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Transcription of Rat-Liver Chromatin with Homologous Enzyme

(RNA polymerase/α-amanitin)

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ABSTRACT **The product of transcription of rat-liver chromatin with homologous rat-liver Form-B polymerase *in vitro* is high molecular weight RNA with sedimentation coefficients principally in the range of 18–45 S. The average size is somewhat smaller than that of the heterogeneous high molecular weight RNA synthesized by the endogenous enzyme *in vivo* (or in isolated nuclei). We have excluded the possibility that this difference can be attributed to degradation of the nascent RNA occurring under the conditions of our experiments. The size of the RNA produced by the homologous enzyme nevertheless approaches that of the natural transcripts much more closely than does that of the RNA produced by bacterial RNA polymerases, which we, in addition to other authors, have found to sediment around 10 S.**

Chromatin can be transcribed by bacterial polymerases *in vitro* yielding a product that bears some similarity to RNA synthesized *in vivo*. In particular, sequence homology of the RNA synthesized by the endogenous enzyme *in vivo* and that made by the bacterial enzyme *in vitro* has been demonstrated by hybridization studies (1–5). However, in several other respects, the RNA made by the bacterial enzyme does not appear to resemble the natural RNA products of transcription. The molecular weight of the RNA made *in vitro* is low (corresponding to 10S RNA), as evidenced by polyacrylamide-gel electrophoresis (6). It is known that the immediate product of chromatin transcription *in vivo* is high molecular weight, heterogeneous RNA with sedimentation coefficients ranging from 6 to 100 S (7–10). Moreover, nuclei incubated *in vitro* yield products of similar sizes to those made *in vivo* (11). The experiments of Butterworth *et al.* (12) indicate that the homologous enzyme transcribes chromatin more efficiently than *Escherichia coli* RNA polymerase (12). Moreover, the two enzymes appear to bind to different regions on the DNA since these authors could not demonstrate competition between them for the template (12). We have examined the size of the RNA transcripts when rat-liver chromatin is incubated with exogenous RNA polymerase (Form B) from the same source. Our results provide the first direct evidence that the homologous enzyme produces a more natural RNA product than that produced by the hitherto extensively used bacterial enzymes.

METHODS

Preparation of chromatin

All operations were performed between 0 and 4°. Nuclei were prepared from about 20 g of rat liver by the method of Blobel and Potter (13). The nuclei were resuspended by hand homogenization in 25 ml of homogenizing medium [0.32 M sucrose in TKM: 50 mM Tris·HCl (pH 7.5)–25 mM KCl–5 mM $MgCl_2$] and pelleted by centrifugation at 1020 × *g* for 5 min. Treatment with 25 ml of 0.5% Triton X-100 in homogenizing medium was followed by three successive washes in homogenizing medium. The nuclei were then washed with 25 ml of salt solution [0.01 M Tris·HCl–0.14 M NaCl (pH 7.9)] (14) three times, stirring for 30 min each time. After homogenization with 25 ml of water, the chromatin was pelleted by centrifugation at 23,300 × *g* for 10 min. Approximately an equal volume of water was added to bring the DNA concentration to between 1.0 and 1.6 mg/ml.

Preparation of RNA polymerase

All operations were performed between 0 and 4°. Nuclei from rat liver were prepared essentially as described by Chesterton and Butterworth (15) and stored at −70° in nuclei storage buffer [70% glycerol–2.5 mM K phosphate–5 mM $MgCl_2$ (pH 7.0)] (16) until needed. RNA polymerase was prepared according to the method of Roeder and Rutter (17) with slight modifications. Nuclei from between 200 and 400 g of liver were thawed, washed with 100 ml of TKM, and spun down at 4080 × *g* for 5 min. The nuclei were resuspended in about 50 ml of TSMD [0.01 M Tris·HCl (pH 7.9)–1.0 M sucrose–5 mM $MgCl_2$–5 mM dithiothreitol], and 3 M ammonium sulfate was added to a final concentration of 0.3 M. The resulting viscous solution was sonicated for 90 sec in 15-ml lots in a Mullard Sonicator. Two volumes of TGMED [25% glycerol–0.05 M Tris·HCl–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol (pH 7.9)] were added, followed by centrifugation at 47,000 × *g* for 30 min. To the supernatant was added 0.42 g/ml of solid ammonium sulfate. The mixture was stirred for 30 min and then centrifuged at 47000 × *g* for 30 min. The precipitate was redissolved in TGMED, and dialyzed against 100 volumes of TGMED containing 0.05 M ammonium sulfate overnight. The dialysate was centrifuged at 105,650 × *g* for 60 min and the supernatant was loaded on a 10 cm × 2 cm column of DEAE-cellulose that had previously been equilibrated with TGMED containing 0.05 M ammonium sulfate. The column was washed with the same buffer until a protein peak had been eluted, and a linear gradient, consisting of 30 ml each of 0.1 M ammonium sulfate in TGMED and 0.5 M ammonium sulfate in TGMED, was applied at a rate of 40 ml/hr. 2.5-ml Fractions were collected into tubes containing 0.5 ml of glycerol and assayed immediately for RNA polymerase activity. Fractions containing activity were divided into 0.5-ml aliquots and stored at −70°. Fractions containing Form-B RNA polymerase were identi-

Abbreviations: SDS, sodium dodecyl sulfate. The Form-B polymerase is also called RNA polymerase II in certain other laboratories.

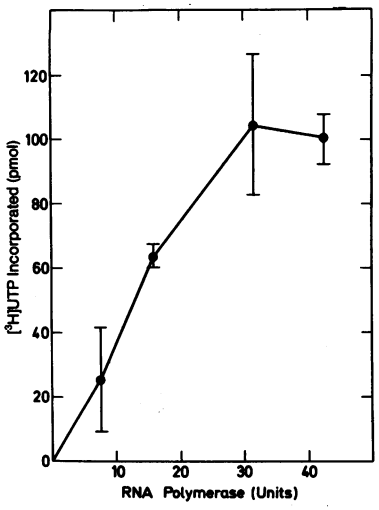

FIG. 1. Saturation of chromatin by Form-B RNA polymerase. Incubations of 0.5 ml were performed as described in *Methods*. The mixtures contained 36 μg of chromatin DNA and were terminated after 40 min. The endogenous polymerase activity of the chromatin (12.59 pmol of [3H]UTP) has been subtracted from the plotted values to give net exogenous RNA synthesis.

fied by inhibition of their activity by α-amanitin. The Form-B enzyme was used in all experiments.

RNA Polymerase Assay. Assay mixtures contained 0.05 M Tris·HCl (pH 7.9), 6 mM $MgCl_2$, 1.6 mM $MnCl_2$, 0.1 M ammonium sulfate, 30 μg of calf-thymus DNA, 0.6 mM each ATP, CTP, GTP, 0.1 mM [3H]UTP (1 μCi), enzyme, and water in a total volume of 200 μl. Assays were performed at 30° for 10 min. One unit of activity is defined as the ability to incorporate 1 pmol [3H]UTP/min into acid-insoluble material. Incubation was terminated by the addition of 0.1 ml of ice-cold 0.1 M $Na_4P_2O_7$ containing 2 mg of bovine-serum albumin per ml and the assay mixtures were frozen and stored at −20° before processing.

Incubations with chromatin

The normal assay mixture was scaled up to 500 μl, calf-thymus DNA was replaced by rat-liver chromatin, and ammonium

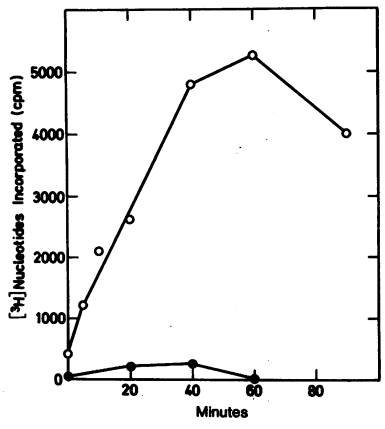

FIG. 2. Time-course of chromatin and DNA transcription by RNA polymerase. Incubations were performed as described in *Methods* and terminated at the times indicated on the graph. 32.5 μg of chromatin DNA in a total volume of 0.5 ml (*solid circles*). 32.5 μg of chromatin DNA plus 18 units of enzyme in a total volume of 0.5 ml (*open circles*).

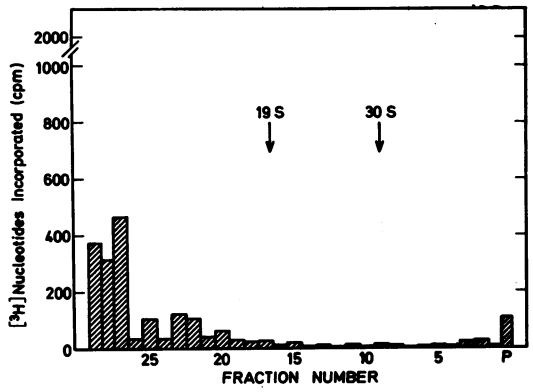

FIG. 3. Transcription products of endogenous RNA polymerase activity of chromatin. Mixtures containing 96 μg of chromatin were incubated for 60 min and analyzed on a sucrose gradient as described in *Methods*.

sulfate by KCl. The concentration chosen was 0.26 M KCl, as this was the optimum ionic strength for enzyme activity, while endogenous chromatin activity remains very low (12).

Incubation mixtures to be analyzed on sucrose gradients contained 0.6 mM each [3H]CTP and [3H]GTP (50 μCi/ml of mixed nucleotides) as well as [3H]UTP. Four incubation mixtures of 0.5 ml each were prepared for each sample, to make a total of 2 ml. At the end of the incubation period, the mixtures were pooled, and two 0.1-ml aliquots were removed for counting. 0.2 ml of 10% sodium dodecyl sulfate (SDS) was added, and the precipitate that formed was collected by centrifugation at 4000 × *g* for 5 min. The precipitate was washed with 1 ml of 1% SDS, the wash was added to the previous supernatant, and the mixture was loaded on the sucrose gradient.

Extraction of RNA. RNA was extracted by the hot phenol–SDS method of Penman (18). Since the incubation mixtures did not contain large amounts of DNA, treatment with DNase was omitted.

Zonal Centrifugation in Sucrose Gradients. Isokinetic gradients calculated to separate RNA molecules with sedimentation coefficients ranging up to 42 S were prepared from sucrose solutions containing 0.01 M Tris·HCl–0.01 M $LiCl_3$–1 mM EDTA–0.1% SDS (pH 8.1). The gradients were centrifuged in an SW27 rotor at 27,000 rpm for 15 hr at 15°. Fractions were collected from the bottom of the tube by means of a probe and a peristaltic pump, which passed the gradient at a constant rate through a flow cell providing a continuous absorbance trace at 260 nm. The fractions were frozen and stored at −20° until processing.

Processing of Samples for Radioactive Counting. Sucrose-gradient fractions required the addition of 500 μg of bovine-serum albumin before acid precipitation. Both assay mixtures and sucrose-gradient fractions were precipitated by addition of 2 volumes of 10% Cl_3CCOOH containing 5% $Na_4P_2O_7$. The mixtures were allowed to stand on ice for 30 min and were then filtered through glass-fiber filters (Whatman GF/C) previously wetted with 5% Cl_3CCOOH containing 3% $Na_4P_2O_7$. The filters were washed with 35 ml of this solution, 25 ml of water, and 5 ml of ethanol, and placed in a 60° oven to dry. 1 ml of toluene, diluted 1:2 with scintillation fluid (5.5 g of Permablend per liter of toluene), was added to each filter, and the filters were left to stand for at least 2 hr. 10 ml

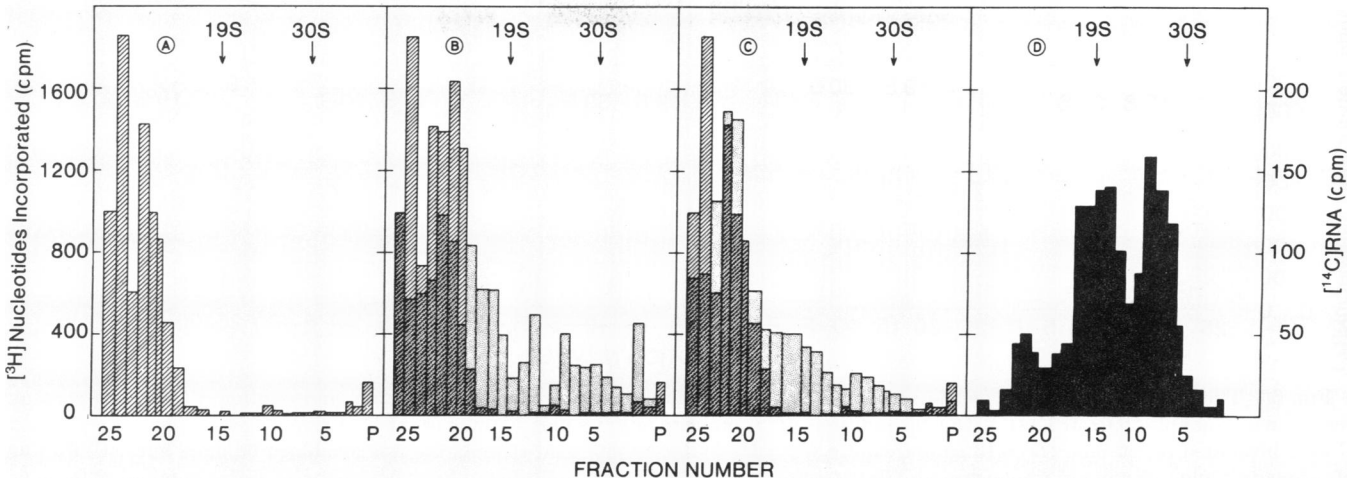

FIG. 4. Analysis of the products of transcription of chromatin by RNA polymerase by zonal centrifugation in sucrose gradients. Mixtures were incubated for 60 min, as described in *Methods*, except that three incubation mixtures of 1 ml each, containing a total of 229 μg of chromatin DNA and 224 units of enzyme, were prepared for each set of conditions and pooled after the incubation. (*A*) EDTA control: complete mixture plus 0.02 M EDTA. (*B*) No additions. (*C*) and (*D*) Complete mixture plus 3 mg of ^{14}C-labeled ribosomal RNA (3312 cpm). (*C*) Shows ^{3}H counts and (*D*) shows ^{14}C counts.

of scintillation fluid containing 1 ml of glacial acetic acid per liter (19) were added, and the samples were counted for 10 min in a Nuclear Chicago Mark II Liquid Scintillation Counter.

RESULTS AND DISCUSSION

Preliminary experiments were performed in order to determine the optimal conditions for obtaining a high yield of RNA in the transcription of rat-liver chromatin by the homologous enzyme. We found that 30 units of enzyme were required to saturate 36 μg of chromatin DNA (Fig. 1). This differs from the results obtained by Butterworth *et al.* (12), who were unable to saturate their chromatin template with much higher amounts of enzyme. We cannot account for this difference.

The time-course of RNA synthesis is shown in Fig. 2. The maximum yield of RNA was obtained after 60-min incubation, after which no further net synthesis occurred. That endogenous RNA polymerase activity of the chromatin was negligible is shown in Fig. 3.

The product of RNA synthesis in a 60-min incubation mixture was characterized by zonal centrifugation in sucrose gradients containing SDS. The distribution of radioactivity in the sucrose gradient of a control sample incubated in the presence of 0.02 M EDTA (which totally inhibits RNA synthesis) is shown in Fig. 4*A*. Since some unincorporated nucleotides are evidently retained by the filters, this pattern is superimposed on the test-gradient patterns to indicate RNA synthesis above the control level. The synthesis of high molecular weight RNA, sedimenting at rates equal to or greater than the 19S and 30S ribosomal RNA markers, is shown in Fig. 4*B*.

To assess the extent of nuclease activity, a third experiment was performed in which ^{14}C-labeled rabbit reticulocyte ribosomal RNA was added to the incubation mixture. The dis-

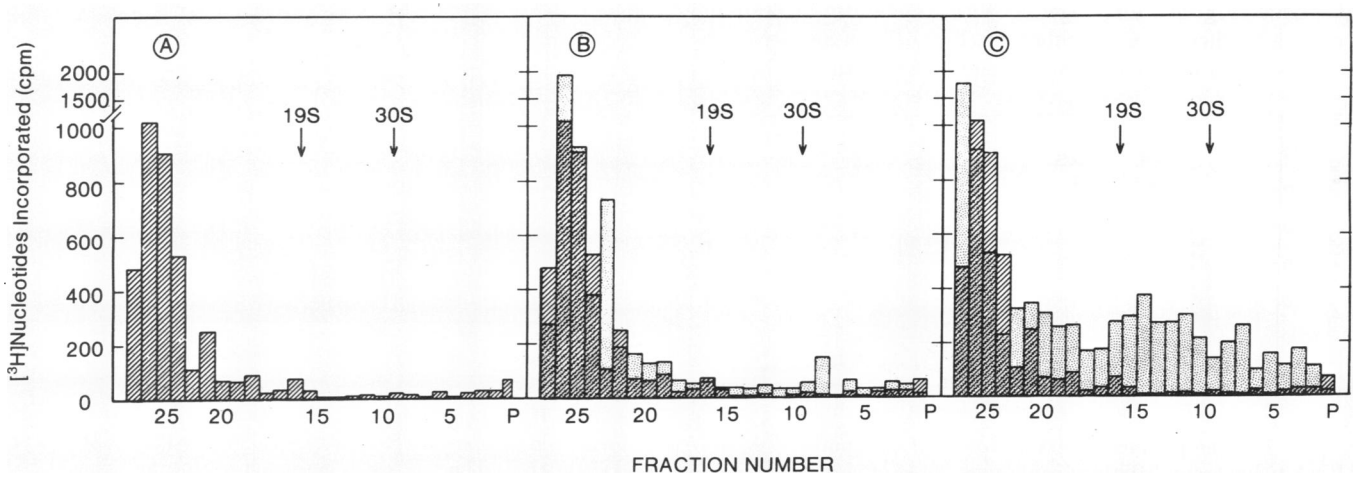

FIG. 5. The effect of α-amanitin on the transcription of chromatin by RNA polymerase. Mixtures containing 96 μg of chromatin DNA and 492 units of enzyme were incubated for 60 min, as described in *Methods*. (*A*) EDTA control: complete mixture plus 0.02 M EDTA. (*B*) Complete mixture plus 0.04 μg of β-amanitin. (*C*) No additions.

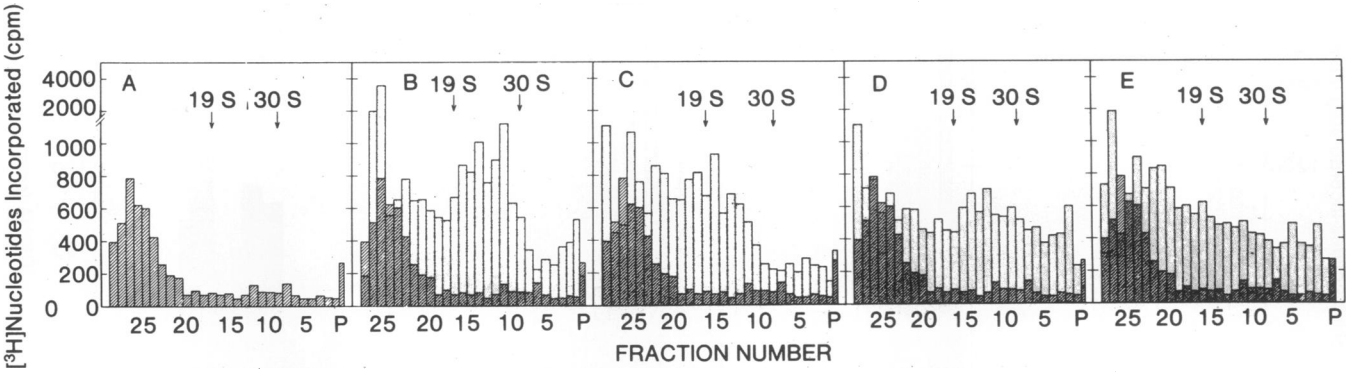

FIG. 6. The effect of rat-liver ribonuclease inhibitor and α-amanitin on the product of chromatin transcription. (*A*) EDTA control: complete mixture plus 0.02 M EDTA. (*B*) No additions. (*C*) Complete mixture plus 10 units of rat-liver ribonuclease inhibitor. *A*, *B*, and *C* were incubated for 60 min. (*D*) 0.13 μg α-amanitin was added after 60 min, and the mixture was incubated 60 more min. (*E*) 0.13 μg of α-amanitin plus 10 units of ribonuclease inhibitor were added after 60 min, and the mixture was incubated 60 more min. Incubations were as described in *Methods*, except that the concentration of UTP was raised to 6 mM. The mixtures contained a total of 96 μg of chromatin DNA and 492 units of enzyme.

tribution of radioactivity due to the synthesized [^{3}H]RNA is shown in Fig. 4*C*, and that due to the added [^{14}C]RNA is shown in Fig. 4*D*. Before incubation, the radioactivity profile of the ^{14}C-labeled RNA resembled the absorbancy profile with the typical ratio of about 2.5:1 for the 30S and 19S components (results not shown). After incubation this ratio is only slightly changed, roughly equal amounts of radioactivity appearing in the two peaks. This result indicates that some degradation of this RNA occurred but that this was very slight. Two breaks in the 30S polynucleotide chain of 5000 nucleotides are required to reduce it to the size of 19S RNA. These breaks could have occurred for only a small fraction of 30S RNA chains to produce the observed result.

A further assay for nucleolytic degradation was made by addition of α-amanitin to the incubation mixture at 60 min and examination of the molecular weight distribution of the RNA after a further 60 min of incubation. α-Amanitin completely inhibits the activity of Form-B polymerase (13) as confirmed by Fig. 5*B* in which the toxin was added at 0 time. By comparison of Fig. 6*D* and *B*, it can be seen that incubation of nascent RNA for 1 hr in the presence of α-amanitin does not appreciably alter the sedimentation profile. Moreover, when rat-liver ribonuclease inhibitor was added both at 0 time (Fig. 6*C*) and together with α-amanitin at 60 min before a further 60-min incubation (Fig. 6*E*), in neither case was a shift to higher molecular weight distribution to be expected, if there was a nuclease susceptible to inhibition present in the system.

Although previous studies had shown that the RNA synthesized by bacterial RNA polymerase on chromatin templates was of low molecular weight, in order to directly compare the size of the transcription products of bacterial and mammalian enzymes it was necessary to perform the transcription reaction using the same chromatin preparation as a template for both enzymes under identical incubation conditions. The results of this experiment are shown in Figs. 6 and 7. All the RNA synthesized by *E. coli* RNA polymerase sedimented below 19 S (Fig. 7*B*), while that synthesized by rat-liver RNA polymerase was heterogeneous with a significant

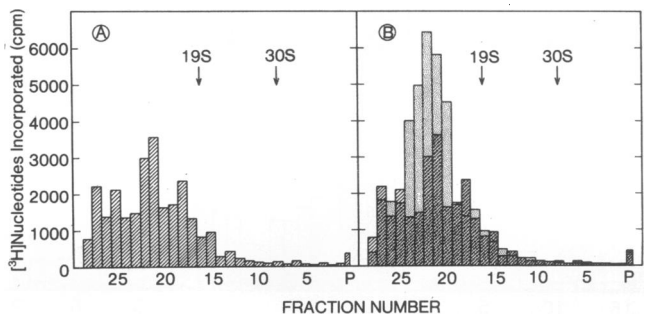

FIG. 7. The products of transcription of chromatin by *E. coli* RNA polymerase. Mixtures containing 96 μg of chromatin DNA and 280 units of *E. coli* RNA polymerase, were incubated for 60 min as described in *Methods*. (*A*) Enzyme alone; (*B*) enzyme plus chromatin.

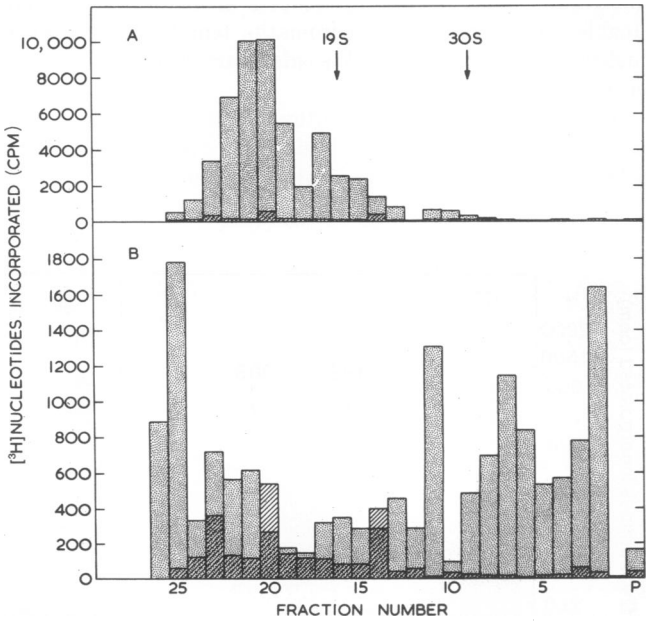

FIG. 8. Comparison of the RNA synthesized by *E. coli* and rat-liver RNA polymerases on rat-liver chromatin. At the end of the 60-min incubation, EDTA (pH 7.5) was added to 0.1 M and SDS to 0.5%. The RNA was extracted by the hot phenol–SDS method. (*A*) 96 μg of chromatin plus 1016 units of *E. coli* RNA polymerase; (*B*) 104 μg of chromatin plus 38 units of rat-liver RNA polymerase.

proportion sedimenting above 30 S (Fig. 6*B*). These results are confirmed by an experiment in which the RNA was extracted from the incubation mixtures before analysis on sucrose gradients (Fig. 8).

We conclude from these experiments that the transcription of DNA on chromatin templates, when the homologous enzyme is used, yields a heterogeneous population of products with sedimentation properties approaching that found *in vivo*. Some degradation of the product formed *in vivo* may occur, but this was scarcely detectable in the three types of assays used. The much smaller size of the product formed when bacterial enzymes transcribe chromatin DNA could be due to the recognition of incorrect initiation and termination sites on the DNA.

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