTP incubated at 70° was located at the top of the gradient (Fig. 4-1A). This radioactivity was due to tritiated water since it was not TCA-precipitable (Fig. 4-1B). The remainder of the radioactivity in this gradient was located at the same position as observed for cDNA labeled with [^{3}H]dGTP that had been stored at -20°. No radioactivity was observed at the top of the gradients for cDNA labeled with [^{32}P]dGTP (Fig. 4-2A) and cDNA labeled with [^{3}H]dCTP (Fig. 4-3A). The profiles of the gradients for these cDNAs, incubated at either temperature, were essentially identical (Figs. 4-2, 4-3).

The loss of tritium, due to exchange, from the cDNA labeled with [^{3}H]dGTP could be avoided by altering the conditions used for hybridization analysis. McConaughy et al. (1969) have used formamide to reduce the temperature of hybridization (1% formamide reduces the T_m by 0.72°). The addition of 50% formamide to buffer A made it possible to lower the incubation temperature to 43° (Harrison et al., 1974). Under this condition, tritium exchange did not occur (Table IV-5).

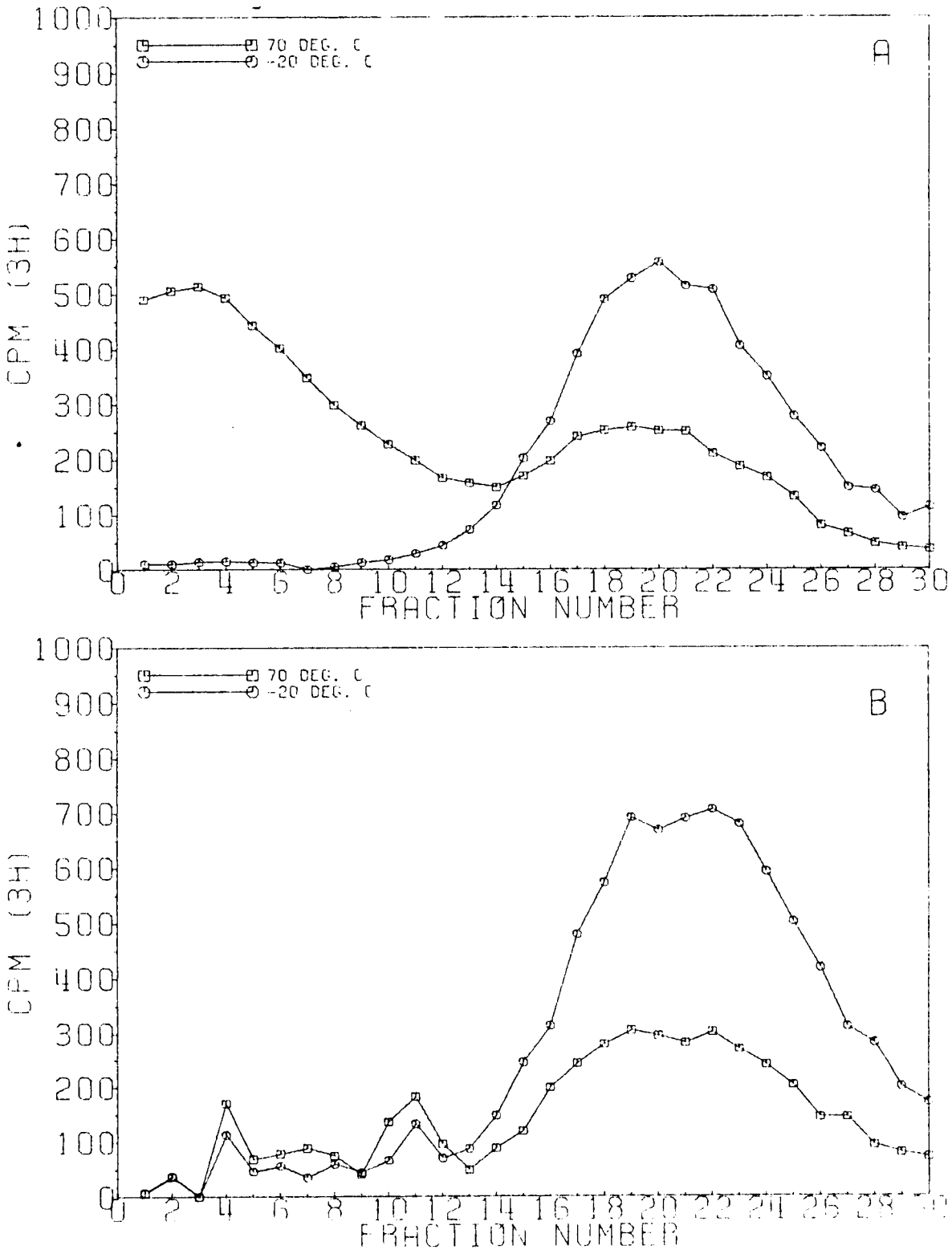


Figure 4.1. Sucrose gradient analysis of cDNA labeled with [$^{3}_{H}$] dGTP incubated at 70° or -20°. Sedimentation was from left to right and centrifugation was as described in Materials and Methods. Fractions were assayed for total radioactivity (A) or TCA-precipitable radioactivity (B).

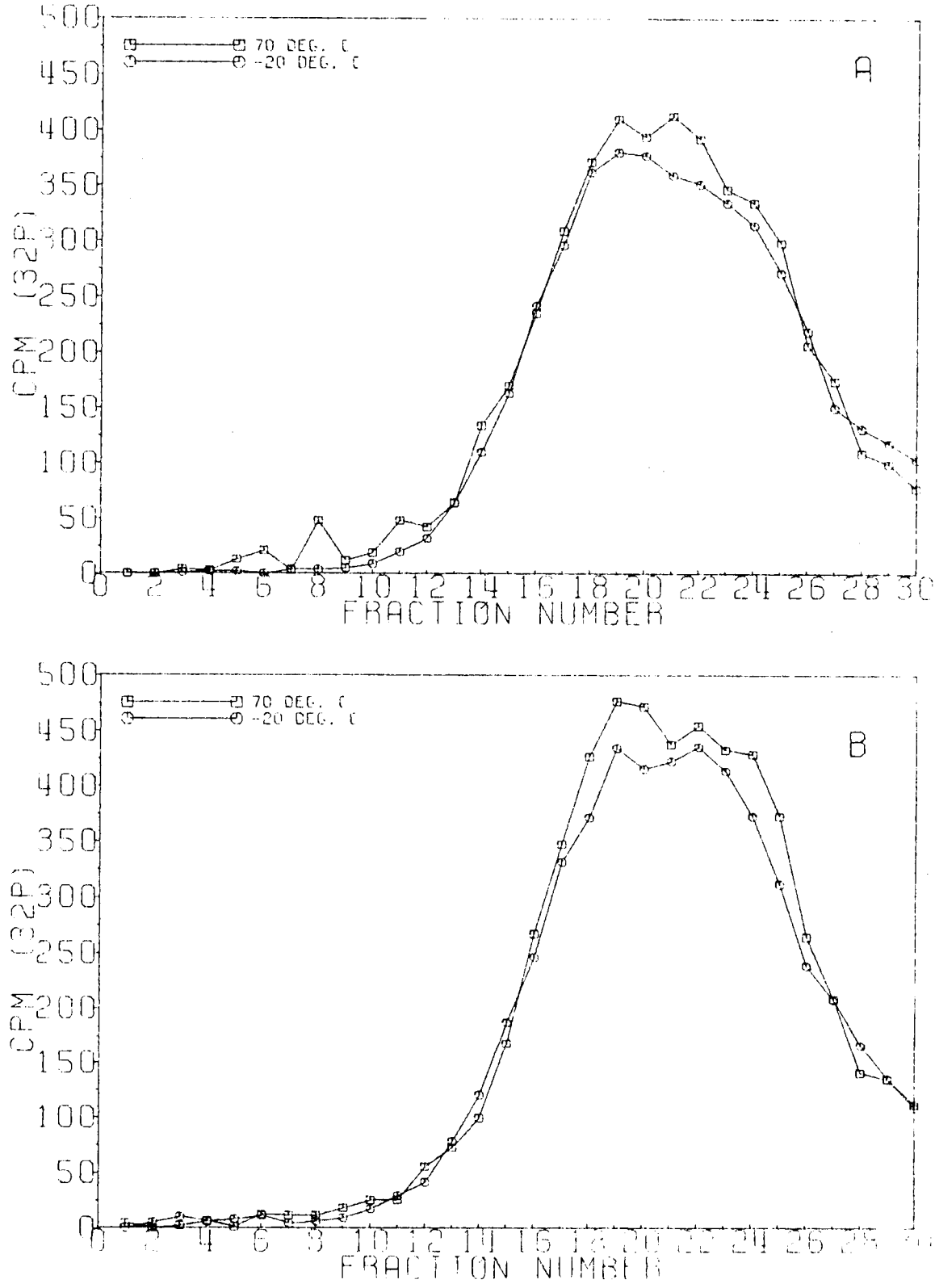


Figure 4-2. Sucrose gradient analysis of cDNA labeled with [32P] dGTP incubated at 70° or -20°. Sedimentation was from left to right and centrifugation was as described in Materials and Methods. Fractions were assayed for total radioactivity (A) or TCA-precipitable radioactivity (B).

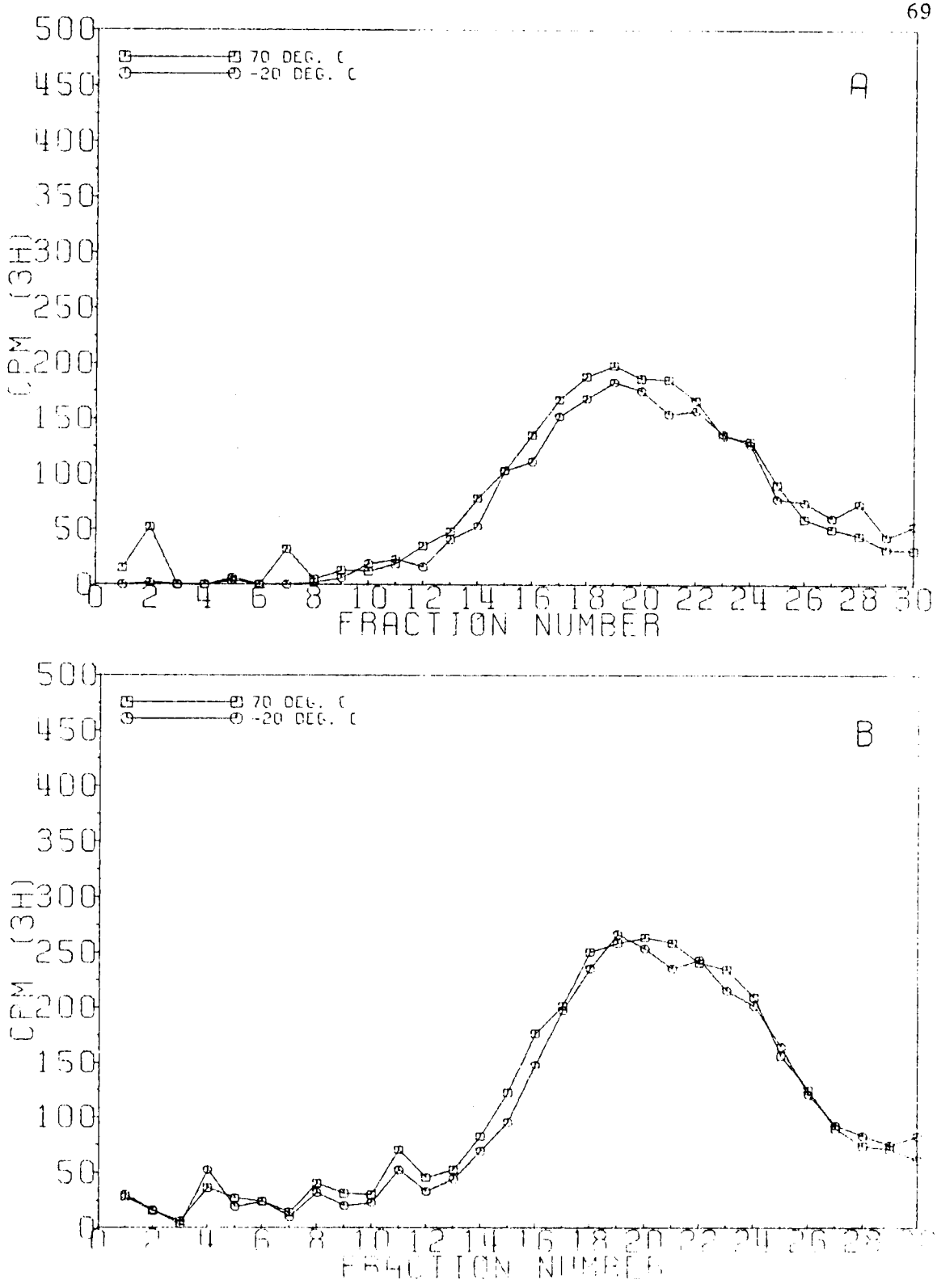


Figure 4-3. Sucrose gradient analysis of cDNA labeled with [$^{3}_{H}$] dCTP incubated at 70° or -20°. Sedimentation was from left to right and centrifugation was as described in Materials and Methods. Fractions were assayed for total radioactivity (A) or TCA-precipitable radioactivity (B).

Table IV-5. Effect of incubation conditions on loss of TCA-precipitable radioactivity.

Source of label	[^{3}H]dGTP				[^{32}P]dGTP			
Incubation temperature	43°	-20°	70°	-20°	43°	-20°	70°	-20°
Formamide (50% v/v)	+	+	-	-	+	+	-	-
TCA-precipitable cpm	1927	1959	849	1949	1316	1289	1263	1287
Percent loss of TCA-precipitable radioactivity	2	-	56	-	0	-	2	-

Reactions were performed as described in Materials and Methods. Incubation was for 20 hr.

Discussion

The use of [^{3}H]dGTP as a precursor for the synthesis of labeled cDNA should be avoided since this cDNA will be susceptible to tritium exchange under the conditions used for nucleic acid hybridization studies. On the basis of other studies concerning the stability of [^{3}H] adenine under various conditions (Evans et al., 1970), the use of [^{3}H]dATP as a precursor should not be considered since the tritium in the 8-position will also be subject to exchange. The tritiated precursor of choice for the synthesis of DNA complementary to poly(A)-containing mRNA is dCTP. No apparent exchange occurred where cDNA labeled with [^{3}H]dCTP was incubated in buffer A at 70° for 20 hr.

For occasions when it is desirable to use highly labeled cDNA, cDNA can be synthesized using [^{3}H]dGTP, [^{3}H]dATP and [^{3}H]dCTP. Tritium

exchange of this cDNA, during hybridization reactions, can be avoided by using formamide and lowering the incubation temperature.

CHAPTER V

THE DISTRIBUTION OF GLOBIN GENES IN FRACTIONATED CHROMATIN

Introduction

The initial step in all procedures used to fractionate chromatin into transcriptionally active and inactive components involves fragmenting the chromatin, either by sonication, mechanical shear or enzyme digestion. Two of the criteria used to designate the active component (increased template activity when assayed with E. coli RNA polymerase and lower thermal stability) will also detect damaged chromatin (i.e., nicked or protein-free regions of DNA resulting from the fragmentation process). The criterion of localization of labeled nascent RNA with the active component has also recently been challenged (Seidman and Cole, 1977). Because the use of these criteria in assaying fractionated chromatin is subject to criticism, a more stringent assay is desirable.

A clever approach used by Howk et al. (1975) involved fractionating chromatin from mouse cells infected with the Moloney strain of murine leukemia virus on ECTHAM cellulose (Reeck et al., 1972) and by differential sedimentation in glycerol gradients (Murphy et al., 1973). They used cDNA for type C virus to assay for the presence of active genes and cDNA for type B virus to assay for the presence of inactive genes in the chromatin fractions. No difference in the distribution of sequences homologous to Moloney type C virus or type B virus was observed in the active and inactive components obtained by either technique. This report

has recently been criticized for the choice of type C and type B viral sequences as representing chromosomally located active and inactive genes since the percentage of cells active in the transcription of integrated viral genomes was not known (Krieg and Wells, 1976).

Gottesfeld et al. (1974) reported an enrichment of nonrepetitive DNA sequences complementary to total cellular RNA in the active fraction (SII) obtained by DNase II digestion and differential $MgCl_2$ precipitation. No information is available concerning the distribution of specific genes in chromatin fractionated by this procedure.

Because of the choice of probes by Howk et al. (1975) in evaluating the ECTHAM-cellulose and differential sedimentation procedures and the lack of information concerning the distribution of specific genes in chromatin fractionated by the DNase II technique, it was of interest to use these methods to fractionate chromatin from a source that was actively transcribing a specific gene and to determine the distribution of this gene in the various fractions. Mouse globin genes were chosen because of the relative ease in isolating their messenger RNAs, and because initially the use of the mouse light chain gene as an inactive gene was anticipated. Livers obtained from mouse fetuses during the 16th day of gestation were chosen as the source of chromatin since 55-70% of these cells are erythropoietic (Paul et al., 1969).

Materials and Methods

Animals. Timed pregnant BALB/c(Cum) mice were obtained from Cumberland View Farms, Clinton, TN. The morning after mating is

designated day zero of the gestation. Pregnant mice were killed by cervical dislocation on the 16th day of gestation. Chromatin was prepared from livers which were dissected out of the fetuses.

Reagents. The following reagents were used: RNase-free sucrose (Schwarz-Mann); S1 nuclease (Sigma); Proteinase K (EM Laboratories, Inc.); E. coli RNA polymerase (Miles); DNase II (Worthington, HDAC).

Isolation of Nuclei

Nuclei were isolated from fetal livers by a modification of the procedure described by Humer and Kuff (1964). Details are given in Appendix A.

Preparation of Chromatin

Chromatin was prepared from nuclei isolated from fetal livers essentially as described by Shaw and Huang (1970). Details are given in Appendix A.

Sonication of Chromatin

This procedure is described in Appendix A.

Fractionation of Chromatin by ECTHAM-cellulose Chromatography

Sonicated fetal liver chromatin was fractionated on ECTHAM cellulose as described by Reeck et al. (1972). Details are given in Appendix A.

Fractionation of Chromatin on Sucrose Gradients

Sonicated fetal liver chromatin was fractionated on 0.17-1.7M sucrose gradients as described by Murphy et al. (1973). Details are given in Appendix A.

Fractionation of Chromatin with DNase II

Fetal liver chromatin was fractionated by DNase II digestion followed by differential precipitation with $MgCl_2$ as described by Gottesfeld et al. (1974). Details are given in Appendix A.

Assay of Template Activity of Chromatin with E. coli RNA Polymerase

This procedure is described in Appendix A.

Extraction of DNA from Chromatin

DNA was extracted from chromatin as described in Appendix A. The DNase II SII DNA was further purified by chromatography on G-50 Sephadex.

Thermal Analysis of Chromatin or DNA

This procedure is described in Appendix A.

Purification of Globin Messenger RNA

Mouse globin mRNA was prepared as described in Chapter II.

Synthesis of Globin cDNA

The synthesis of globin cDNA was essentially as described by Kacian and Myers (1976a). Details are given in Appendix A.

DNA-cDNA Hybridization Analysis

This procedure is described in Appendix A.

S1 Nuclease Assay of cDNA-DNA Hybrids

This procedure is described in Appendix A.

Results

ECTHAM-cellulose Fractionation

The elution profile of sonicated mouse fetal liver chromatin from ECTHAM cellulose is shown in Fig. 5-1. On the basis of previous experience with this procedure, fractions 50-56 (designated early), 57-67 (designated middle) and 68-92 (designated late) were pooled. Some of the properties of these fractions are shown in Table V-1. The greater template activity when assayed with E. coli RNA polymerase and and the lower Tm of the late fraction are consistent with results reported by Reeck et al. (1972) and Simpson (1974).

Sucrose Gradient Fractionation

Sonicated mouse fetal liver chromatin was fractionated by centrifugation on 0.17-1.7M sucrose gradients. The gradients were fractionated and pooled into three components: A(fractions 5-9), B(fractions 10-13) and C(fractions 14-18) (Fig. 5-2). Some of the characteristics of these components are shown in Table V-1. The increased template capacity for E. coli RNA polymerase and the lower Tm for the slowly sedimenting component are in agreement with other reports

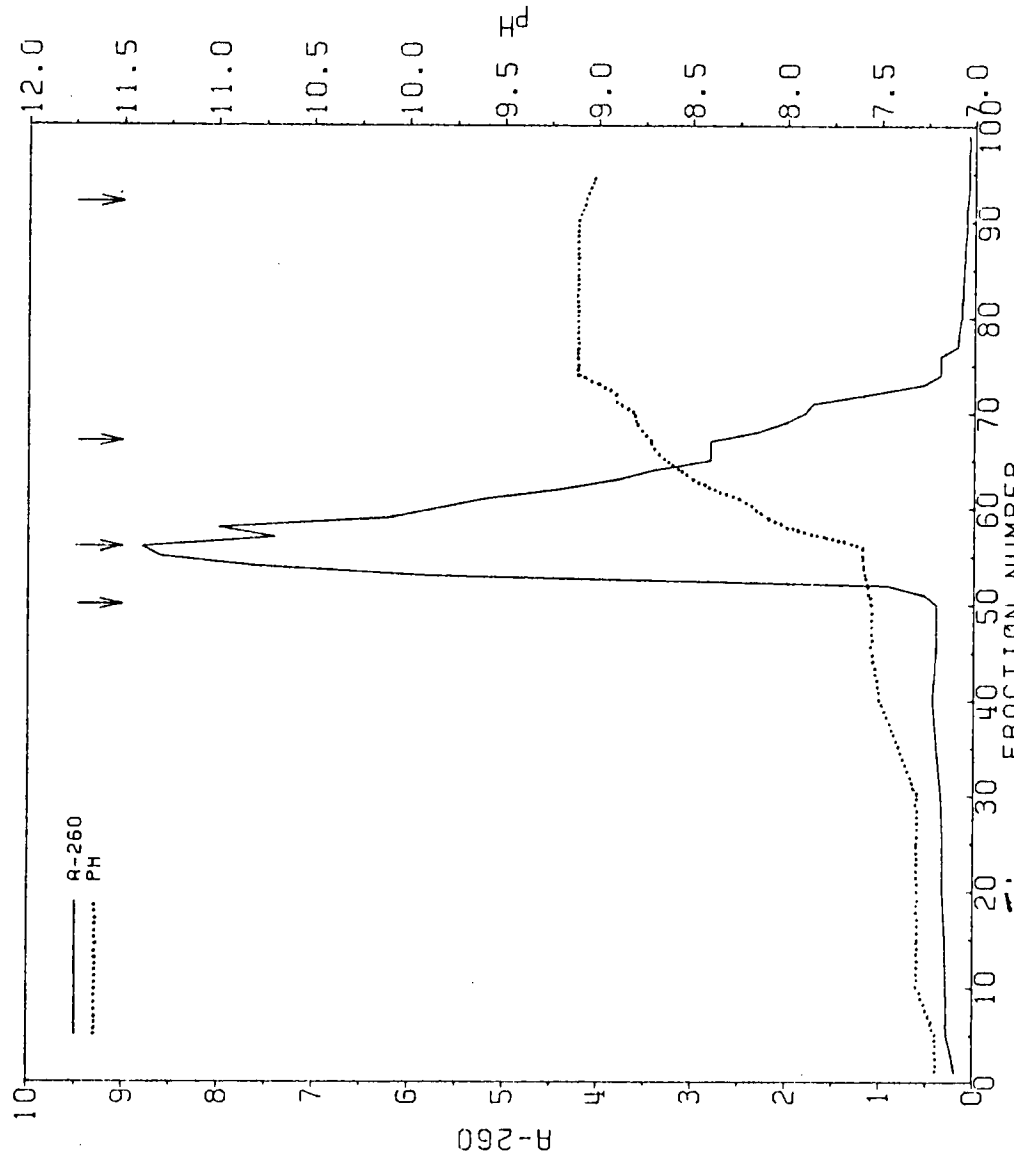


Fig. 5-1. ECTHAM-cellulose frationation of fetal liver chromatin.

Table V-1. Characteristics of fractionated chromatin.

	Template activity of chromatin (^{3}H UMP cpm incorporated X 10^{-3}/A260)[a]	T_m value of chromatin[b]	Percent hyperchromicity	T_m value of DNA extracted from chromatin	Percent hyperchromicity
ECTHAM Early	42	77.1	37.7	81.2	41.0
" Middle	136	75.2	36.8	81.5	40.2
" Late	300	73.1	35.3	81.6	39.0
Sucrose A	271	72.9	30.6	81.1	39.5
" B	228	75.1	37.8	81.7	40.8
" C	99	76.4	34.8	82.0	41.2
DNase II PI	81	79.0	35.3	79.6	38.8
" PII	113	78.4	33.1	77.4	37.6
" SII	182	70.0	24.1	70.2	25.7
Fetal liver chromatin (unfractionated)	93	77.0	36.6	81.9	39.8
Fetal liver chromatin (unfractionated, sonicated)	190	75.9	35.0	81.3	41.2

[a]Assayed with E. coli RNA polymerase as described in Appendix A.

[b]T_m values were determined as described in Appendix A.

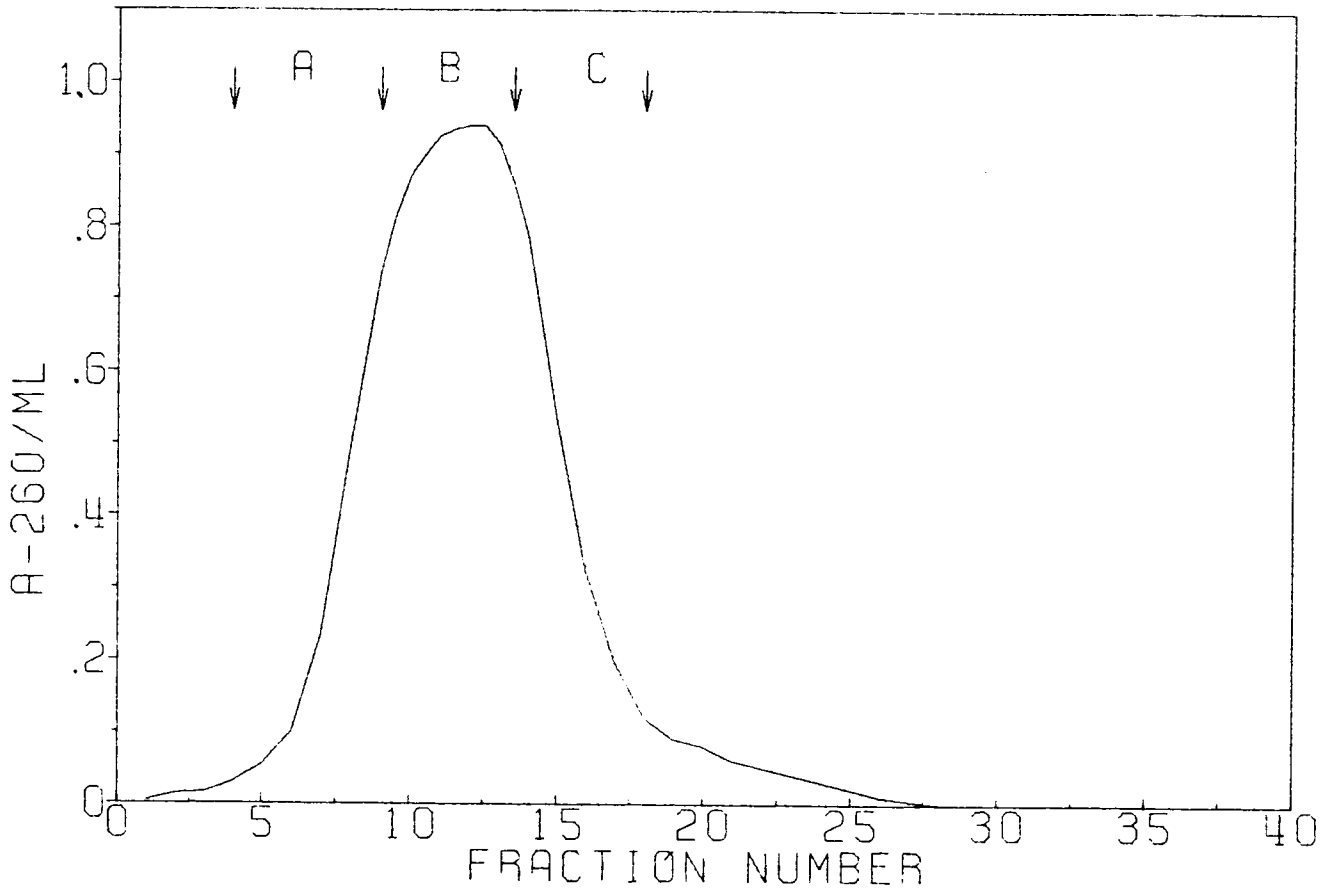


Fig. 5-2. Fractionation of fetal liver chromatin on 0.17 - 1.7 M sucrose gradients. Sedimentation was from left to right and centrifugation was as described in Materials and Methods.

concerning this fractionation technique (Murphy et al., 1973); McCarthy et al., 1973; Berkowitz and Doty, 1975).

DNase II Fractionation

The procedure of Gottesfeld et al. (1974) which consists of digestion of chromatin with DNase II followed by differential precipitation with $MgCl_2$ was used to fractionate mouse fetal liver chromatin. The fractionation scheme was the same as shown in Fig. 1-4, p. 10). The SII fraction has the highest template activity when assayed with E. coli RNA polymerase and also has a considerably reduced Tm when compared with the PI and PII fractions or unfractionated chromatin (Table V-1). The increased template activity observed for the SII fraction is in agreement with studies reported by Bonner et al. (1973).

Thermal Denaturation Analysis of DNA from Chromatin Fractions

DNA was extracted from the chromatin fractions obtained by the different fractionation methods described above. The Tm values for these DNAs are essentially the same with the exception of the PII and SII DNA which are lower (Table V-1). The DNA from the SII fraction has a considerably lower Tm and the percent hyperchromicity is greatly reduced compared to DNA from the other fractions.

Reassociation Kinetics of Globin cDNA with DNA from Chromatin Fractions

Mouse globin cDNA was reassociated to DNA from the various fractions under conditions of DNA to cDNA excess ($8x10^6$ to $1x10^7$: 1). The

reassociation kinetics of globin cDNA to DNA extracted from unfractionated mouse fetal liver chromatin are shown in Fig. 2-10, p. 33. The $C_o t$ 1/2 for this curve is approximately 100. Reassociation kinetics of globin cDNA to DNA from ECTHAM-cellulose fractions are shown in Fig. 5-3. These curves are essentially the same and have a $C_o t$ 1/2 of approximately 100. Curves for the reassociation of globin cDNA to DNA from sucrose gradient fractions are very similar (Fig. 5-4). The $C_o t$ 1/2 value for these curves (approximately 140) is slightly slower than that observed for cDNA to fetal liver DNA. The reassociation kinetics of globin cDNA to DNA from DNase II fractions are shown in Fig. 5-5. The $C_o t$ 1/2 value for the PI fraction is approximately 100. The PII and SII fractions have $C_o t$ 1/2 values of approximately 110. Under the conditions employed, the SII DNA protects only about 50% of the cDNA from S1 nuclease digestion at saturation compared to 60-65% protection for the PI and PII fractions.

Discussion

On the basis of increased template activity for E. coli RNA polymerase and lower thermal stability, all of the fractionation methods employed seem to yield an active fraction. However, within experimental error, all the curves obtained for the reassociation of globin cDNA with DNA from the various chromatin fractions are the same. Globin genes are not preferentially located in the active fractions obtained by fractionation on ECTHAM cellulose or by differential sedimentation on sucrose gradients. Although the report by Howk et al. (1975) has been criticized

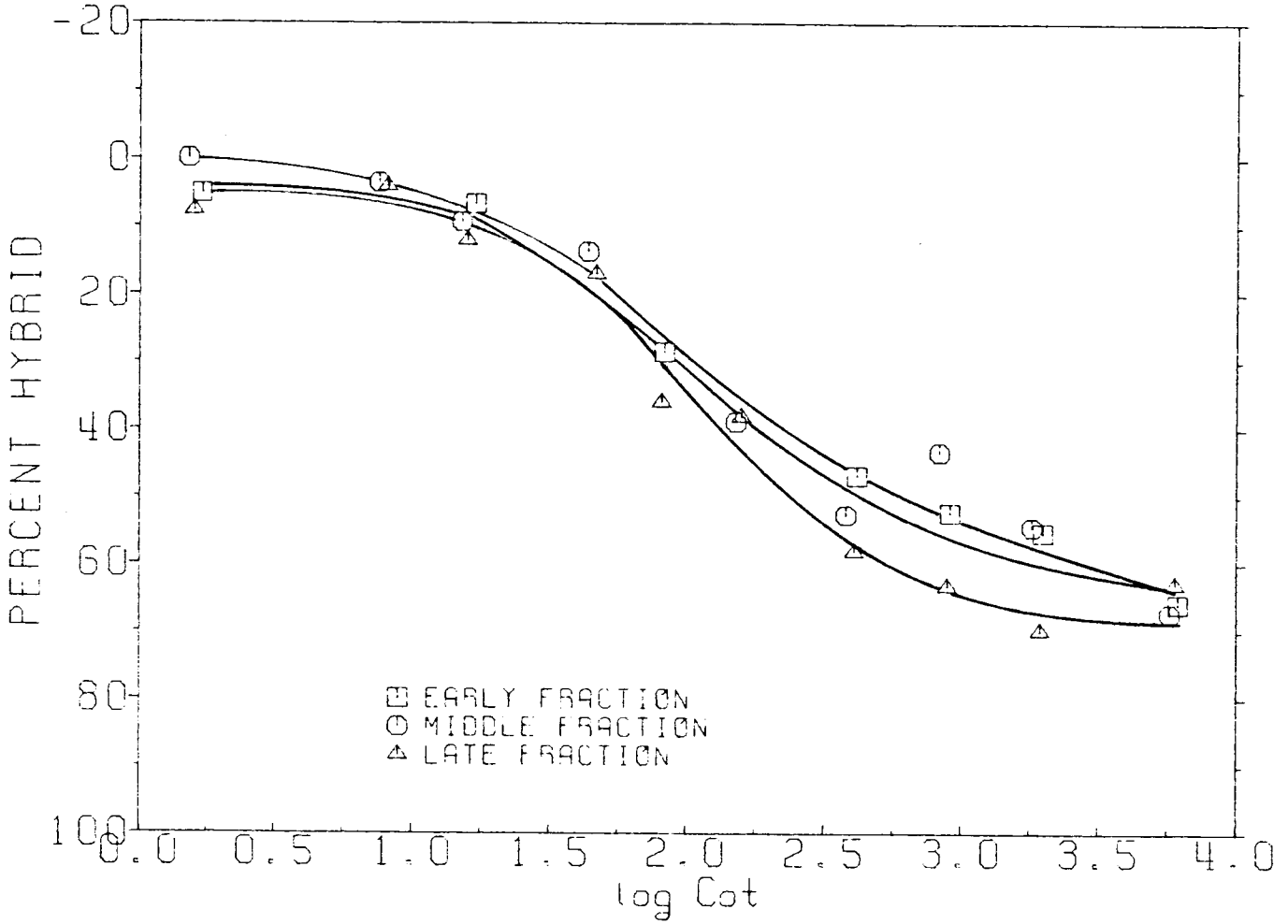


Fig. 5-3. Hybridization kinetics of globin cDNA with DNA from chromatin fractionated on ECTHAM cellulose.

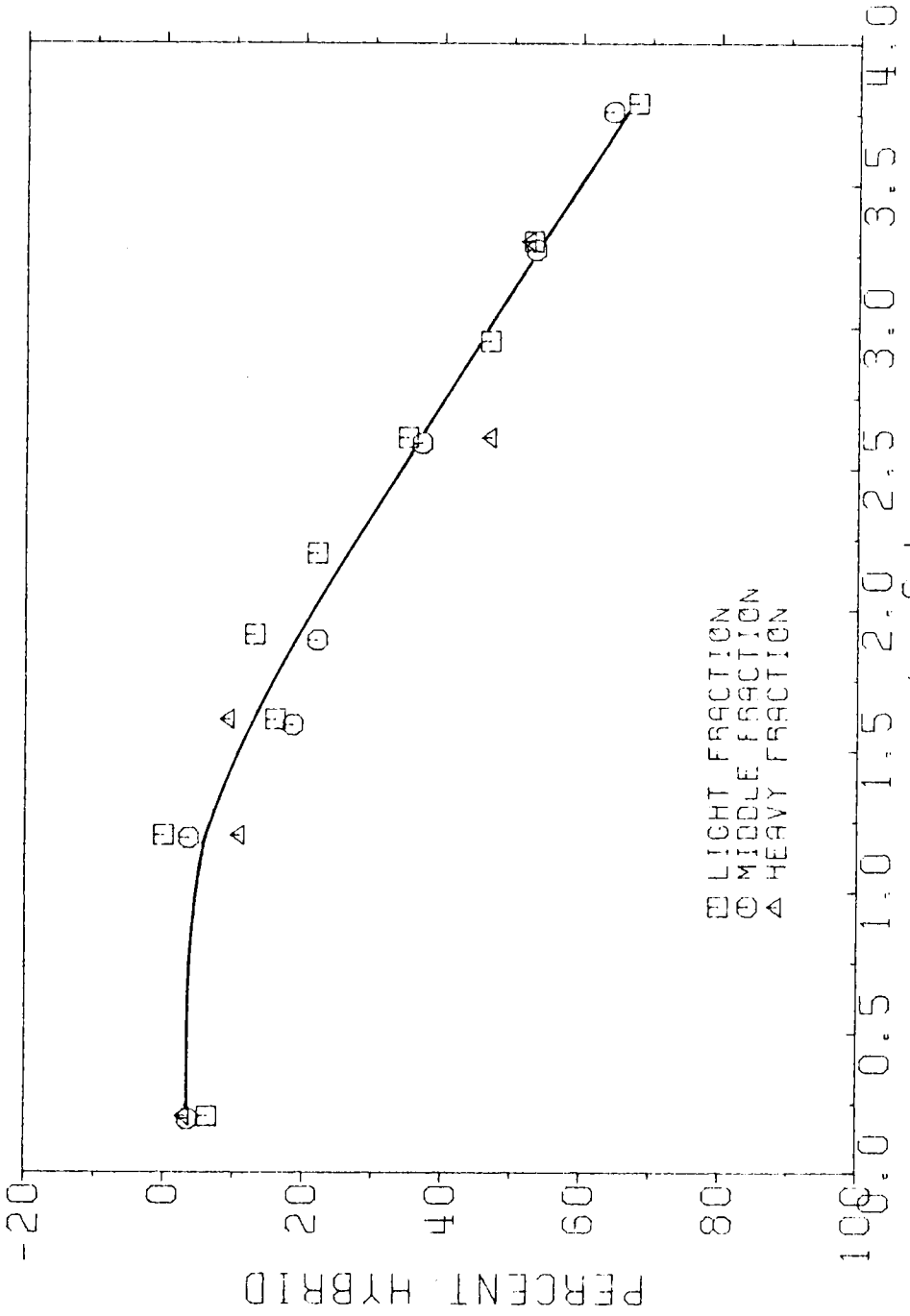


Fig. 5-4. Hybridization kinetics of globin cDNA with DNA from chromatin fractionated on sucrose gradients.

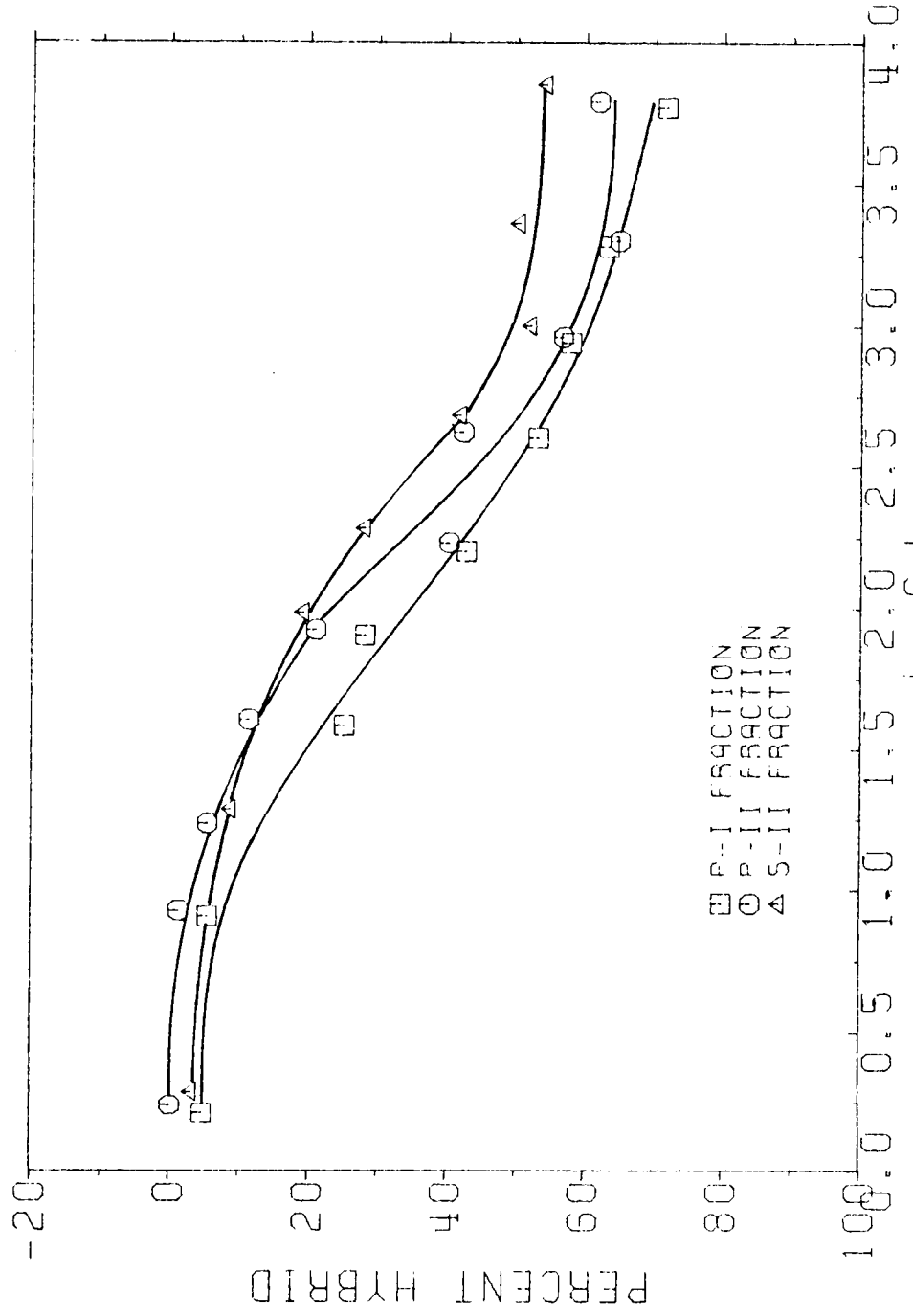


Fig. 5-5. Hybridization kinetics of globin cDNA with DNA from chromatin fractionated with DNase II.

for the choice of probes for active and inactive genes, the results of this study support their earlier findings.

Gottesfeld et al. (1974) have reported a 6- to 7-fold enrichment of single-copy sequences in the active fraction (SII) obtained by DNase II digestion that are homologous to total RNA synthesized in rat liver. The results of this study do not suggest an enrichment of globin genes in the SII fraction. It is interesting that the SII DNA appears to protect less globin cDNA from digestion with S1 nuclease at saturation than the DNA of the PI and PII fractions. Weintraub and Groudine (1976) and Garel and Axel (1976) have reported that treatment of nuclei from tissues actively transcribing globin or ovalbumin genes with DNase I results in the preferential digestion of these genes. It is not known if DNase II is capable of this same action.

Recently Krieg and Wells (1976) have reported that there was no preferential localization of globin genes in the active fraction of chicken reticulocyte chromatin fractionated by the gel-exclusion technique developed by Janowski et al. (1972). They also assayed for the presence of an inactive gene (keratin) in these chromatin fractions and found that they were equally distributed among the fractions. This technique appeared to fractionate chromatin into active and inactive components as judged by the distribution of labeled nascent RNA and the thermal denaturation profiles of the fractions.

Willetts and Wells (1976) have also reported that on the basis of globin gene distribution, minimal fractionation of chicken reticulocyte chromatin may occur when it is sheared and fractionated by buoyant density centrifugation in urografin solutions.

The bimodal distribution of highly sheared chromatin from Friend cells (uninduced or induced with dimethylsulphoxide) on metrizamide gradients has been reported (Rickwood et al., 1974). These fractions, whether from induced or uninduced cells, exhibited no difference in globin gene content. Unlike other separation techniques, fractions obtained with this technique showed little difference in template activity when assayed with E. coli RNA polymerase.

On the basis of the gene distribution data obtained in this study, the ability of the three methods examined to fractionate chromatin into transcriptionally active and inactive components must be questioned. This finding is supported by several published reports discussed above. Since the homogeneity of the cell populations used in these types of experiments is extremely important, caution should be used in interpreting the data.

CHAPTER VI

TRANSCRIPTIONAL SPECIFICITY OF FRACTIONATED CHROMATIN

Introduction

The criteria routinely used in assaying fractionated chromatin for the transcriptionally active component are subject to criticism. For this reason it was desirable to develop more stringent criteria for evaluating chromatin fractionation procedures. One method employed, an assay for the distribution of globin genes in fractionated fetal liver chromatin, was discussed in Chapter V. Another potentially useful method consists of assaying fractionated chromatin for the ability to support the synthesis of specific genes. Homologous RNA polymerase is the enzyme of choice for this assay; however, it is difficult to obtain sufficient quantities of this enzyme with purification methods presently available. Jendrisak and Burgess (1975) have recently described a method for the large-scale purification of wheat germ RNA polymerase II. Because of the relative ease of this purification procedure, this enzyme was chosen for these studies. The ECTHAM-cellulose and DNase II fractionation procedures were chosen for evaluation.

Materials and Methods

Animals. Male BALB/c (Cum) mice, 14 wk-old, were obtained from Cumberland View Farms, Clinton, TN.

Timed pregnant BALB/c (Cum) mice were obtained from Cumberland View Farms, Clinton, TN. The morning after mating was designated day zero of gestation. Pregnant mice were killed by cervical dislocation on the 16th day of gestation. Chromatin was prepared from livers which were dissected out of the fetuses.

Reagents. The following reagents were used: S1 nuclease (Sigma); Proteinase K (EM Laboratories, Inc.); DNase II (Worthington HDAC); 5-Mercuri-Uridine 5'-triphosphate (PL Biochemicals, Inc.); ATP, UTP, CTP, and TTP (Sigma); wheat germ (donated by Dixie Portland Flour Mills, Chattanooga, TN); Polymin P (donated by BASF, Rhein, Germany); Affi-Gel 401 (Bio-Rad Laboratories); [^{3}H] GTP 13.8 Ci/mmol (New England Nuclear); [^{32}P] GTP 6.17 Ci/mmol (Amersham/Searle); [^{3}H] UTP 16 Ci/mmol (Schwartz/Mann).

Methods

Isolation of Nuclei

Nuclei were isolated from adult or fetal livers by a modification of the procedure described by Hymer and Kuff (1964). Details are given in Appendix A.

Preparation of Chromatin

Chromatin was prepared from nuclei isolated from adult or fetal liver essentially as described by Shaw and Huang (1970). Details are given in Appendix A.

Sonication of Chromatin

This procedure is described in Appendix A.

Fractionation of Chromatin by ECTHAM-cellulose Chromatography

Sonicated fetal liver chromatin was fractionated on ECTHAM cellulose as described by Reeck et al. (1972). Details are given in Appendix A.

Fractionation of Chromatin with DNase II

Fetal liver chromatin was fractionated by DNase II digestion followed by differential precipitation with $MgCl_2$ as described by Gottesfeld et al. (1974). Details are given in Appendix A.

Concentration of Chromatin Fractions

This procedure is described in Appendix A.

Purification of Wheat Germ RNA Polymerase II

Wheat germ RNA polymerase II was purified by the method of Jendrisak and Burgess (1975) except that storage conditions were modified as recently described by them (Jendrisak and Burgess, 1977). The specific activity of the enzyme was 260 units/mg when assayed as described by Jendrisak and Burgess (1975). Protein concentration was by spectral analysis, $E_{280}^{1\%}$ =7.7 (Jendrisak et al., 1976).

Assay of Template Activity of Chromatin with Wheat Germ RNA Polymerase II

The wheat germ RNA polymerase II assay was incubated at 25° for 1 hr. It contained in a volume of 1 ml: 0.01M Tris, pH 7.9; 2mM $MnCl_2$; 50mM $(NH_4)_2 SO_4$; 0.4mM each GTP, CTP, and ATP; 4 uCi [^{3}H]UTP (14Ci/mmol); 100 ug BSA; 5 units of wheat germ RNA polymerase II; and 0.05 OD_{260} units of chromatin. Reactions were stopped by the addition of 0.5 ml of cold

20% TCA containing 0.01 M sodium pyrophosphate. After 10 min at 0°, the reactions were filtered on membrane filters (Millipore,HA), washed with cold 5% TCA, dried and counted in toluene-based scintillator containing 0.4% BBOT.

In Vitro Synthesis of RNA

RNA was synthesized from chromatin as follows: 50mM Tris (pH 7.9); 2mM $MnCl_2$; 10mM 2-mercaptoethanol; 150mM KCl; 1mM ATP, GTP and CTP; 0.5mM UTP; 0.5mM HgUTP; chromatin 130ug/ml (concentration determined from A_{260} in 0.9N NaOH, 27 units/mg (Gottesfeld et al., 1974)); and wheat germ RNA polymerase II 50 ug/ml. Incubation was 4 hr at 25°C. Reactions were immediately treated with 0.5% SDS and 50 ug/ml Proteinase K for 30 min at 37°C. RNA was extracted with phenol:chloroform:isoamyl alcohol (50:50:1) twice, and with chloroform:isoamyl alcohol (50:1) once. The aqueous phase was precipitated with 0.1 volume of 20% CH_3COOK (pH 5.5) and 2.5 volumes of 95% ethanol overnight at -20°C.

Purification of the in Vitro Synthesized RNA

The precipitated nucleic acids were dissolved in 1.5 ml of water and made 10mM Tris (pH 7.5), 50mM NaCl, 0.1mM EDTA. Triphosphates were separated from the higher molecular weight material by chromatography on Sephadex G-50 (coarse). The excluded material was precipitated by the addition of 0.1 volume of 20% CH_3COOK (pH 5.5) and 2.5 volumes of 95% ethanol and stored at -20°C overnight. The precipitated nucleic acid was dissolved in 1.5 ml of water, heated at 80°C for 2 min and quick cooled. It was then made TNE (10mM Tris (pH 7.5) - 100mM NaCl-0.1mM EDTA)

and loaded onto a sulfhydryl affinity column (Bio-Rad Affi-Gel 401) at 4°C. The 10 ml column was washed thoroughly with TNE to remove DNA and endogenous RNA. The HgUTP-containing RNA was then eluted from the column with TNE containing 0.1M 2-mercaptoethanol and precipitated with 0.1 volume of 20% CH_3COOK (pH 5.5) and 2.5 volumes of ethanol at -20°C.

Purification of Globin Messenger RNA

Mouse globin mRNA was prepared as described in Chapter II.

Synthesis of Globin cDNA

The synthesis of globin cDNA was essentially as described by Kacian and Myers (1976a). Details are given in Appendix A.

RNA-cDNA Hybridization Analysis

This procedure is described in Appendix A.

S1 Nuclease Assay of RNA-cDNA Hybrids

This procedure is described in Appendix A.

Results

Conditions for in Vitro Transcription

Conditions similar to those described by Towle et al. (1977) were used for the *in vitro* transcription of chromatin with wheat germ RNA polymerase II. They have observed that lowering the enzyme to chromatin ratio increased the fidelity of *in vitro* transcription. In order to eliminate contaminating DNA and endogenous RNA in the *in vitro* transcripts, the mercuration technique of Dale et al. (1975) and Dale and Ward (1975) was employed.

The *in vitro* transcription kinetics from fetal liver DNA (single-stranded) and chromatin as template are shown in Fig. 6-1. The enzyme to template ratio was 0.4. Conditions are as described in *Materials and Methods* except that reactions included 0.05mM [^{3}H]GTP (13.8 Ci/mmol). Under these conditions synthesis of RNA continued for 4 hr. Chromatin acts as a restricted template compared with single-stranded DNA for wheat germ RNA polymerase II. The presence of Hg UTP in the reaction mixture reduced the rate of synthesis from DNA and chromatin. This is in agreement with published reports (Smith and Huang, 1976; Dale and Ward, 1975; Biessmann et al., 1976). Chromatin used in these experiments has no endogenous polymerase activity (Fig. 6-1).

The specificity of binding of HgUTP-containing RNA to the sulfhydryl affinity column (Affi-Gel 401) is shown in Fig. 6-2. RNA was synthesized from single-stranded DNA as described in *Materials and Methods* except that one reaction contained 1mM UTP and 0.05mM[^{3}H]UTP (16 Ci/mmol) the other contained .5mM UTP, .5mM HgUTP and 0.05mM[^{32}P]GTP. After 1 hr incubation at 25°, the *in vitro* synthesized RNA was purified as described in *Materials and Methods.* Approximately 50,000 cpm of [^{3}H]RNA containing no mercury substitutions and 50,000 cpm of [^{32}P]RNA containing HgUTP were mixed together in TNE and subjected to chromatography on the sulfhydryl affinity column as described in *Materials and Methods* except that the mixture was not heated prior to application. Approximately 90% of the [^{3}H]RNA failed to bind to the column in TNE. The amount of Hg-RNA binding to the column was estimated to be approximately 80%, however, this may be an underestimate because of quenching due to 2-mercaptoethanol. The bound

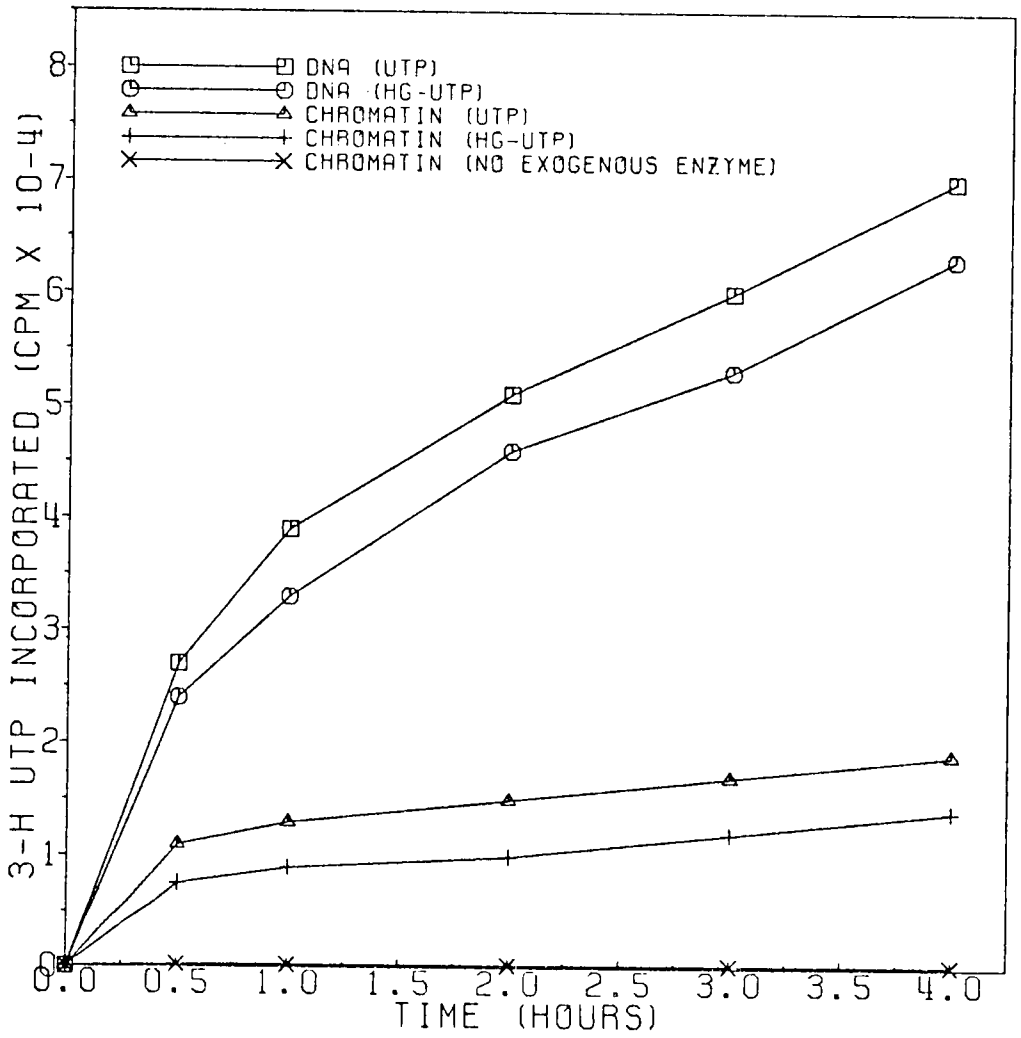


Fig. 6-1. Kinetics of RNA synthesis in vitro.

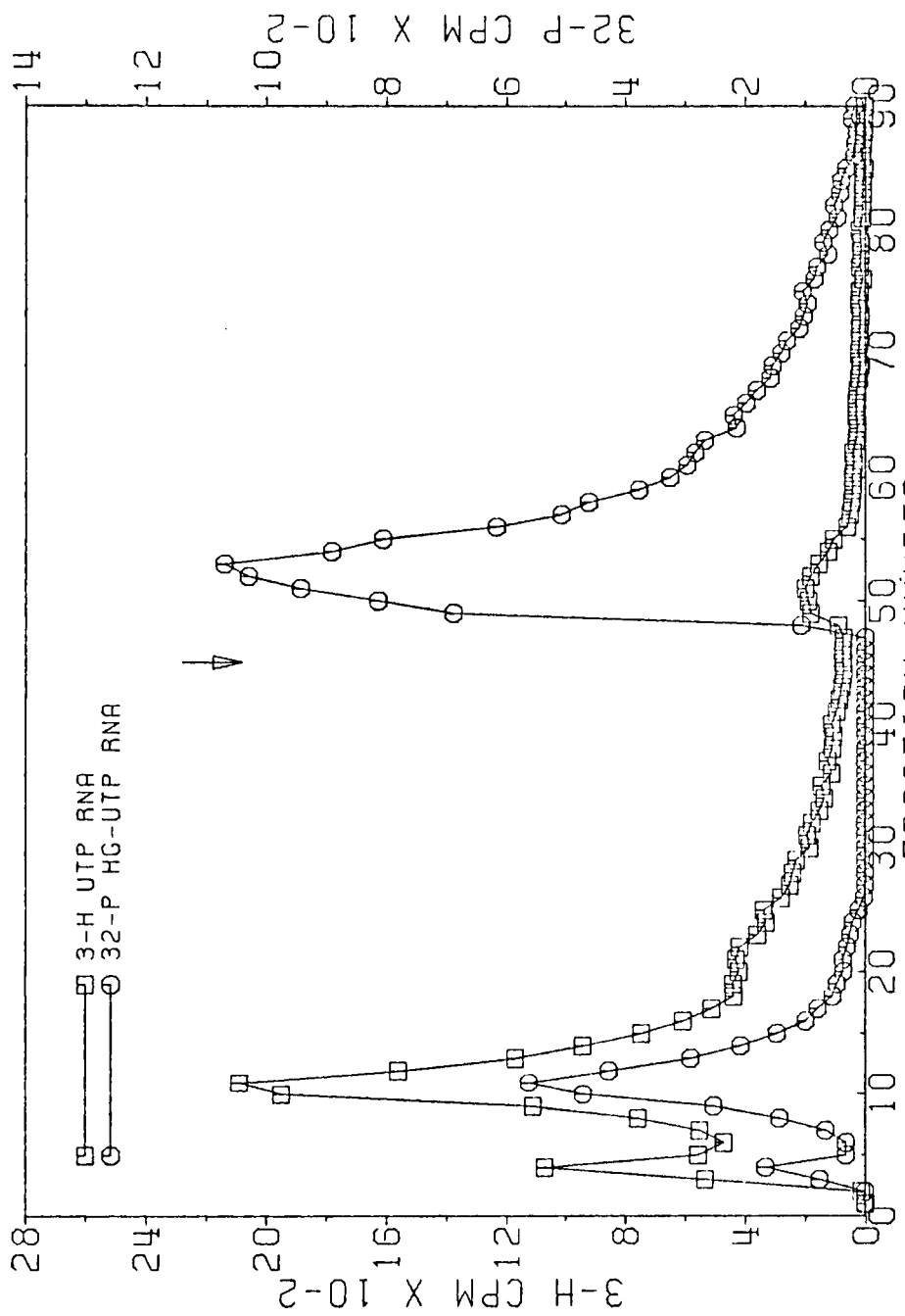


Fig. 6-2. Separation of mercurated RNA from non-mercurated RNA by sulfhydral affinity chromatography.

[^{32}P]Hg RNA was eluted from the column with 0.1M 2-mercaptoethanol along with some contaminating [^{3}H]RNA (approximately 10%).

Specificity of in Vitro Transcription

Before using fractionated chromatin as a template for wheat germ RNA polymerase II, it was necessary to determine if globin mRNA transcripts could be observed with unfractionated chromatin as template. Chromatin preparations from adult mouse livers and fetal mouse livers were used as templates. The hybridization of globin cDNA to increasing concentrations of globin mRNA is shown in Fig. 6-3. As can be seen in Fig. 6-4, globin mRNA sequences are transcribed from fetal liver chromatin to a greater extent than from adult liver chromatin. In this type of hybridization assay, originally described by Young et al. (1974), the initial slope of the hybridization curve is proportional to the concentration of globin mRNA sequences. The percentage of globin mRNA sequences present in the transcripts can be estimated by comparing the initial slope of the titration curve to that observed for the hybridization of globin cDNA to purified globin mRNA from which it was transcribed (Fig. 6-3). The concentration of globin mRNA sequences transcribed from adult liver chromatin was 0.0005%. The transcripts from fetal liver chromatin contained 0.013% globin mRNA sequences.

ECTHAM-Cellulose Fractionation

The elution profile of sonicated mouse fetal liver chromatin from ECTHAM cellulose is shown in Fig. 6-5. Various fractions across the profile were assayed with wheat germ RNA polymerase II to determine

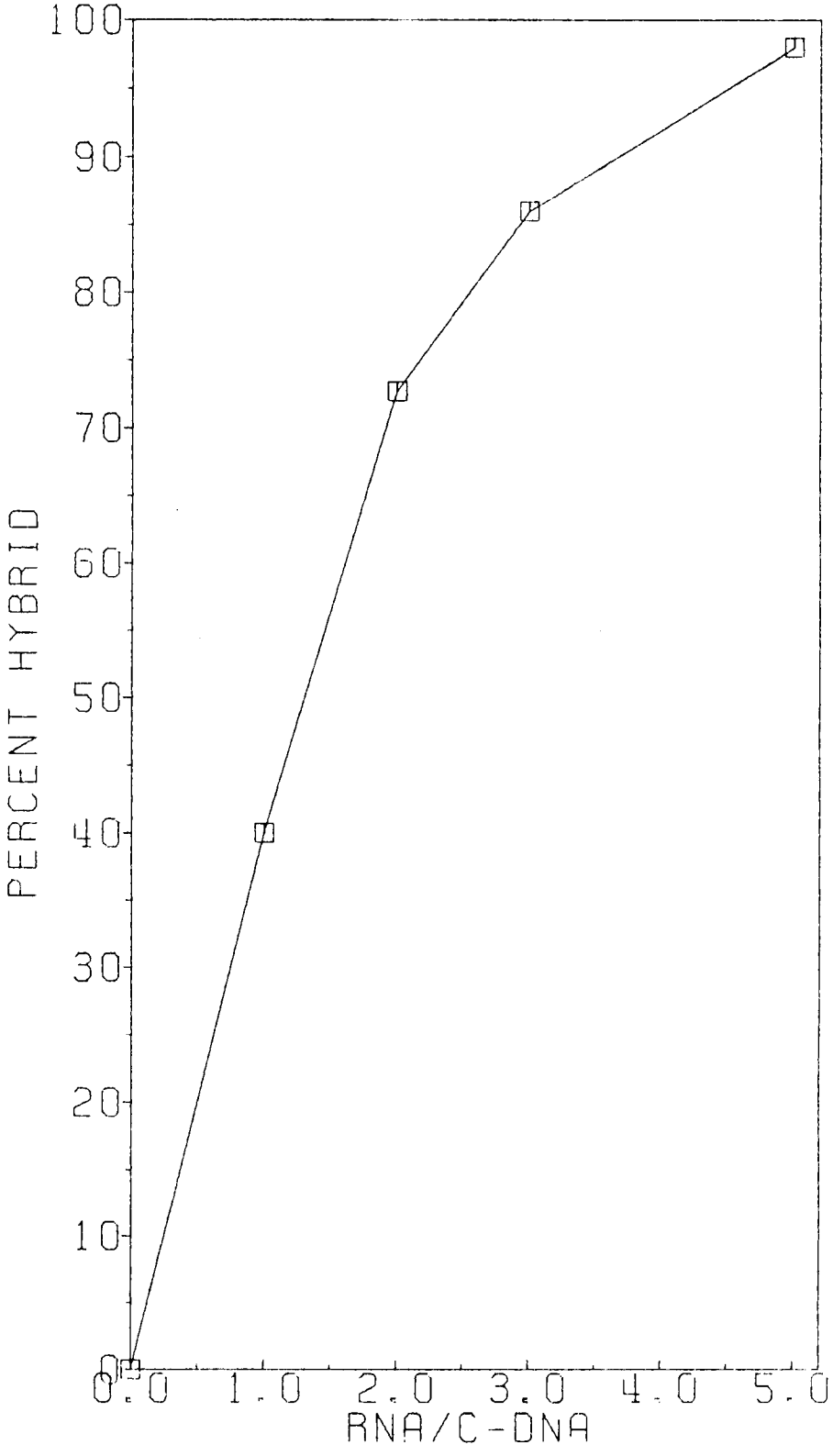


Fig. 6-3. Hybridization of globin mRNA with globin cDNA.

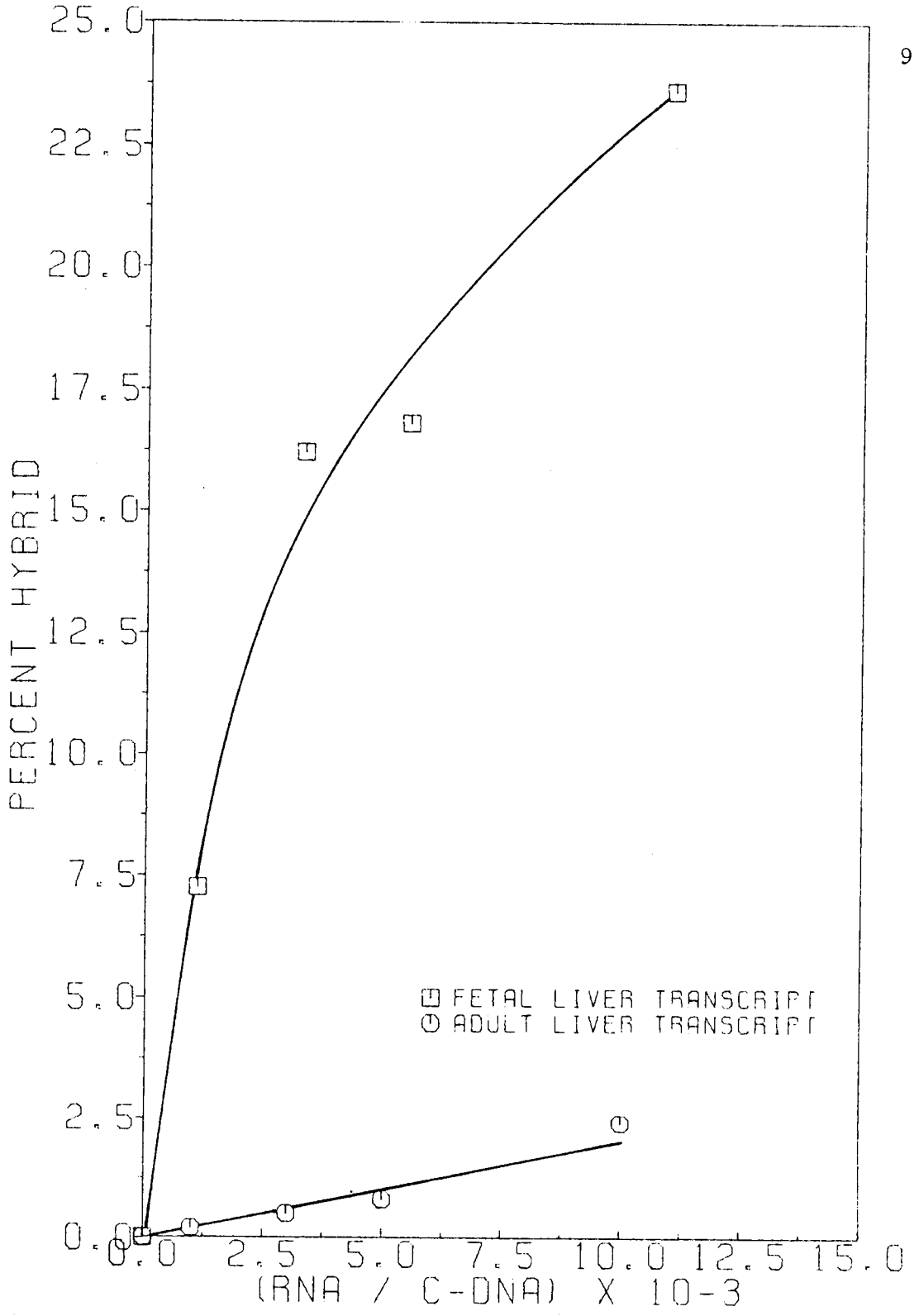


Fig. 6-4. Hybridization of RNA transcribed from adult liver and fetal liver chromatin with globin cDNA.

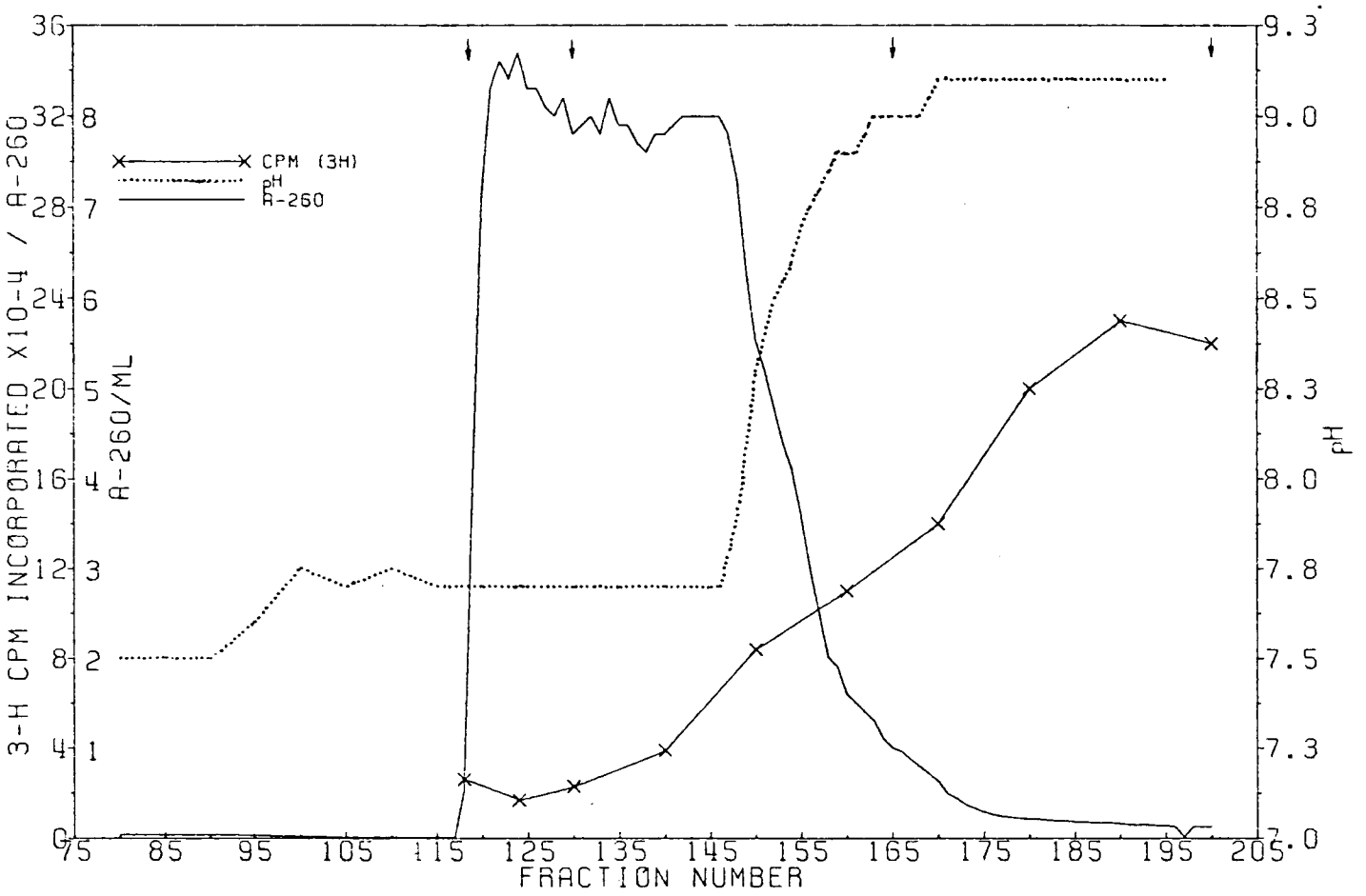


Fig. 6-5. Fractionation of fetal liver chromatin on ECTHAM cellulose (assayed with wheat germ RNA polymerase II).

template activity. Fractions 118-130 (designated early) were pooled and used as the inactive chromatin source. Fractions 165-200 (designated late) were pooled and used as the active chromatin source.

DNase II Fractionation

Fetal liver chromatin was fractionated by digestion with DNase II followed by differential precipitation with $MgCl_2$. The fractionation scheme was the same as shown in Fig. 1-4, p. 10. These fractions were assayed for template activity using wheat germ RNA polymerase II (Table VI-1). The PI fraction was used as the inactive chromatin source, and the SII fraction was used as the active chromatin source.

Table VI-1. Template activity of DNase II fractionated chromatin (assayed with wheat germ RNA polymerase II).

Fraction	Percent of total chromatin present in fraction	^3H CPM incorporated x $10^{-3}/A_{260}$
PI	84.7	106
PII	9.0	149
SII	6.3	211

Transcription of Fractionated Chromatin

The chromatin fractions described above were concentrated as described in the Materials and Methods and dialyzed extensively against 1mM Tris, pH 8.0. These fractions were then used as templates for wheat

germ RNA polymerase II. There is approximately a 10-fold difference in the amount of globin mRNA sequences transcribed from the ECTHAM-cellulose late fraction (0.005%) compared to the ECTHAM-cellulose early fraction (0.0005%) (Fig. 6-6). Approximately a 2-fold difference in globin mRNA sequences is observed for transcripts from the DNase II SII fraction (0.0028%) compared to the DNase II PI fraction (0.0015%) (Fig. 6-7).

Discussion

Wheat germ RNA polymerase II appears to be able to transcribe specific sequences from chromatin *in vitro* as judged by the 25-fold difference in globin mRNA sequences in transcripts of fetal liver chromatin compared to transcripts of adult liver chromatin. This is in agreement with the only other published report concerning *in vitro* transcription with wheat germ RNA polymerase II, that of Towle et al. (1977) who demonstrated specific transcription of chick oviduct and reticulocyte chromatin using this enzyme.

On the basis of the *in vitro* transcription of globin mRNA sequences, the ECTHAM-cellulose fractionation technique is judged superior to the DNase II procedure in fractionating fetal liver chromatin. A 10-fold difference in globin mRNA sequence content is observed for the active and inactive fractions from ECTHAM cellulose compared to a 2-fold difference observed for the active and inactive DNase II fractions. The percent globin mRNA sequences detected in transcripts of the DNase II fractions (0.0015% for PI and 0.0028% for SII) are intermediate values between those observed for transcripts of ECTHAM-cellulose fractions (0.0005% for early and 0.005% for late).

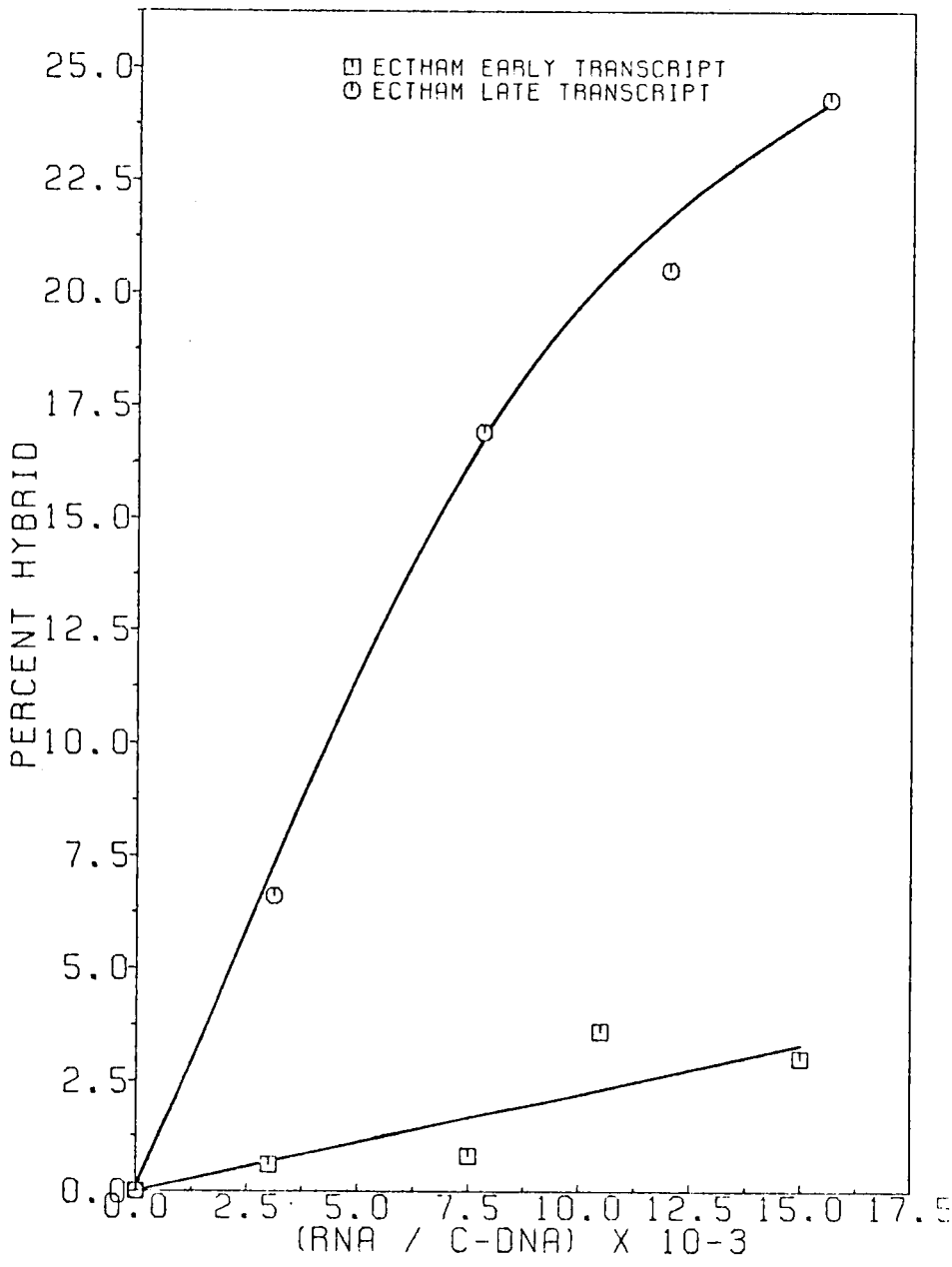


Fig. 6-6. Hybridization of RNA transcribed from fetal liver chromatin (fractionated on ECTHAM cellulose) with globin cDNA.

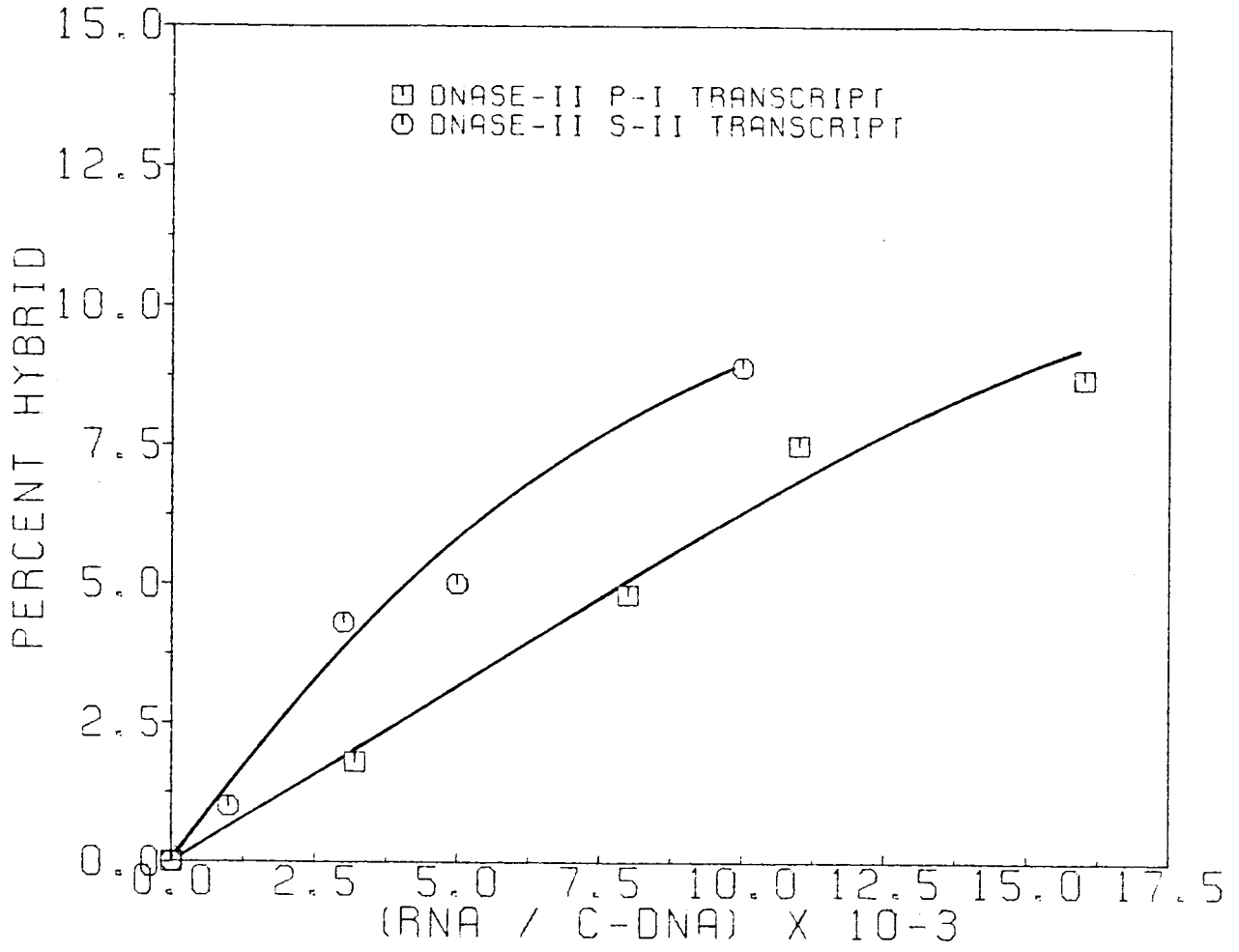


Fig. 6-7. Hybridization of RNA transcribed from fetal liver chromatin (fractionated with DNase II) with globin cDNA.

It is also interesting to note that the 10-fold difference in the percentage of globin mRNA sequences in transcripts of early and late ECTHAM-cellulose fractions seems to correlate with the difference observed in template activity between these fractions (7- to 8-fold). This also appears to be the case for DNase II fractions since there is a 2-fold difference in globin mRNA sequences in transcripts from the PI and SII fractions and there is a 2-fold difference in template activity for these fractions.

Zasloff and Felsenfeld (1977) have recently reported that the use of mercury-substituted ribonucleoside triphosphates can lead to artifacts in the analysis of in vitro chromatin transcripts. They claim this arises because the endogenous RNA acts as template for E. coli RNA polymerase and forms a duplex with the Hg-substituted complementary strand that is made. When the HgRNA is purified on sulfhydral columns, the endogenous RNA is also purified. It is not known if wheat germ RNA polymerase II is capable of using RNA as template. Even if this enzyme is able to use RNA as template, this should not complicate the interpretation of the data in this study since all RNA transcripts were denatured prior to chromatography on the sulfhydral affinity column.

Superficially there is a disagreement between these results and those obtained in the gene distribution studies described in Chapter V. This difference is not difficult to resolve, however. It seems reasonable that the assay for the ability to support the synthesis of globin genes with wheat germ RNA polymerase II is a more sensitive assay for transcriptional activity than assaying for the presence of globin

genes. The population of cells used in these experiments is not homogeneous. Paul et al. (1969) have reported that 55-70% of the cells in fetal livers at this period of gestation are erythropoietic; however, probably not all of these cells are active in the synthesis of globin mRNA. Although globin genes in the inactive fraction of chromatin may be repressed transcriptionally, they will nevertheless be detected in the gene-distribution experiments. This is not the case in experiments where wheat germ RNA polymerase II is used to determine the ability of fractions to serve as template for globin mRNA synthesis.

CHAPTER VII

SUMMARY

The fractionation of chromatin by the three methods discussed (ECTHAM-cellulose chromatography, differential sedimentation on sucrose gradients and DNase II digestion followed by differential precipitation with $MgCl_2$) yields a component with characteristics that are consistent with it being transcriptionally active *in vivo* (increased template activity when assayed with *E. coli* RNA polymerase, lower thermal stability and association of labeled nascent RNA). The fractionation procedures do produce components which are similar when assayed by the criteria listed above; however, close examination reveals that characteristics of these components differ to some extent.

First, consider the assay for template activity using *E. coli* RNA polymerase. Routinely, there is a 6-7 fold difference in template activity for ECTHAM-cellulose fractions, 3-4 fold difference for sucrose gradient fractions and 2-3 fold difference for DNase II fractions (Table I-1, p. 6 and Table V-1, p. 75). Similar results were obtained in assays for template activity with wheat germ RNA polymerase II (a 6-fold difference for ECTHAM-cellulose fractions (Fig. 6-5, p. 95) and a 2-fold difference for DNase II fractions (Table VI-1, p. 96).

Comparison of the Tm values of these various components reveals little difference except for DNase II fractions. The ECTHAM-cellulose and sucrose gradient fractions differ over a range of about 4°; however,

the DNase II fractions differ over a range of about 9° (Table I-1, p. 6 and Table V-1, p. 75). The greater difference in Tm for the DNase II fractions probably results from digestion by the enzyme since the percent hyperchromicity for the PII and SII fractions are also reduced. This is supported by the fact that DNA extracted from PII and SII fractions also has lower Tm values and percent hyperchromicity. It is of interest to note that DNA extracted from the ECTHAM-cellulose and sucrose gradient fractions all have very similar Tm values (Table V-1).

A comparison of the active and inactive components on the basis of association of labeled nascent RNA reveals little difference. In general, the amount of labeled RNA associated with the active fraction is 5 to 6 times greater than that associated with the inactive fraction; this is true for all the fractionation procedures (Fig. 1-2, Fig. 1-3 and Table I-1, pp. 7, 8 and 6, respectively).

Although the ECTHAM-cellulose procedure appears to resolve active and inactive components better than the other procedures based on template activity, it is impossible to evaluate the procedures on the basis of thermal stability or association of labeled nascent RNA. For this reason, and because the criteria discussed above have been criticized (see Chapter V for discussion), more stringent assays were developed.

One of the assays developed consists of fractionating total liver chromatin, which is active in the synthesis of globin genes, by the three fractionation methods and determining the distribution of globin genes in these fractions. Essentially no difference in globin gene content was observed in the various fractions. This result was

unexpected. However, after considering the importance of the homogeneity of cell population in this type of experiment, it was not surprising. Fetal livers at this stage of gestation contain 55-70% erythropoietic cells; however, not all of these cells are active in the synthesis of globin genes. For this reason, genes not actively transcribing globin mRNA will be detected in the inactive chromatin fractions and little difference in gene content will be observed in the various fractions.

Fetal liver chromatin was fractionated by the ECTHAM-cellulose chromatography and DNase II digestion, and the ability of these fractions to serve as template for globin mRNA sequences was determined using wheat germ RNA polymerase II. The sucrose gradient fractionation procedure was not used in these experiments since fractionation of a large amount of chromatin with this method is more difficult. A 10-fold difference in globin mRNA sequences was observed for the ECTHAM-cellulose fractions; however, only a 2-fold difference was observed for the DNase II fractions. Better resolution of active and inactive components seems to have been obtained with the ECTHAM-cellulose procedure because of the greater difference in globin mRNA sequences in those transcripts, and because the values observed for the DNase II fractions (0.0015%(PI) and 0.0028%(SII) are between the ECTHAM-cellulose values (0.0005% (early) and 0.005% (late)) indicating that fractionation with the DNase II procedure was only minimally achieved.

Since the assay with wheat germ RNA polymerase II will only detect genes that are actively transcribing globin mRNA, it is the most sensitive assay for evaluating chromatin fractionation procedures. On

the basis of this assay, chromatography on ECTHAM cellulose is the method of choice for this purpose. Because of the high resolution achieved with this procedure, it would be of interest to examine the active fraction for the presence of components which may regulate transcription of chromatin. It would also be interesting to isolate the DNA from this fraction and determine if possible regulatory sequences are present.

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APPENDIX

APPENDIX

Synthesis of cDNA

Reverse transcriptase reaction mixtures contained the following: 50mM Tris HCl (pH 8.3), 6mM $MgCl_2$, 1mM dithiothrietol, 40mM KCl, 0.2mM dTTP, 0.2mM dATP, 0.2mM dGTP, 0.2mM [^{3}H]dCTP (20Ci/mmol), 4mM sodium pyrophosphate, 10 ug/ml of $(dT)_{10}$, 60 ug/ml of RNA (globin or myeloma), and 45 units/ml of AMV reverse transcriptase.

The [^{3}H]dCTP was lyophilized to dryness prior to addition of the other reagents. Reactions were incubated at 38° for 2 hr. The reaction was terminated by adding SDS to a final concentration of 0.5%. Alkaline hydrolysis of the RNA was accomplished by making the reaction 0.2N NaOH, after the volume was adjusted to 1ml, and incubating at 70C for 10 min. The reaction was neutralized and 150 ug of yeast RNA was added. This mixture was then chromatographed on Sephadex G-50 (0.8x50cm) in glass-distilled water. The excluded fractions were pooled, made 0.2M NaCl and precipitated with 2.5 volumes of 95% ethanol.

The precipitated cDNA was collected by centrifugation for 1 hr in a SW27 rotor at 27,000 rpm. The cDNA was dissolved in 0.1 ml of 0.2N NaOH, 0.9M NaCl, 5mM EDTA and layered onto a 5-20% linear sucrose gradient in the same solution. The gradient was centrifuged for 24 hr at 41,000 rpm in a SW41 rotor. Fractions were neutralized and counted in Aquasol. The peak fraction and all fractions sedimenting faster than this fraction were pooled, neutralized, and precipitated with 2.5 volumes of 95% ethanol after the addition of 100 ug of yeast RNA.

The specific activity of the cDNA was 2.4×10^7 dpm/ug.

Size Determination of cDNA

The size of cDNA preparations was determined by centrifugation in 5-20% sucrose gradients containing 10mM Tris (pH 7.5), 100mM KCl and 1mM EDTA. The gradients were centrifuged for 15 hr at 41,000 rpm in a SW41 rotor. Marker 4S, 18S and 28S RNAs were centrifuged in a parallel gradient.

RNA-cDNA Hybridization Analysis

Hybridizations were performed in 20 ul volumes in 40 ul glass capillaries. Glass capillaries (Microcaps) were treated as described by Wilson et al. (1975). The reaction mixture contained 0.6M NaCl, 0.01M Hepes (pH 7.0), 0.002M EDTA, 8 ug yeast RNA, 0.5 ng of cDNA and varying amounts of the RNA to be assayed. Reactions were heated at 100° for 2 min and incubated at 70° for 96 hr. Reactions were terminated by freezing.

DNA-cDNA Hybridization Analysis

Hybridizations were performed at a DNA to cDNA excess (7.7×10^6 to 9.5×10^6). DNA samples were sonicated so that the average length was approximately 500 nucleotides. Reactions were performed in 0.6M NaCl, 0.01M Hepes (pH 7.0), 0.002M EDTA and contained unlabeled DNA at concentrations ranging from 7.7 to 9.5 mg/ml. Paraffin oil was layered over the reactions which were carried out in polypropylene tubes. The reactions were heat denatured and incubated at 70°C. At intervals (from

1.2 minutes to 74 hours), 50 ul samples of the reaction mixture were pipetted into 450 ul of S1 nuclease reaction mixture and frozen until analysis with S1 nuclease. Each 50 ul sample contained 0.1 ng of cDNA ($1x10^7$ cpm/ug).

S1 Nuclease Assay of cDNA-RNA Hybrids

Assays of cDNA-RNA hybrids with S1 nuclease were performed by a modification of the method of Sutton (1971). Hybridization reactions either 10 ul or 25 ul were expelled into 500 ul of reaction mixture containing 1mM $ZnSO_4$, 100mM NaCl, 30mM sodium acetate, pH 4.5, and 12.5 ug of calf thymus DNA (sonicated and denatured). This mixture was mixed and two 250 ul aliquots were taken. To one aliquot 4 units of S1 nuclease was added; the other served as an incubated control without nuclease. After incubation at 37° for 1 hr, 400 ug of yeast RNA was added to each aliquot and TCA precipitable radioactivity was collected on glass-fiber filters. After the filters were dried, they were placed in 0.5 ml of NCS reagent (diluted 1:3 with toluene scintillation fluid (6g/l PPO, 75mg/l POPOP)) to dissolve the precipitate. After incubation at room temperature for 30 min, 10 ml of toluene scintillation fluid containing 1ml/l of glacial acetic acid was added.

S1 Nuclease Assay of cDNA-DNA Hybrids

Aliquots of 50 ul were removed from the reaction mix and placed directly into 450 ul of S1 nuclease reaction mixture (same as described above). All steps were as described above except 40 units of S1 nuclease were used / 250 ul aliquot. The TCA precipitable radioactivity was collected on glass-fiber filters which were treated as described above.

Wheat Germ Cell-Free Protein Synthesis System

The final volume of the cell-free reaction mixture was 100 ul and contained: 25 ul S-30, 20mM Hepes (pH 7.6 adjusted with KOH), 2mM DTT, 1mM ATP, 20mM GTP, 8mM creatin phosphate, 40 ug/ml creatin phosphokinase, 25mM of the appropriate unlabeled amino acids, 80mM KCl, 3.0mM Mg acetate, 50mM spermine, 7.5 ug wheat germ tRNA, 5uCi [^{3}H]-leucine (60 Ci/mmole) and 3.4 ug of the RNA to be assayed. The reaction was incubated at 25° for 90 min.

Extraction of DNA from Chromatin or Nuclei

1. Make chromatin or nuclei 0.5% SDS, 0.05M Tris, pH 7.5, 5mM EDTA; add 50 ug/ml Proteinase K and incubate for 1 hr at 37°. (Proteinase K was dissolved at a concentration of 5 mg/ml in 0.01M Tris, pH 8.0, 5mM $CaCl_2$, self-digested for 30 min at 37° and stored at -20°.)

2. Make 0.2M NaCl and extract with phenol-chloroform-isoamyl alcohol (50:50:1) 2 times and once with chloroform-isoamyl alcohol (50:1).

3. Precipitate the DNA by the addition of 2 volumes of 95% ethanol and store overnight at -20°.

4. Collect the precipitated DNA by centrifugation 30 min at 10,000 g.

5. Dissolve the DNA pellet in 0.01M Tris, pH 7.5, 0.02M NaCl and incubate 30 min at 37° with 20 ug/ml of RNase A. (RNase A was dissolved at a concentration of 1 mg/ml in 0.1M Tris, pH 7.5, 0.15M NaCl, 1mM EDTA, boiled for 10 min and stored at -20°).

6. Make 0.2M NaCl and 0.5% SDS and extract once with phenol-chloroform-isoamyl alcohol (50:50:1) and once with chloroform-isoamyl alcohol (50:1).

7. Precipitate the DNA by the addition of 2 volumes of 95% ethanol and store overnight at -20°.

Procedure for Isolating Poly(A)-Containing RNA by Affinity Chromatography on Poly(U)-Sepharose

1. Swell 2.5 g of poly(U)-Sepharose 4 B (Pharmacia) in 1.0M NaCl-0.01M Tris pH 7.5 for 5 min and pack in a water-jacketed column. This results in approximately 12 ml of swollen gel.

2. Equilibrate column with high salt buffer (0.5M NaCl, 0.5% SDS, 0.01M Tris, pH 7.5).

3. Dissolve RNA (200-1000 OD units) in glass-distilled water (as small a volume as possible). Heat 10 min at 65 C; cool in ice-water bath. Make the RNA solution 0.5M NaCl, 0.5% SDS, 0.01M Tris, pH 7.5 using 5M NaCl, 10% SDS, 1.0M Tris, pH 7.5. Since SDS will precipitate at this temperature, it will be necessary to warm the RNA solution slightly to get the SDS into solution. Apply this solution to the column. Initially, apply slowly to insure binding. After the RNA is applied, the elution rate is increased to approximately 20 ml/hr.

4. Elute with high salt buffer while monitoring the absorbance at 254 nm. When the absorbance has returned to baseline, begin elution with low salt buffer (0.1M NaCl, 0.5% SDS, 0.01M Tris, pH 7.5). Continue elution until the absorbance again returns to baseline. Increase the temperature to 50 C (we use a Haake constant temperature circulator) and begin elution with glass-distilled water. Collect 1-3 ml fractions, depending on the amount of RNA applied.

5. Precipitate the desired fractions with 0.1 volume of 20% potassium acetate, pH 5.5 and 2.5 volumes of 95% ethanol.

6. Collect the RNA precipitated out of the water fraction and repeat steps 3-5.

All glassware used in the preparation of buffers was heated at 200° for at least 2 hr to avoid contamination with RNase.

Sucrose Gradient Centrifugation

The precipitated RNA obtained in the water fraction after the second poly(U)-Sepharose column was dissolved in glass-distilled H_2O, heated for 10 min at 70C and quick-cooled in an ice-water bath. This RNA was then applied to 5-22% sucrose (RNase-free) gradients (35ml) containing 0.01M Tris -Cl(pH 7.8) and centrifuged in a SW27 rotor at 27,000 rpm for 24 hr. Successive centrifugation steps were employed until RNA of the desired size had been resolved.

Isolation of Nuclei

Minced tissue was homogenized with a Dounce homogenizer at 4C in 9 volumes of 0.25M sucrose, 0.2mM PMSF, 3mM $CaCl_2$, 0.01M Tris, pH 8.0. The homogenate was filtered through a single layer of cheese cloth and centrifuged at 1000 g for 10 min. The nuclear pellet was suspended in the above buffer, containing 0.5% Triton X-100, and centrifuged as above. This step was repeated two times.

Preparation of Chromatin

Nuclear pellets isolated as described above were suspended in 0.075M NaCl, 0.024M EDTA, pH 8.0, 0.2mM PMSF and centrifuged at 7700 g for

10 min. This step was repeated two times. The pellet was suspended in 0.05M Tris, pH 8.0, 0.2mM PMSF, and centrifuged as above. This wash was repeated once, and followed by two washes in each of the following: 0.01M Tris, pH 8.0, 0.2mM PMSF, 0.001M Tris, pH 8.0, 0.2mM PMSF. The gelatinous pellet obtained after this last wash was resuspended in 0.001M Tris; it is this material that will be referred to as chromatin.

Sonication of Chromatin

Chromatin was fragmented by exposure to ultrasonic radiation generated by a Branson W 185 ultrasonic disruptor. The microtip was used, and the instrument was calibrated by measuring the downward force transmitted by the instrument when the tip was immersed halfway down in 80 ml of water in a 100 ml beaker. The force was measured with a Mettler P 1200 balance.

Fractionation of Chromatin by ECTHAM-Cellulose Chromatography

ECTHAM cellulose was prepared by the method of Petterson and Kuff (1969), and columns were packed as described by them, except that no pressure was applied except the hydrostatic head of the slurry. Column chromatography of sonicated chromatin was carried out as described by Reeck et al. (1972). The flow rate was approximately 4 ml/hr, and 2.0 ml fractions were collected. The pH of each fraction was measured with a Fisher Accumet pH meter, and the A_{260} of the fractions was monitored with a Beckman Acta C III spectrophotometer. Further analysis was carried out either on individual fractions or on pools of contiguous

fractions. After each elution, the column was purged of uneluted material by extensive washing with 0.05 M NaOH, and was regenerated by equilibration with 0.01 M Tris, pH 7.2.

Fractionation of Chromatin on Sucrose Gradients

Sonicated chromatin was layered onto 0.17-1.7M sucrose gradients (35 ml), containing 0.01M Tris, pH 8.0. The gradients were formed using an ISCO Model 570 gradient former. Gradients were centrifuged for 16 hr at 25,000 rpm at 4C in a Beckman L3-50 centrifuge, using the SW-27 rotor. Gradients were fractionated with an ISCO Model 183 gradient fractionator, A_{254} was monitored with an ISCO Type 4 absorbance monitor. Fractions were collected and pooled for further analysis.

Fractionation of Chromatin Using DNase II

This fractionation procedure was carried out as described by Gottesfeld et al. (1974a, 1974b). Briefly, chromatin prepared as described above was homogenized with a motor-driven glass-Teflon homogenizer (20 strokes at 10 strokes per minute) and dialyzed overnight vs. 0.025M sodium acetate (pH 6.6). After dialysis, the chromatin was homogenized as before with the motor-driven glass-Teflon homogenizer. The volume of the chromatin solution was adjusted to give 10 A_{260} units/ml when the absorbance of an aliquot was measured in 0.9N NaOH. DNase II was added to 10 units of enzyme/A_{260} unit of chromatin. Digestion was for 5 min at 24C. At the end of the incubation period, the pH of the chromatin suspension was brought to 7.5 with 50mM Tris-HCl (pH 9.0). The chromatin suspension was cooled on ice and then centrifuged at 27,500 g

for 20 min at 4°. To the supernatant, 0.2M $MgCl_2$ was added drop wise while stirring to give a final concentration of 0.002M . After stirring 30 min at 0°, the solution was centrifuged again at 27,500 g for 20 min.

Thermal Analysis

Thermal dissociation of chromatin was carried out in a Beckman Acta C III spectrophotometer equipped with automatic sample changer. Temperature was monitored with a platinum probe immersed in an otherwise unused cuvette in the sample chamber. Temperature was controlled with a programmable Haake circulating bath (Model PG 11) containing a mixture of ethylene glycol and water. The temperature was increased at a rate of 18C/hr. A_{260} was recorded at intervals of approximately 1 min and values were read from the profile at intervals of 0.5C. A_{260} values were corrected for thermal expansion (Mandel and Marmur, 1968) by a computer program.

Assay of Template Activity of Chromatin with E. coli RNA Polymerase

The E. coli RNA polymerase assay was incubated at 37° for 1 hour. It contained in a volume of 1 ml: 0.01M Tris, pH 7.9; 1mM $MnCl_2$; 5mM $MgCl_2$; 0.8mM each GTP, CTP and ATP; 12.5mCi [^{3}H]UTP (14Ci/mmol); 5 units of E. coli RNA polymerase; and 0.05 OD_{260} unit of chromatin. After the addition of 300 ug of carrier yeast RNA, the reactions were stopped by the addition of 0.5ml of cold 20% TCA containing 0.01M sodium pyrophosphate. After 10 minutes at 0°, the reactions were filtered on membrane filters (Millipore, HA), washed with cold 5% TCA, dried and counted in toluene-based scintillator containing 0.4% BBOT.

Labeling of Nascent RNA

MOPC-31C tumor fragments were suspended in 10ml of Eagle's medium and placed in a 25ml flask. The suspension was stirred vigorously for 5 min at 37°. Large fragments were allowed to settle and the supernatant was filtered through a 100 mesh stainless steel screen. The cells in the filtrate were washed twice in Eagle's medium and resuspended in the same medium at a concentration of 2 x 10^6 cells/ml. 0.1 mCi of [^{3}H] uridine (14Ci/mmol) was added per 10ml of cells. Usually 100ml of cells were used. After the addition of the [^{3}H] uridine, the cells were incubated at 37° for 10 min. At the end of the incubation period, the cells were collected by centrifugation at 1000g for 10 min and washed with 0.25M sucrose - 0.2mM PMSF - 3mM $CaCl_2$ - 0.01M Tris, pH 8.0. Chromatin was then prepared from these cells.

VITA

The author was born February 26, 1947, in Jonesville, Virginia. He was graduated from Jonesville High School in June 1965. He entered Berea College, Berea, Kentucky, in the fall of 1965 and received a Bachelor of Arts degree in biology in June 1969. In September 1969 he received a research assistantship supported by a NIH grant administered by his major professor and began study toward a Master's degree in microbiology at the University of Tennessee. In August 1971 he was awarded a NIH predoctoral traineeship. He received the M.S. degree in August 1972 and immediately entered the Ph.D. program in the same department. He received the Ph.D. degree in microbiology in August 1977. He became a postdoctoral fellow in the laboratory of Bert W. O'Malley at Baylor College of Medicine in March 1977.

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