Transcription of Isolated Mouse Liver Chromatin†

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ABSTRACT: Analysis of RNA transcription from isolated mouse liver chromatin has been undertaken by means of RNA-excess hybridizations with small amounts of radioactive DNA. This analysis indicates that mouse liver chromatin is a restricted template for the in vitro synthesis of RNA complements to repetitive DNA, but more RNA species are synthesized than are found in the RNA isolated from mouse liver nuclei. Extraction with 0.5 M NaCl destroys the template restriction of isolated chromatin. RNA synthesized in vitro from DNA or chromatin templates by *Escherichia coli* RNA polymerase, as well as in vivo mouse liver nuclear RNA, were each hybridized to ^{125}I-labeled DNA of high, intermediate, or low reiteration frequency. Chromatin-primed and nuclear RNA saturate a smaller portion of each DNA fraction than does DNA-primed RNA. However, chromatin-primed RNA saturates more high and low reiteration frequency DNA than does nuclear RNA. Simultaneous hybridization of nuclear- and chromatin-primed RNA with ^{125}I-labeled DNA indicates that chromatin-primed RNA contains all of the sequences present in nuclear RNA. Extraction of chromatin with 0.5 M NaCl leads to removal of histone F_1, as well as a wide variety of non-histone proteins. When used as a template for in vitro RNA synthesis, such salt-extracted chromatin produced RNAs that hybridize as large a portion of each DNA fraction as does DNA-primed RNA.

Studies on the structure and function of chromatin rest on the assumption that this isolated nucleoprotein complex retains many of the properties of chromosomes in vivo. A large number of procedures are available for the isolation of chromatin (Pomerai et al., 1974). A number of potential artifacts exist for each method, and the likelihood of protein exchange between chromatin components is great in all. A number of attempts have been made to determine the extent to which isolated chromatin retains its in vivo activity by demonstrating that RNA transcribed in vitro from isolated chromatin retains the sequence-specific pattern of transcription found in vivo. Early attempts to demonstrate sequence-specific transcription in eukaryotes relied on the hybridization of RNA to DNA immobilized on filters. As is generally recognized (McCarthy and Church, 1970), filter hybridization experiments are subject to several reservations. (1) They reveal hybridization to reiterated DNA sequences only and, thus, distinguish between groups of molecules transcribed from similar but not necessarily identical loci rather than demonstrate strict sequence-specific transcription. (2) The RNA concentrations and incubation times employed limit hybridization to the more prevalent RNA sequences. (3) Since the rate of hybridization is controlled by the concentration of the hybridizing RNA, variations in the concentration of a given RNA sequence in different RNA populations can lead to variations in the detection of that sequence. Within these limitations, the set of sequences transcribed from chromatin in vitro appears to reflect the pattern of transcription in the tissue from which the chromatin was prepared (Paul and Gilmour, 1968; Smith et al., 1969; Tan and Miyagi, 1970).

Several recent studies have reported the specific in vitro transcription of a known gene from isolated chromatin. Globin-specific sequences are found among the in vitro RNA transcripts of chromatin isolated from erythropoietic tissues, using either bacterial RNA polymerases (Axel et al., 1973; Gilmour and Paul, 1973) or mammalian polymerases (Steggles et al., 1974), but are not detected among RNA transcripts from chromatin isolated from other tissues or from purified DNA. In SV 3T3 cells the same region of the early strand of SV 40 DNA is transcribed in vitro from isolated chromatin as is observed in vivo, while this restriction is not observed using a purified DNA template (Astrin, 1973, 1975). The demonstration of specific in vitro transcription of a few genes does not, of course, demonstrate that all sequences are transcribed specifically. In at least one case the aberrent transcription of known genes from isolated chromatin has been demonstrated. Honjo and Reeder (1974) have shown that while transcription of *Xenopus laevis* 28, 18, and 5s RNAs occur in vivo from only one strand of the appropriate gene region, transcription of these same genes from isolated chromatin occurs from both strands of both the gene and spacer regions.

We report here an analysis of the in vitro transcription of reiterated DNA sequences from mouse liver chromatin by exogenous *E. coli* RNA polymerase. RNA-excess hybridization to labeled DNA probes in solution was used in order to minimize the effect of varying RNA concentrations and DNA reiteration frequency inherent in filter hybridization experiments. Nuclear RNA from mouse liver has been compared to the RNA transcribed in vitro from mouse liver chromatin, partially extracted mouse liver chromatin, and purified mouse DNA.

Materials and Methods

(*1*) *Reagents.* Concentrated stock solutions were filtered through 0.45 μpore diameter nitrocellulose filters (Millipore Corp., Bedford, Mass.) and Chelex-100 resin (Bio-Rad Laboratories, Richmond, Calif.) to remove divalent cations. Hydroxylapatite was purchased from Bio-Rad Laboratories (Bio-Gel HTP) and from Clarkson Chemical Co., Williamsport, Pa. (Hyapatite C). Liquified phenol free of preservative (Mallinckrodt Chemical Works, St. Louis, Mo.) was purified by fractional distillation and stored as a solid at −20 °C or as a water saturated liquid at 4 °C. Formamide was purchased

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from Eastman Kodak (Rochester, N.Y.). Ribonucleotide triphosphates, as the tetralithium salts, were purchased from Schwarz/Mann (Organgeburg, N.Y.). ^{125}I (carrier free) in 0.1 N NaOH and adenosine-[8-^{14}C]ribonucleotide triphosphate were purchased from New England Nuclear (Boston, Mass.). Thallium trichloride was the generous gift of Dr. Bill Hoyer.

(*2*) *Animals.* Randomly bred male and female Swiss Webster mice (*Mus musculus*) from a colony maintained in this laboratory were used in all experiments. Chromatin and nuclear RNA were prepared from the livers of 3–6 month-old animals.

(*3*) *Enzymes.* Pancreatic ribonuclease (RNase) free of deoxyribouuclease (DNase) and DNase I free of RNase were purchased from Worthington Biochemical Corp. (Freehold, N.J.). Pronase (Type IV fungal protease, B grade) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA dependent RNA polymerase was prepared from frozen *E. coli*, either mid-log strain D-10 (RNase 1$^-$) grown in high peptone medium in a fermenter in this laboratory, or *E. coli* K12 (λ) late log, high peptone media cells purchased from General Biochemicals (Chagrin Falls, Ohio). The purification procedure of Burgess (1969) was followed, using filtration on Bio-Gel A-5m and A-1.5m to purify the active fraction obtained by chromatography on DEAE-cellulose. The purified polymerase was at least 100-fold dependent on added DNA template and contained sigma factor as determined by sodium dodecyl sulfate gel electrophoresis (Burgess and Travers, 1971).

S1 nuclease was prepared according to the method of Sutton (1971) from α-amylase type IV A (Sigma Corp.).

Exonuclease I from *E. coli* was prepared as a by-product of the RNA polymerase isolation. The proteins insoluble in 33% $(NH_4)_2SO_4$ were redissolved in 0.02 M potassium phosphate buffer, pH 7.2, 1 mM β-mercaptoethanol, and 5% glycerol. Exonuclease I activity was precipitated by the addition of 16 g/100 ml of $(NH_4)_2SO_4$, redissolved in the 0.02 M potassium phosphate buffer, and further purified by DEAE chromatography according to the procedure of Lehman (1966).

(*4*) *Chromatin.* (a) Isolation. Nuclei for the preparation of chromatin were isolated by modification of the method of Schmeckpeper and Smith (1972). Nuclei were kept in buffers of low-ionic strength (<0.015 M) and purified by centrifugation through 2.3 M sucrose. The final nuclear pellet was resuspended in saline-EDTA (0.075 M NaCl, 0.024 M EDTA, pH 8.0) and washed by centrifugation at 7700*g* for 10 min at 4 °C. Chromatin was released from the nuclei by lysis in 0.01 M Tris, pH 8.0, and then washed successively with buffers of decreasing ionic strength (0.01 Tris, three times; 0.005 M Tris, three times; and 0.001 M Tris, one time) as described by Paoletti and Huang (1969). The final clear gelatinous pellet was sheared in a 37-ml "Minicontainer" for the Waring blender at 70 V for 2 min.

(b) Molecular Composition. DNA concentrations were determined by A_{260} (1 mg/ml = 22 A_{260}, in 0.001 M Tris, pH 8.0) or the diphenylamine reaction (Burton, 1968). Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as a standard. Acid soluble proteins were obtained by two 30-min extractions with 0.2 M H_2SO_4 at 4 °C followed by centrifugation to pellet the precipitated chromatin. RNA was extracted with cold 0.3 M perichloric acid and its concentration estimated by the Orcinol reaction (Ashwell, 1957) following hydrolysis for 18 h at 37 °C in 0.3 M NaOH.

(c) Total Chromatin Proteins. Chromatin proteins were extracted by a modification of the method described by Shaw and Huang (1970). Samples for gel electrophoresis were lyophilized, dissolved in 10 M urea, 0.9 N acetic acid, 0.5 M β-mercaptoethanol, and incubated overnight at room temperature or for 1 h at 37 °C. Electrophoresis of proteins in 15% acrylamide gels in 2.5 M urea, 0.9 N acetic acid, pH 2.7, was according to the procedure of Panyim and Chalkly (1969). The gels were stained in Buffalo blue black and destained and stored in 0.9 N acetic acid.

(d) 0.5 M NaCl Soluble Chromatin Proteins. Chromatin dissolved in 0.001 M Tris, pH 8.0, at 1–2 mg of chromatin DNA/ml was brought to a final concentration of 0.5 M NaCl by the gradual addition of solid NaCl with stirring at 4 °C. After stirring for 1 h, the protein depleted chromatin was separated from the extracted proteins by filtration on Bio-Gel (Bio-Rad) in 0.5 M NaCl, 0.001 M Tris, pH 8.0. The salt extracted proteins (included on this column) were dialyzed against 0.05 M acetic acid and lyophilized prior to gel electrophoresis. The proteins remaining with the excluded chromatin were extracted as outlined above.

(*5*) *Nucleic Acids.* (a) Isolation of Nuclei. Freshly excised mouse livers were minced in ice-cold 0.32 M sucrose, 0.01 M Tris, 0.005 M $NgCl_2$, 0.5% Triton X-100, pH 8.0. The minced tissue was homogenized by three strokes of a motor-driven Teflon-glass homogenizer, filtered through eight layers of cheese cloth and two layers of Miracloth. The homogenate was centrifuged at 2000*g* for 15 min at 4 °C and the crude nuclear pellet was resuspended in the sucrose–Triton buffer and pelleted as before.

(b) DNA. DNA from washed mouse liver nuclei and from *E. coli* cells was isolated as previously described (Schmeckpeper and Smith, 1972).

(c) Nuclear RNA. Mouse liver nuclei were resuspended in 0.28 M LiCl, 0.1 M sodium acetate, 0.05 M $MgCl_2$, pH 5.1, and stored frozen at −70 °C. RNA from the combined nuclear suspensions of 100 livers was purified by extraction with phenol at 4 °C according to the procedure developed by Angerer (1973). This procedure combines the use of both DNase I and exonuclease I to ensure the complete removal of DNA from RNA preparations used in RNA excess hybridizations. The purified RNA was concentrated by partial lyophilization, dialyzed against distilled water, and stored at −70 °C.

(d) Synthesis and Extraction of Complementary RNA. RNA was synthesized in vitro from mouse liver DNA, chromatin, and salt-extracted chromatin templates using *E. coli* DNA dependent RNA polymerase. The reaction mixture for the synthesis of complementary RNAs contained: 200 μg/ml of DNA template (either as purified DNA or as chromatin), 0.01 M Tris, pH 8.0, 0.004 M $MgCl_2$, 0.001 M $MnCl_2$, 0.012 M β-mercaptoethanol, 0.8 mM each of ATP, CTP, UTP, GTP, and 40–80 units/ml of *E. coli* RNA polymerase (one unit polymerizes 1 nmol of AMP in 10 min at 37 °C, Burgess, 1969). High-salt reaction mixtures contained in addition 0.15 M NaCl. Radioactive complementary RNA (cRNA) was prepared from reaction mixtures containing 2–4 μCi/ml of [^{14}C]ATP. Synthesis was carried out at 37 °C for up to 1 h for chromatin templates and up to 5 h for DNA templates. At the end of the incubation the reaction mixture was made 0.05 M EDTA, 0.5% sodium dodecyl sulfate, and incubated for 30 min at 37 °C with 50–100 μg/ml of predigested pronase. As in the case of nuclear RNA, cRNA was purified by phenol extraction

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

at pH 5.1, chloroform extraction, DNase digestion, exonuclease I digestion, and G-75 gel filtration.

A number of the experiments reported here utilize nonradioactive in vitro synthesized RNA to drive hybridization reaction to ^{125}I-labeled DNA probes. In such experiments, endogenous chromatin associated RNA is not differentiated from in vitro synthesized RNA. An estimation of the contribution of endogenous chromatin RNA to the final RNA products was made by carrying chromatin through the RNA synthesis and extraction procedure, in the absence of RNA polymerase. 30.6 μg of RNA/mg of input chromatin DNA were recovered as material excluded from G-75 after DNase treatment of the extracted RNA. Since recovery at this stage from chromatin synthesis reactions was at least 300 μg of RNA/mg of chromatin DNA, the in vitro synthesized RNA represents at least 90% of the isolated RNA. Since these experiments were designed to detect the presence, in the in vitro synthesized transcripts from isolated chromatin, of *additional* sequences not found in vivo, the presence of a small fraction of in vivo transcripts in the isolated RNA product will not affect the interpretation of the experimental results.

(e) DNA Contamination in RNA Preparations. Nuclear RNA and cRNA preparations to be used in RNA excess hybridization experiments were tested for DNA contamination by measuring their ability to accelerate the rate of DNA reassociation following alkaline digestion of the RNA. Contaminated preparations were retreated with exonuclease I and retested for contamination.

(f) Iodination of Nucleic Acids in Vitro. ^{125}I was incorporated into DNA by modification (Bacheler, 1974) of the procedure described by Commerford (1971). DNA was iodinated at 80 °C which is above the T_m of native mouse DNA in the reaction mixture and was chosen to maximize isotope incorporation and to minimize variation in the specific activity of the isolated DNA C_0t fractions.Since single-stranded DNA is labeled 20 times as effectively as double-stranded DNA, any DNA sequences that reassociated during the labeling period would be under labeled. Cytosine poor regions, such as the AT rich mouse satellite, would also be poorly labeled by this method. Greater than 96% of the label was 10% Cl_3CCOOH soluble after digestion with S_1 nuclease and specific activities of 1.2–2.3 $\times$ 10^5 cpm/μg were obtained.

^{125}I was incorporated into RNA by a combination of the methods of Getz et al. (1972) and Altenberg et al. (1973). Greater than 99% of the incorporated label was rendered acid soluble by digestion with pancreatic RNase and a specific activity of 1.5 $\times$ 10^5 cpm/μg was obtained. All of the counts were excluded from G-75 both before and after incubation for 48 h at 37 °C in 2× SSC, 50% formamide, 1 mM EDTA.

(6) *Measurement of Radioactivity.* Radiolabeled molecules hybridized to DNA fibers or precipitated on glass fiber filters were counted by liquid scintillation. Aqueous samples containing ^{125}I were counted either by liquid scintillation or in a Packard γ counter (Model 578).

(7) *Nucleic Acid Hybridization.* (a) Filter Hybridization. Filters were prepared and hybridizations were carried out as previously described (Schmeckpeper and Smith, 1972). Low temperature hybridizations in formamide (2× SSC, 50% formamide, 1 mM EDTA at 37°C for 48 h) are equivalent to those carried out in 2× SSC at 68 °C for 18 h and improve retention of filter-bound DNA. Nonspecific binding was monitored by including a filter containing *E. coli* DNA, equal in amount to the experimental filter, for each individual point. The *E. coli* filters were carried through the same washes as the experimental filters and the counts bound were subtracted from the experimental filters to determine the level of specific hybridizations.

(b) Solution Hybridization. (1) Shearing of DNA: the molecular weight of DNA to be used for reassociation was reduced by sonication in a Sonifier cell disrupter fitted with a microtip (Heat Systems Ultrasonics, Inc., Model W140) for 5 min at a setting of 28. Ten milliliters of DNA at a concentration of 1 mg/ml in 1× SSC was sonicated in a glass scintillation vial. After sonication, the DNA was digested at 60 °C for 3 h in 0.3 N NaOH. Alkaline digestion eliminates contaminating materials labeled during iodination.

The molecular weights of the sonicated DNA fragments were determined by sedimentation in alkaline sucrose using a marker of known molecular weight (Studier, 1956).

(2) DNA reassociation: all solution reassociations were performed at 60 °C in 0.12 M sodium phosphate buffer at pH 6.8. C_0t values were calculated according to the convention of Britten and Kohne (1968) as the product of the concentration of nucleic acid in mol/l. and the time of incubation in seconds. Equivalent C_0ts for isolated reiteration fractions were calculated by dividing whole genome C_0t values by the percentage of the genome represented by the isolated reiteration fraction. Reassociation was initiated by heat denaturation and rapid cooling to 60 °C.

The extent of reassociation was monitored by binding DNA duplexes to hydroxylapatite columns in 0.12 M phosphate buffer at 60 °C (Britten and Kohne, 1968). Reassociated DNA duplexes were eluted from hydroxylapatite columns with 0.3 M phosphate buffer at 60 °C. Fractions were monitored for radioactivity and, where appropriate, aliquots of each fraction were counted and A_{260} determined in 0.5 M NaOH. The concentration of DNA in 0.5 M NaOH was calculated assuming 1 mg/ml = 26.5 A_{260}.

(3) RNA–DNA hybridizations: hybridization reactions were carried out in 0.12 M PB containing 0.06% sodium dodecyl sulfate at 60 °C. Hybridized samples were passed over hydroxylapatite columns equilibrated at 60 °C with the reaction buffer. Hybrids bound to the hydroxylapatite columns were eluted with 0.3 M phosphate buffer. Hybridization reactions were loaded onto the hydroxylapatite columns at concentrations not exceeding 100 μg of RNA/ml of HPA. Experiments to be directly compared were chromatographed on the same lot of hydroxylapatite.

(4) Thermal elutions from hydroxylapatite: RNA–DNA hybrids or DNA–DNA duplexes were bound to hydroxylapatite in 0.12 M phosphate buffer, 0.06% sodium dodecyl sulfate at 60 °C. After washing through the unreacted sequences and the sodium dodecyl sulfate with 0.12 M phosphate buffer, the temperature of the column was raised in 4–5 °C increments. Following 10–15 min to allow for temperature equilibration, the dissociated molecules were washed out of the column with 0.12 M phosphate buffer at the same temperature. Nucleic acids remaining on the column at 95 °C were eluted with 0.3 M PB.

Results

(1) *Preparation of Templates and the In Vitro Synthesis of RNA.* The molecular compositions of the chromatin and salt-extracted chromatin used as templates for in vitro synthesis of RNA are given in Table I. The extracted chromatin is depleted in acid soluble proteins (20–25% extracted), acid insoluble proteins (about 50% extracted), and RNA (60–70% extracted).

As judged by acrylamide gel electrophoresis (Figure 1), extraction with 0.5 M NaCl results in a chromatin that is de-

TABLE I: Chemical Composition of Chromatin (Mass Relative to DNA).

	DNA	Protein			RNA
		Total	Acid Soluble	Acid Insoluble	
Mouse Liver Chromatin	1	2.6	–	–	0.09
	1	2.5	1.4	1.00	0.18
	1	2.5	1.3	0.9	0.09
	1	2.1	1.0	0.7	–
Salt-Extracted Mouse Liver Chromatin	1	1.6	1.1	0.54	0.04
	1	1.5	0.83	0.31	–

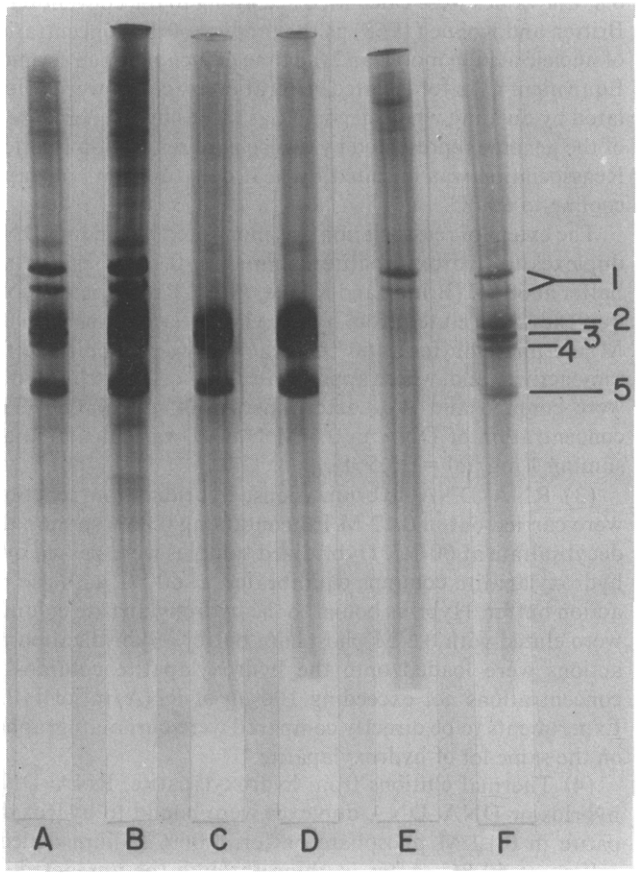

FIGURE 1: Polyacrylamide gel electrophoresis of chromatin proteins. Proteins from intact and salt-extracted chromatin were electrophoresed in 2.5 M urea, 0.9 N acetic acid, pH 2.7, in 15% acrylamide gels. Gels, from left to right are: (A) mouse liver total chromatin proteins, 200 μg; (B) mouse liver total chromatin proteins, 400 μg; (C) proteins remaining in 0.5 M NaCl extracted chromatin, 200 μg; (D) proteins remaining in 0.5 M NaCl extracted chromatin, 400 μg; (E) proteins extracted from chromatin by 0.5 M NaCl, 400 μg; (F) purified mouse liver histone, 20 μg. Histone fractions F_{1-5} are identified according to Panyim and Chalkley (1969).

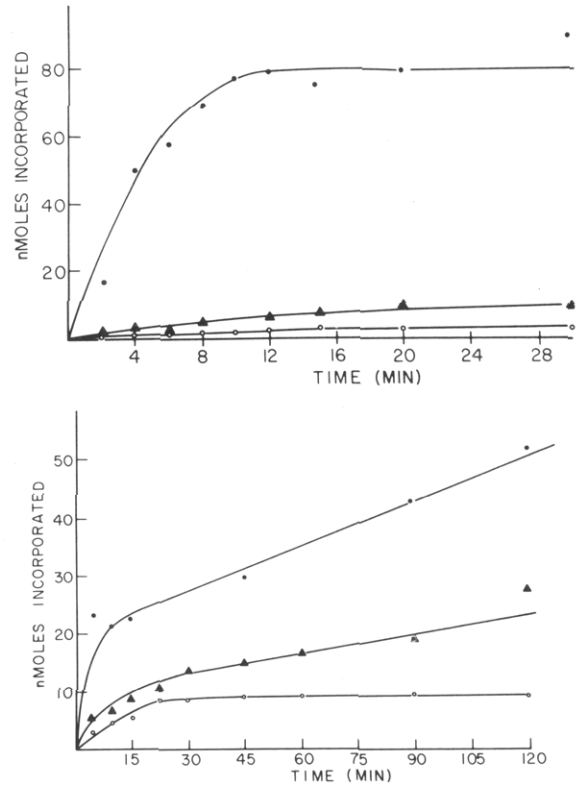

FIGURE 2: Kinetics of RNA synthesis from chromatin and DNA templates. Incorporation of [^{14}C]ATP into 10% Cl_3CCOOH insoluble material in an in vitro RNA synthesis reaction containing 40 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 1 mM $MnCl_2$, 0.8 mM β-mercaptoethanol, 0.8 mM β-mercaptoethanol, 0.8 mM each of ribonucleatide triphosphate, 200 μg/ml of template and 0.2 μCi/ml of [^{14}C]ATP. Incorporation of nucleotides/20 μg of template is plotted as a function of time of incubation at 37 °C. (A) "Low" salt conditions. Saturating amounts (1 unit/5 μg of template) of *E. coli* RNA polymerase were used. (B) "High" salt conditions. Reactions contained 150 mM KCl and 1 Burgess unit of *E. coli* RNA polymerase/5 μg of template. (●) DNA template; (○) chromatin template; (▲) salt-extracted chromatin template.

void of the lysine-rich F_1 histones and retains major portions of all the other histone fractions. In addition, a wide variety of non-histone proteins are extracted. Examination of the proteins remaining with the salt-extracted chromatin reveals a simplified pattern of the non-histone proteins suggesting that many but not all of the non-histone proteins are removed.

Figure 2 illustrates the kinetics of in vitro RNA synthesis in low and high salt. Low-salt conditions (Figure 2A) have been previously used for analysis of chromatin template specificity (Paul and Gilmour, 1968; Smith et al., 1969). High-salt conditions (Figure 2B) have been reported to promote reinitiation by *E. coli* RNA polymerase (Fuchs et al., 1967; Richardson, 1970). Under both conditions, chromatin and salt-extracted chromatin are much less efficient templates for RNA synthesis than is purified DNA. In low salt, the bulk of RNA synthesis terminates after 15 min for all three templates examined. In high salt, synthesis from purified DNA and salt-extracted chromatin continues for at least 120 min. However, the synthesis primed by chromatin again ceases after about 15 min. The constant level of incorporation measured up to 120 min in chromatin-primed reactions suggests that the degradation of synthesized RNA by chromatin-associated nucleases is not responsible for the early cessation of net incorporation.

High-salt synthesis conditions were utilized for the majority of experiments reported here, since the yield of RNA per unit of RNA polymerase was greater for DNA and salt-extracted chromatin. Also, hybridization competition experiments (discussed below) failed to demonstrate differences in the RNA population produced from chromatin templates under low- and high-salt incubation conditions.

(2) *Filter Hybridization Competition Experiments.* Filter hybridization competition experiments were performed in order to compare our chromatin and RNA preparations to those previously reported to demonstrate sequence specific in

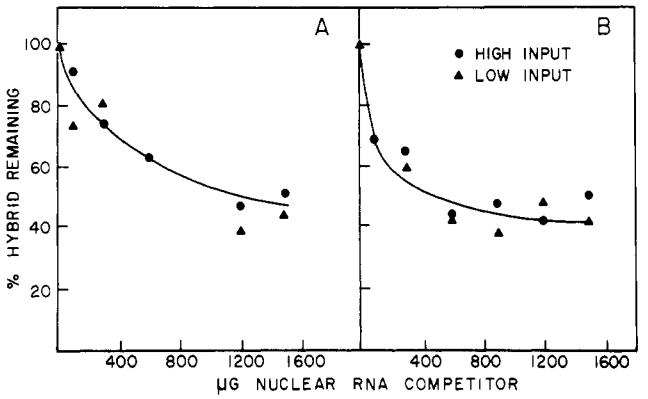

FIGURE 3: Competition of liver nuclear RNA for the hybridization of [^{14}C]DNA-primed RNA. Hybridization was for 48 h at 37 °C in 2× SSC, 50% formamide, 1 mM EDTA to filter bound mouse DNA (8.35 μg/filter). (A) DNA-primed RNA synthesized under high-salt conditions: (●) 19.8 μg of RNA input/0.2 ml, 100% = 9400 cpm = 1.14 μg of RNA; (▲) 1.77 μg of RNA input/0.2 ml, 100% = 876 cpm = 0.105 μg RNA. (B) DNA-primed RNA synthesized under low-salt conditions: (●) 19.60 μg of RNA input/0.2 ml, 100% = 4560 cpm − 0.585 μg of RNA; (▲) 1.99 μg of RNA input/0.2 ml, 100% = 398 cpm − 0.051 μg of RNA.

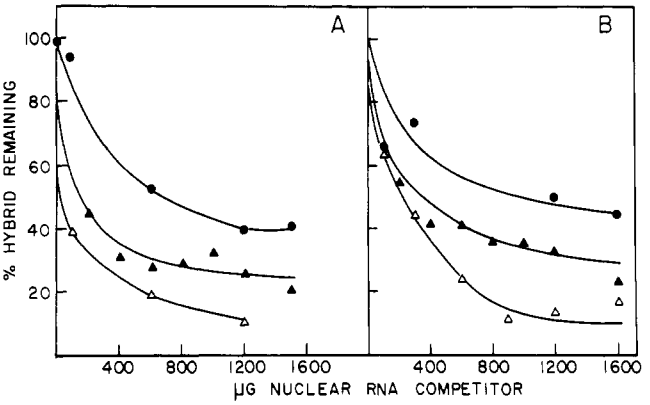

FIGURE 4: Competition of liver nuclear RNA for the hybridization of mouse liver chromatin-primed [^{14}C]RNA. Hybridization was for 48 h at 37 °C in 0.2 ml of 2× SSC, 50% formamide, 1 mM EDTA to filter bound mouse DNA (8.34 μg/filter). (A) Chromatin-primed RNA synthesized under high-salt conditions: (●) 17.5 μg of RNA input, 100% = 1310 cpm = 0.750 μg of RNA; (▲) 2.60 μg of RNA input, 100% = 1048 cpm = 0.176 μg of RNA; (△) 1.75 μg of RNA input, 100% = 154 cpm = 0.088 μg of RNA. (B) Chromatin-primed RNA synthesized under low-salt conditions: (●) 15.3 of μg RNA input, 100% = 873 cpm = 0.310 μg of RNA; (▲) 3.64 μg of RNA input, 100% = 1525 cpm = 0.386 μg of RNA; (△) 1.84 μg of RNA input, 100% = 125 cpm = 0.043 μg of RNA.

vitro transcription from reiterated DNA sequences (Paul and Gilmour, 1968; Smith et al., 1969; Tan and Miyagi, 1970).

Increasing amounts of mouse liver nuclear RNA were used to compete for the hybridization of radiolabeled RNA synthesized in vitro from DNA and chromatin templates. Figures 3 and 4 compare the competition by nuclear RNA for the hybridizaton of cRNA synthesized under low- and high-salt conditions at several different amounts of input cRNA. For cRNA transcribed from DNA templates (Figure 3a,b) the extent of competition by nuclear RNA is independent of the input cRNA concentration and the conditions of synthesis. The results obtained with chromatin-primed cRNA synthesized under high (Figure 4a) and low (Figure 4b) salt conditions are indistinguishable and indicate that by this criterion the same populations of sequences are synthesized in both reaction mixtures. As previously observed, low concentrations of input cRNA result in greater than 80% competition by nuclear RNA. However, as the concentration of input cRNA is increased, the extent of competition decreases. The highest concentrations of chromatin-primed cRNA used (75–85 μg/ml of hybridization buffer) showed no greater competition by nuclear RNA than did DNA-primed cRNA (Figure 3). In both cases, nuclear RNA competes for about 50% of the cRNA, suggesting that mouse liver chromatin-primed cRNA contains a wide variety of sequences not found in nuclear RNA and, in fact, is no more restricted than purified DNA with respect to the kinds of sequences synthesized in vitro that can be detected by the filter hybridization assay.

In order to demonstrate that the inability to achieve greater than 50% competition at high RNA inputs was not an artifact of the hybridization system, the ability of nuclear RNA to compete with itself was measured at comparable low- and high-input RNA concentrations. A portion of the nuclear RNA competitor preparation was iodinated in vitro and used as the radiolabeled input test RNA. Figure 5 demonstrates that nuclear RNA competes effectively for the hybridization of an identical nuclear RNA sample at both high- and low-input concentrations.

(3) *Preparation of ^{125}I-Labeled DNA Probes.* The results of filter hybridization experiments reported above indicated that the sequence specificity of in vitro transcription from chromatin was comparable to previous reports, but that this

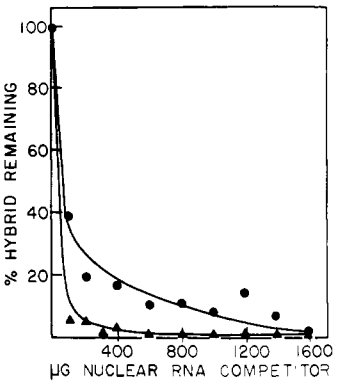

FIGURE 5: Competition of liver nuclear RNA for the hybridization of ^{125}I liver nuclear [^{125}I]RNA. Hybridization was for 48 h at 37 °C in 0.2 ml of 2× SSC, 50% formamide, 1 mM EDTA to filter bound mouse DNA: (●) 25 μg of mouse liver nuclear [^{125}I]RNA/0.2 ml, 100% = 1825 cpm; (▲) 2.5 μg of mouse liver nuclear [^{125}I]RNA/0.2 ml, 100% = 571 cpm.

sequence specificity was not absolute. Therefore, further analysis of the RNA populations transcribed was undertaken by RNA excess hybridization to radiolabeled DNA. ^{125}I-labeled DNA probes of high, intermediate, and low reiteration frequency were prepared as outlined in Figure 6. The DNA fractions used as probes are indicated in the boxes in Figure 6 and were collected as duplex molecules after the final fractionation in order to maximize hybridizability.

The properties of the various iodinated DNA C_0t fractions are given in Table II. The difference in specific activity among the fractions is approximately twofold (see methods). The piece size of the DNA, as measured by sedimentation in alkaline sucrose, was reduced from about 600 nucleotides to 200–300 nucleotides after labeling and C_0t fractionation. This may be the result of slight depurination during the course of iodination at high temperature and acid pH. Also, the reassociability of each fraction was reduced following labeling and C_0t fractionation. The maximum reassociation to excess driver DNA ranged from 60 to 74%.

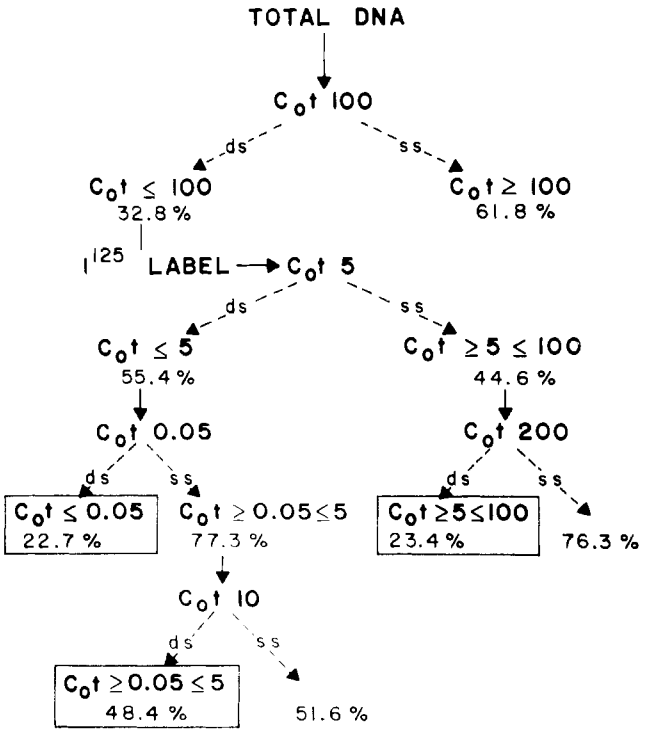

FIGURE 6: Preparation of ^{125}I labeled DNA probes. Purified mouse DNA was reassociated to C_0t 100, the reassociated DNA isolated by chromatography on hydroxylapatite and the purified repetitive DNA sequences iodinated in vitro at 80 °C. The labeled DNA was further fractionated by additional cycles of reassociation and fractionation on hydroxylapatite. Solid arrow indicate reassociation steps. Broken arrows indicate subsequent fractionation into duplex (d_s) and single strand (ss) molecules.

TABLE II: Characteristics of Fractionated ^{125}I-Labeled DNA.

C_0t Fraction	% DNA	Initial Sp Act.	Piece Size (Average Number of Nucleotides)	% Hybridizable to DNA
≤0.05	3.86	1.28×10^5 cpm/μg	210	60
≥0.05 ≤ 5	13.15	2.24×10^5 cpm/μg	326	67
≥ 5 ≤ 100	17.0	1.66×10^5 cpm/μg	245	74

The reassociation properties of the iodinated DNA are not measurably different from unlabeled DNA. The reassociation kinetics in 0.12 M PB of whole genome iodinated DNA do not differ from those of unlabeled mouse DNA when monitored optically (data not shown). The reiteration frequency distribution of each probe was measured by reassociating trace amounts with excess unlabeled whole genome DNA (Figure 7). Since the whole genome DNA is present in vast excess, the rate of reassociation of each trace probe is determined by the reiteration frequency of its complements in the unlabeled DNA. Each probe is complementary to a narrower range of reiteration frequencies than that present in unfractionated DNA and reassociated at the rates expected for high ($C_0t \leq 0.05$), intermediate ($C_0t \geq 0.05 \leq 5$), or low ($C_0t \geq 5 \leq 100$) reiteration frequency. Finally, iodinated $C_0t \leq 100$ DNA was reassociated in the presence of excess unlabeled $C_0t \leq 100$ DNA and the thermal stability of the reassociated DNA measured by thermal elution from hydroxylapatite. Figure 8

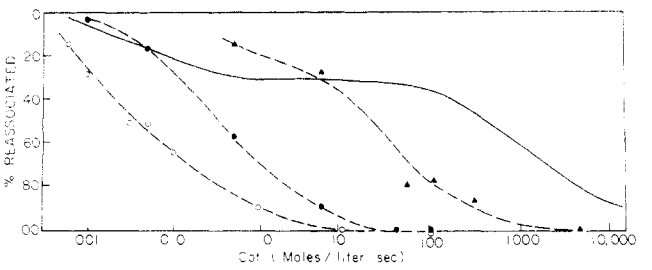

FIGURE 7: Reassociation of whole genome mouse DNA and trace [^{125}I]DNA fractions. Reassociation was at 60 °C in 0.12 M phosphate buffer. Reassociated at each point were 250–400 μg/mouse DNA and 3000–5000 cpm of trace [^{125}I]DNA. Reassociation of trace DNAs are normalized to 100%. (—) Whole genome DNA, average of all experiments; (○) $C_0t \leq 0.05$ ^{125}I DNA; (●) $C_0t \geq 0.05 \leq 5.0$ ^{125}I DNA; (▲) $C_0t \geq 5.0 \leq 100$ ^{125}I DNA.

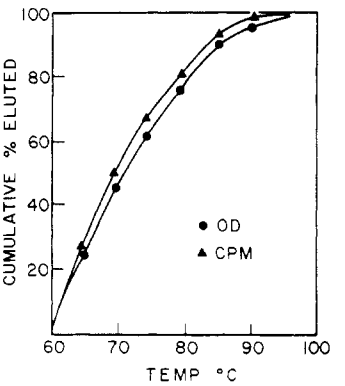

FIGURE 8: Thermal elution of ^{125}I and unlabeled DNA duplexes from hydroxylapatite. 493 μg of $C_0t \leq 100$ mouse DNA and 3.06 μg of ^{125}I $C_0t \leq 100$ mouse DNA were reassociated to an equivalent C_0t 200, bound to hydroxylapatite at 60 °C, and eluted with 0.12 M phosphate buffer at the indicated temperatures. (●) Unlabeled DNA; (▲) trace [^{125}I]DNA.

shows that the T_m of the ^{125}I-labeled DNA was only 1.5 °C lower than that of the unlabeled DNA. This lowered T_m probably is a result both of the reduction in piece size that accompanies the iodination reaction and the iodine substitution itself, calculated to be 1% of the cytosine residues.

(4) *RNA Excess Hybridization to Isolated DNA Probes.* In order to assay the presence of complements to the iodinated DNA probes in a variety of RNA samples, a trace amount of each DNA was hybridized in the presence of a large excess of RNA. The method of Davidson and Hough (1971) as modified in this laboratory (Angerer, 1973) was used to measure RNA/DNA hybrid formation by binding to hydroxylapatite. The concentration of the DNA is kept as low as possible to limit DNA–DNA duplex formation. The rate and extent of hybrid formation are thus determined by the excess RNA present. The hybridization reaction is passed over hydroxylapatite at 60 °C in 0.12 M phosphate buffer, 0.06% sodium dodecyl sulfate. Under these conditions, a large, though variable, fraction of the RNA is bound to the column, as well as the RNA/DNA hybrids and the DNA/DNA duplexes, while unreassociated DNA is not retained.

The ability of hydroxylapatite to retain the mismatched RNA–DNA hybrids formed with reiterated DNA was tested by hybridizing sheared mouse DNA (8.9 μg) with [^{14}C]cRNA (634 μg) synthesized in vitro from a DNA template to C_0t 2.4 $\times 10^{-2}$. Sixty-five percent of the RNA passed through a hydroxylapatite column in 0.12 M phosphate buffer, 0.06% sodium dodecyl sulfate at 60 °C, while 35% was retained and subsequently eluted with 0.3 M phosphate buffer. The amount

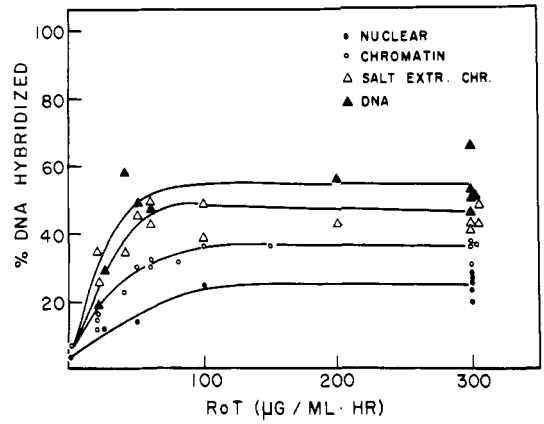

FIGURE 9: Hybridization of RNAs to $C_0t \leq 0.05$ DNA. Increasing amounts of RNA were hybridized to 2-5 × 10^{-3} μg of $C_0t \leq 0.05$ [^{125}I]DNA in 0.12 M phosphate buffer at 60 °C for 30 min and assayed on hydroxylapatite. (●) Nuclear RNA; (○) chromatin-primed RNA; (▲) DNA-primed RNA; (△) salt-extracted chromatin-primed RNA.

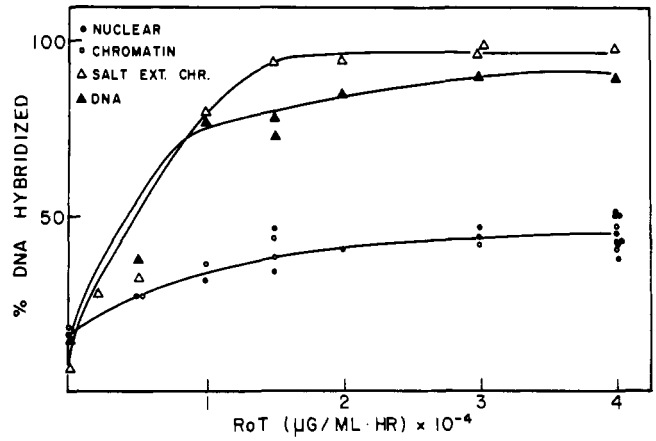

FIGURE 10: Hybridization of RNAs to $C_0t \geq 0.05 \leq 5$ DNA. Increasing amounts of RNA were hybridized to 2-4 × 10^{-3} μg of $C_0t \geq 0.05 \leq 5$ [^{125}I]DNA in 0.12 M phosphate buffer at 60 °C for 6-12 h and assayed on hydroxylapatite. (●) Nuclear RNA; (○) chromatin-primed RNA; (▲) DNA-primed RNA; (△) salt-extracted chromatin-primed RNA.

of RNA/DNA hybrid in each fraction was then estimated by its resistance of RNase (20 μg/ml in 0.24 M PB at room temperature for 2 h (Davidson and Hough, 1969). The RNA excluded from Bio-Gel A-5M after RNase digestion was judged to be in hybrid. Ninety-four percent of the RNA resistant to RNase was found in the fraction retained on hydroxylapatite. Thus, hydroxylapatite detects at least those RNA hybrids to reiterated DNA that are sufficiently well base-paired to be resistant to RNase.

Each of the three iodinated DNA probes was hybridized with nuclear RNA or cRNA primed by chromatin, salt-extracted chromatin, or DNA templates. The values reported for the fraction of DNA in hybrid have been corrected for zero time binding of the DNA to hydroxylapatite (2-12% depending on the DNA fraction) and for the fraction of the iodinated probes capable of forming duplexes with total genome DNA (Table II), according to the following formula: (% DNA bound to hydroxylapatite − % bound at zero time)/(% DNA reassociatable to total DNA − % bound at zero time). In Figures 9, 10, and 11 the percent of the probe hybridized is plotted as a function of R_0t (μg of RNA/ml × h of incubation at 60 °C). Increasing R_0ts were achieved by hybridizing for a constant period of time with increasing concentrations of RNA. The value plotted at zero R_0t represents the fraction of DNA that reassociated during the incubation period in the absence of RNA. Since the time of incubation was constant for all samples, this value represents the maximum degree of DNA/DNA duplex formation and is probably reduced in cases where hybridization to complementary RNA removes a portion of the DNA from the selfing reaction.

Figure 9 presents the results with the most reiterated DNA probe ($C_0t \leq 0.05$), which includes the mouse satellite. DNA-primed RNA hybridizes to a large fraction (55-65%) of this probe. Nuclear RNA contains complements to a much lower fraction (25%) of this probe, as might be expected from previous reports (Flamm et al., 1969) that failed to demonstrate transcription of satellite DNA in vivo. Chromatin-primed RNA consistently saturates a higher percentage of the highly reiterated DNA (38%) than does nuclear RNA. Salt-extracted chromatin serves as the template for an even wider diversity of RNA sequences (47% of the $C_0t \leq 0.05$ probe saturated).

Figure 10 presents the results with the probe of intermediate reiteration frequency ($C_0t \geq 0.05 \leq 5$). Ninety-one-hundred

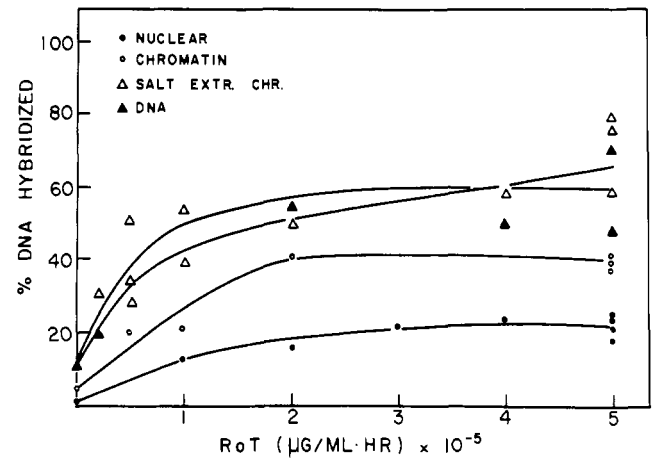

FIGURE 11: Hybridization of RNAs to $C_0t \geq 5.0 \leq 100$. Increasing amounts of RNA were hybridized to 2-4 × 10^{-3} μg of $C_0t \geq 5.0 \leq 100$ [^{125}I]DNA in 0.12 M phosphate buffer at 60 °C for 100 h and assayed on hydroxylapatite. (●) Nuclear RNA; (○) chromatin-primed RNA; (▲) DNA-primed RNA; (△) salt-extracted chromatin-primed RNA.

percent of the DNA fragments in this fraction have complements in DNA and salt-extracted chromatin-primed RNAs. Nuclear and chromatin-primed RNA each contain complements to 45% of the probe.

Figure 11 presents the results with the least reiterated probe studied ($C_0t \geq 5 \leq 100$). Again, salt-extracted chromatin-primed RNA contains complements to as many DNA sequences as does DNA-primed RNA (50-60%). A low percentage of the DNA in this probe is represented in nuclear RNA (20%), while chromatin-primed RNA includes complements to substantially more DNA sequences (up to 40%).

The saturation values from Figures 9, 10, and 11 are summarized in Table III. It is not clear from the saturation values obtained to what extent the populations of chromatin-primed and nuclear RNA overlap. To assess the degree of sequence similarity between these two RNA populations, addition experiments were performed. Saturating amounts of chromatin-primed and nuclear RNA were hybridized to each DNA probe independently and in combination. The data from these experiments are summarized in Table IV. For the $C_0t \leq 0.05$ fraction, the combination of nuclear RNA and chromatin-

TABLE III: Saturation of ^{125}I DNA Probes with RNA.

Source of RNA	Fraction of DNA Probe Hybridized (%)		
	$C_0t \leq 0.05$	$C_0t \geq 0.05 \leq 5$	$C_0t \geq 5 \leq 100$
In vitro DNA-primed	55	100	60
In vitro salt-extracted chromatin-primed	50	90	60
In vitro chromatin-primed	32	45	40
In vivo mouse liver nuclear	25	45	20

primed RNA does not hybridize more DNA fragments than does in vitro chromatin-primed RNA alone, suggesting that all the hybridizing sequences represented in nuclear RNA are also found in chromatin-primed RNA. For the $C_0t \geq 0.05 \leq 5.0$ DNA fraction, the same fraction of the DNA is hybridized to the combination of nuclear and chromatin-primed RNAs as to either RNA alone, indicating that the same families of intermediate repetition frequency are transcribed in vitro from isolated chromatin as are found in nuclear RNA preparations. Addition experiments with the $C_0t \geq 5.0 \leq 100$ DNA fraction show greater hybridization with combinations of nuclear and chromatin-primed RNA than for either RNA alone.

Discussion

Filter Hybridization. While chromatin-primed RNA apparently contains a preponderance of molecules that reflect the tissue origin of the chromatin (Smith et al., 1969), it also contains low concentrations of a wide variety of sequences not detectable in the nuclear RNA from the same tissue.

While nuclear RNA is an effective competitor against itself at both low and high concentrations of test RNA (Figure 5), its ability to compete for the hybridization of chromatin-primed RNA is dependent on the concentration of the input-chromatin-primed RNA. As the concentration of the chromatin-primed RNA increases, the extent of competition decreases (Figure 4). At low concentrations of test RNA the extent of competition is indistinguishable from the competition with homologous nuclear RNA, while at the highest input concentration tested, the extent of competition with nuclear RNA is no better for chromatin-primed RNA than it is for DNA-primed RNA (Figure 3). Thus, chromatin-primed RNA contains, in addition to the sequences present in nuclear RNA, additional sequences which only hybridize at high concentrations of test RNA.

The population of RNA sequences synthesized in vitro from chromatin could arise in several ways. (1) The majority of in vitro transcripts are the same as those transcribed in vivo, but a low level of random transcription also occurs. At low RNA inputs, the randomly transcribed sequences are not present in sufficient concentration to hybridize to filter bound DNA under the conditions of these experiments. Thus, the hybridized RNA belong to the majority class of correctly transcribed sequences and are efficiently competed by in vivo nuclear RNA. At high input RNA concentrations the randomly transcribed sequences are able to hybridize, lowering the ability of nuclear RNA to compete. (2) Chromatin transcription by *E. coli* polymerase in vitro is a faithful representation of in vivo transcription, but the nuclear RNA population to which it is being compared has been altered by post-transcriptional pro-

TABLE IV: Additive Hybridization of RNA to DNA Probes.

DNA Fraction	RNA	% DNA Hybridized
	$C_0t \leq 0.05$	
Sample A[a]	(i) Nuclear (150)[b]	30
	Chromatin-primed (150)	41
	Nuclear (150) + chromatin-primed (150)	40
	(ii) Nuclear (300)	36
	DNA-primed (300)	55
	Nuclear (300) + DNA-primed (300)	76
Sample B	Nuclear (300)	17
	Nuclear (600)	15
	Chromatin-primed (300)	41
	Nuclear (300) + chromatin-primed (300)	40
	$C_0t \geq 0.05 \leq 5$	
Sample A	Nuclear (2×10^4)	39
	Nuclear (4×10^4)	33
	Chromatin-primed (2×10^4)	38
	Nuclear (2×10^4) + chromatin-primed (2×10^4)	39
Sample B	Nuclear (4×10^4)	49
	Nuclear (6×10^4)	51
	Chromatin-primed (4×10^4)	47
	Nuclear (3×10^4) + chromatin-primed (3×10^4)	50
	$C_0t \geq 5.0 \leq 100$	
Sample B	Nuclear (4×10^5)	
	Chromatin-primed (4×10^5)	41
	Nuclear (4×10^5) + chromatin-primed (4×10^5)	71
	Nuclear (4×10^5) + chromatin-primed (4×10^5)	76

[a] Samples A and B are two independent [^{125}I]DNA probe preparations and were assayed on different batches of hydroxylapatite. [b] The values in parentheses represents the R_0t ($\mu g/ml \times h$) achieved for each RNA sample.

cessing. Differential degradation and/or selective transport to the cytoplasm could result in a nuclear RNA population in which the kinds and concentrations of various sequences differ widely from those originally transcribed. The inability of nuclear RNA to compete for chromatin-primed RNA, at high RNA input, could result from the low concentration of certain processed sequences in the nucleus relative to their representation in the nonprocessed transcripts produced by in vitro synthesis from chromatin. Since nuclear RNA is an effective competitor for chromatin-primed RNA, a low input, the majority of sequences in both RNA populations are the same.

RNA Excess Hybridization. RNA excess hybridization to trace amounts of DNA are independent of the concentration difference (at saturation) of RNA sequences over a wide range and allow examination of the alternatives suggested above. They also make possible the study of defined fractions of genome.

(*a*) *Limitations of the Method.* Since the rate of hybrid formation is controlled by the large amounts of RNA used, it is theoretically possible to limit the self-reassociation of any DNA fraction by lowering its concentration and/or limiting the time of hybridization. This method has been successfully applied to the study of unique DNA transcription in a number of eukaryotic organisms (Firtel, 1972; Hough and Davidson, 1972; Liarakos et al., 1973). The use of reiterated DNA fractions increases the practical difficulties of achieving low self-reassociation. The effect of isolating defined C_0t fractions of reiterated DNA is to greatly increase the concentration of complementary sequences by purifying them away from the rest of the genome. In these experiments, it was necessary to accept some self-reassociation, since hybridization to saturating R_0ts in reasonable times would have required the use of excessive amounts of in vitro synthesized RNA, given the specific activity of the DNA available. This problem is especially severe in the case of the highly reiterated fraction ($C_0t \leq 0.05$) where self-reassociation (10–15%) probably represents mouse satellite sequences. Since satellite DNA is reported to be nontranscribed in vivo (Flamm et al., 1969) or from isolated chromatin templates in vitro (Reeder, 1973), its rapid self-reassociation almost certainly results in an overestimation of the portion of this fraction complementary to nuclear and chromatin-primed RNA.

The self-reassociation of the DNA probe measured in the absence of any RNA is a maximum estimate, probably an overestimate, of the actual extent of DNA–DNA duplex formation, since hybridization of the DNA probe to complementary RNA will prevent its self-reassociation. The degree to which self-reassociation of the DNA probe will be reduced is a function of its complementarity to the RNA population. While self-reassociation leads to overestimates of the complementarity between a given DNA fraction and a given RNA population, observed differences in the fraction of a particular DNA sample saturated by different RNA populations are real.

Different batches of hydroxylapatite vary in their capacity to retain RNA–DNA hybrids and in the extent to which such binding is inhibited by excess RNA. Hydroxylapatite lots tested in this laboratory gave saturation values for the hybridization of $C_0t \geq 0.05 \leq 5$ DNA to nuclear RNA ranging from 22 to 55%. While variation in the fraction of total RNA–DNA hybrid bound by different hydroxylapatite preparations can be compensated by standardizing each batch and performing experiments to be directly compared on the same batch, it remains a serious limitation. The relative extents of hybridization between a particular DNA probe and different RNA populations are meaningful, but the absolute value for the amount of RNA–DNA hybrid formed cannot be determined.

(*b*) *Restriction of Chromatin Transcription In Vitro.* Chromation is a less effective template for RNA synthesis than is purified DNA (Figure 2). Saturation of reiterated DNA fractions with excess RNA (Figures 9, 10, and 11) reveals that a smaller portion of each DNA C_0t fraction has complements in chromatin- than in DNA-primed RNA. Since the experiments involve saturating the test DNAs with excess RNA, even low levels of totally random transcription should have been detected. Thus, the low level of in vitro template activity of chromatin reflects a restriction on the kinds of DNA sequences available for transcription.

While all three DNA C_0t fractions demonstrate restricted transcription from chromatin, the high ($C_0t \leq 0.05$) and low ($C_0t \geq 5 \leq 100$) reiteration fractions show a higher saturation value for chromatin-primed than for nuclear RNA (Figures 9 and 11). The $C_0t \geq 0.05 \leq 5$ fraction is saturated to the same extent by both chromatin-primed and nuclear RNA (Figure 10). Addition experiments (Table IV) in which chromatin-primed and nuclear RNA are simultaneously hybridized to trace DNA C_0t fractions indicate that the $C_0t \leq 0.05$ DNA sequences complementary to nuclear RNA are a subset of those complementary to RNA transcribed in vitro from chromatin. The same population of $C_0t \geq 0.05 \leq 5$ DNA is complementary to both nuclear and chromatin-primed RNA.

While RNA-excess hybridization indicates that random transcription of all repetitive DNA sequences does not occur even at low levels during in vitro RNA synthesis from isolated chromatin, the extent to which chromatin retains the template restriction found in vivo remains unclear. Since, at least, for the highly and moderately reiterated DNA sequences in vivo and in vitro synthesized RNA are not additive at saturation when hybridized together, DNA families active in transcription in vivo remain active in vitro. Thus, there is not a rearrangement of structural elements within chromatin that represses the "normal" synthetic function of the chromatin.

Both filter and RNA-excess hybridization experiments indicate the presence of RNA transcripts from reiterated DNA families not detected in liver nuclear RNA. These additional transcripts could be "correct", i.e., identical to in vivo transcripts, but just not detectable in our nuclear RNA preparation or they could be "incorrect", an artifact resulting from the isolation of chromatin and its transcription in vitro by a heterologous polymerase. As these experiments measure the accessibility of DNA sequences within chromatin for transcription and not the accuracy of initiation differences in the frequency of transcription of individual RNA species in vivo and in vitro might result from the use of a heterologous polymerase. Using fractionated calf thymus chromatin, Henner et al. (1975) have shown that in most cases the rate of RNA synthesis with *E. coli* RNA polymerase is greater than the rate with homologous RNA polymerase. The increase in rate of RNA synthesis could reflect a change in the frequency with which "correct" sequences are transcribed or an increase in the number of sequences being transcribed. An example of the latter was discussed in the introduction (page 4, Honjo and Reeder, 1974). Since the "new" families detected in transcripts from isolated chromatin, in this study, are not detected in our intermediate $C_0t \geq 0.05 \leq 5$ DNA fraction, they are derived from a restricted set of reiterated DNA sequences. Although a firm conclusion cannot be reached, we feel that in general these observations are best explained by post transcriptional changes in the nuclear RNA population rather than by tran-

scriptional artifact with isolated chromatin.

Chromatin Transcription after Extraction with 0.5 M NaCl. Chromatin depleted of F_1 histone and some non-histone proteins by exposure to 0.5 M NaCl transcribes quite a different set of RNAs than those transcribed in vitro from intact chromatin. Salt-extracted chromatin-primed RNA saturates a larger percentage of each DNA C_0t fraction than does chromatin-primed RNA. Hybridization of the $C_0t \leq 0.05$ fraction was slightly less efficient with salt-extracted chromatin or DNA. Thus, with the possible exception of a small portion of the highly reiterated DNA, extraction with 0.5 M NaCl destroys the restriction of reiterated DNA sequence transcription observed with intact chromatin. This significant increase in the kinds of sequences transcribed is not reflected by the template activity of 0.5 M NaCl-extracted chromatin, which increases only slightly relative to intact chromatin. The random nature of the transcription from salt-extracted chromatin could be a result either of the removal of the protein elements responsible for blocking reiterated DNA transcription, or from a nonspecific rearrangement of proteins in the extracted chromatin. Georgiev and his colleagues (Juhasy et al., 1970) have observed an increased variety of reiterated DNA transcripts synthesized from chromatin extracted with 0.6 M NaCl and have suggested that histone F_1, the major protein removed by this treatment, is specifically responsible for repressing reiterated DNA transcription. However, several groups (Clark and Felsenfeld, 1972; Jensen and Chalkey, 1968; Varshavky and Georgiev, 1972) have demonstrated that exposure of isolated chromatin to moderate salt concentrations, even lower than 0.5 M, allows exchange of chromatin proteins in the presence of exogenous sources of nucleic acids. This exchange involves primarily F_1 histone, not present in our salt-extracted preparation (Figure 1). Nevertheless, if exchange of other species of chromatin proteins occurs in 0.5 M NaCl in the absence of added nucleic acids, and if this exchange is not sequence specific, then the DNA sequences subsequently available for transcription do not accurately reflect the original protein–DNA sequence association.

These experiments demonstrate that: (1) the transciption of reiterated sequences from isolated chromatin is restricted; (2) all sequences transcribed in vivo are transcribed in vitro from isolated chromatin; (3) the chromatin transcripts not detectable in nuclear RNA are synthesized from a limited set of DNA sequences; (4) extraction with 0.5 M NaCl eliminates the sequence restricted transcription of reiterated DNA observed with intact chromatin.

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