RNA Polymerase Isolated from Bovine Lymphosarcoma by Sequential Low- and High-Salt Extraction

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SUMMARY

Bovine lymphosarcoma tissue has been extracted with low- and high-salt buffers [0.05 M Tris-Cl ± 0.3 M (NH₄)₂SO₄]. Diethylaminoethyl-Sephadex chromatography of both the high-salt and low-salt extracts yields RNA polymerases I and II, although low-salt extraction releases only one-third as much activity. Extraction by high salt of the residue from the low-salt extract, followed by diethylaminoethyl-Sephadex chromatography, yields additional enzyme activity with properties of Form II. Purification of the low-salt extract by protamine precipitation, elution with sodium succinate, and phosphocellulose chromatography yields a preparation of RNA polymerase (RNAP) with hybrid properties, combining the salt optimum of Form I, diethylaminoethyl-Sephadex elution pattern of Form II, and α-amanitin sensitivity of Form III. RNAPₖₗ transcribes native DNA and chromatin efficiently. More RNAPₖₗ is recovered from lymphosarcoma tissue than from calf thymus.

INTRODUCTION

A significant aspect of neoplasia is that of altered gene expression. Cells assume characteristics of other tissues such as ectopic hormone production (21) and of embryonic and fetal tissues (13). Some abnormality in the mechanism of regulation of transcription is thought to be involved in producing pathological patterns of gene expression. Therefore, the study of RNA polymerase is of importance in elucidating the role of the enzyme in the regulation of transcription and possible alterations in the enzyme or its regulation as a result of neoplasia. The study of RNA polymerase is also of interest in view of the possible involvement of RNA as primer in DNA synthesis (2, 14, 20, 26, 29).

RNA polymerase was first isolated from bovine lymphosarcoma in this laboratory in 1965 (8) by low-salt extraction of whole tissue. This enzyme has been purified (9, 10) and some of its properties have been studied (7, 10, 12), especially in comparison with the properties of the analogous enzyme from calf thymus. Since these studies began, work in other laboratories has shown multiple forms of mammalian RNA polymerase [see review by Jacob (15)]. These are obtained by high-salt extraction, sonic extraction, and resolution by chromatography on DEAE-cellulose or DEAE-Sephadex. This procedure yields 3 major forms of RNA polymerase, for which 2 standard nomenclatures exist (18, 23). Form I or A elutes first from DEAE-cellulose and is resistant to α-amanitin. Form II elutes second and is sensitive to α-amanitin. Form III elutes last (25) and has recently been shown to be sensitive to high concentrations of α-amanitin (28). By these criteria, and also by differences in salt optima, metal cofactor optima, and intranuclear localization (3, 16, 23, 24), these enzymes are considered to be different, unrelated forms of RNA polymerase. In addition, differences in molecular weights of pure enzymes have been shown (17).

In this communication we compare the properties of our preparation of RNA polymerase with the forms of RNA polymerase isolated by the procedure of Roeder and Rutter (23) and with RNA polymerase obtained by subsequent high-salt extraction of the residue remaining after the low-salt extraction. The high-salt-extractable enzyme has properties of Form II; the low-salt-extractable enzyme appears to have hybrid properties and has been tentatively designated RNAPₖₗ.⁴

MATERIALS AND METHODS

Materials. ³H-Labeled ribonucleoside triphosphates were purchased from Schwarz/Mann, Orangeburg, N. Y., and ICN, Cleveland, Ohio. α-Amanitin was purchased from Henley and Co., New York, N. Y., and calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N. J. DEAE-Sephadex A-25, Pharmacia Laboratories, Inc., Piscataway, N. J., was swollen in Buffer A containing 1 M (NH₄)₂SO₄ and repeatedly washed with Buffer A containing 0.025 M (NH₄)₂SO₄ until the

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⁴ The abbreviations used are: RNAPₖₗ, low-salt-extractable RNA polymerase; Buffer A, 50 mM Tris-HCl (pH 7.9), 25% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol.

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(NH₄)₂SO₄ concentration was 0.025 M. DEAE-Sephadex was recycled as follows. The used DEAE-Sephadex, as a thick slurry, was mixed with an equal volume of 0.5 N HCl for 30 min. The mixture was filtered and repeatedly washed with distilled water until the pH of the filtrate was neutral. The HCl procedure was repeated. The DEAE-Sephadex was then mixed with an equal volume of 0.5 N NaOH for 30 min, filtered, and washed with distilled water until the pH was neutral. The DEAE-Sephadex was equilibrated by adding Buffer A containing 1 M (NH₄)₂SO₄, followed by repeated washings as described above.

Bovine lymphosarcoma was obtained from animals maintained by the University of Pennsylvania School of Veterinary Medicine and stored at —90° until used.

RNA Polymerase Assay. The standard reaction mixture contained (in 0.5 ml): 0.05 M Tris-maleate (pH 7.7); 2 mM MnCl₂; 2 mM 2-mercaptoethanol; 320 µM each of ATP, CTP, and GTP; 40 µM ³H-labeled UTP (15 to 30 x 10⁶ cpm/nmole); calf thymus DNA (120 µM deoxyribonucleotides); and enzyme. The (NH₄)₂SO₄ concentration in the reaction mixture was 0.05 M in the case of RNP₄ or Form I and 0.08 M for Form II. After 10 min at 37°, incorporation of labeled triphosphate into RNA was determined as described previously (1). One unit of RNP₄ or Form II is defined as the amount of enzyme that converts 1 nmole of ³H-labeled UTP into acid-insoluble material in 10 min, with native and denatured calf thymus DNA as template, respectively.

DNA was denatured by heating in a boiling water bath for 5 min, followed by quick cooling in ice. Protein was determined as described previously, except that bovine serum albumin was weighed and used directly (8).

Preparation of Enzymes. Bovine lymphosarcoma tissue, 400 to 800 g, was suspended in 2 volumes of 50 mM Tris-HCl (pH 7.7), 5 mM MgCl₂, and 5 mM 2-mercaptoethanol; minced with scissors; ground in a Waring Blendor (Model CB-5) for 30 sec at medium speed and 30 sec at high speed; and centrifuged at 10,000 x g for 10 min. The supernatant (441 units/kg tissue; specific activity, 0.01) was used as the source of RNP₄ (10). The pellet from this centrifugation was suspended in an equal volume of 10 mM Tris-HCl (pH 7.9); 50 mM MgCl₂; 1.0 M sucrose; and 5 mM 2-mercaptoethanol; mixed in a blender at low speed; stored at —90°; and used as the source of Form II.

RNP₄ was further purified by high-speed centrifugation, precipitation with protamine sulfate, elution with sodium succinate, concentration with (NH₄)₂SO₄, and chromatography on phosphocellulose as described previously (10). Purified enzyme, 147 units, was obtained with a specific activity of 2.6 units/mg protein.

Form II was prepared from the frozen pellet by a modification of the method of Weaver et al. (27). The pellet described above was thawed slowly, and 3.6 M (NH₄)₂SO₄ (adjusted to pH 7.8 with NH₄OH) was added to bring the final concentration to 0.32 M. The viscous mixture was ground in the Waring Blendor by five 15-sec bursts at low setting. It was then sonically extracted for 10 min in a Branson sonifier (Model LS 75, maximum output), with the temperature maintained below 10° by an alcohol-Dry Ice bath. The sonic extract was blended with twice its volume of Buffer A (30 sec, medium setting) and centrifuged for 30 min at 10,000 x g. The supernatant was stored at —90°.

Further purification of Form II was achieved by DEAE-Sephadex column chromatography (5 to 10 mg protein per ml of bed volume). One-fourth of the DEAE-Sephadex equilibrated in Buffer A containing 0.025 M (NH₄)₂SO₄ was poured into the column base. The remainder of the DEAE-Sephadex was mixed with the enzyme solution and Buffer A was added to reduce the (NH₄)₂SO₄ concentration to 0.05 M. After being stirred for 10 min, the mixture was applied to the column. The column was washed with 1 column volume of Buffer A containing 0.05 M (NH₄)₂SO₄. The enzyme was then eluted with 1 column volume of Buffer A containing 0.3 M (NH₄)₂SO₄. The eluate was mixed with an equal volume of saturated (NH₄)₂SO₄ in 0.05 M Tris-Cl (pH 7.9) containing 5 mM MgCl₂, 0.1 mM EDTA, and 0.5 mM dithiothreitol (at least 1 hr), and the precipitate was collected by centrifugation for 30 min at 10,000 x g. The pellet was suspended in a minimal volume of Buffer A and dialyzed overnight against Buffer A containing 40% (v/v) glycerol and 0.0125 M (NH₄)₂SO₄.

Form I and II were also obtained from DEAE-Sephadex chromatography of the crude extract used for the preparation of RNP₄ (see legend to Chart 2) or by a modification of the procedure of Weaver et al. (27) (see legend to Chart 1).

Escherichia coli RNA polymerase was prepared as described previously (11), followed by chromatography on DEAE-Sephadex. The preparation contained α factor.

Chromatin. Calf thymus chromatin was prepared by the method of Paul and Gilmour (22), slightly modified, and was stored at —90°. Prior to use the chromatin was sheared in a VirTis homogenizer at 60% of the maximum rate for 1 min at 4°. A₂₆₀nm/A₂₈₀nm, A₂₆₀nm/A₂₄₀nm, A₂₆₀nm/A₂₈₀nm ratios were 1.64, 1.49, and 0.036, respectively. The chromatin has no detectable endogenous RNA polymerase activity at the specific activity of radioactive nucleoside triphosphate used.

RESULTS

High-salt extraction of whole lymphosarcoma tissue followed by chromatography on DEAE-Sephadex results in 2 main peaks of enzyme activity, Forms I and II, eluting at 0.08 and 0.2 M (NH₄)₂SO₄ (Chart 1). The fractions of the earlier peak, when combined and concentrated with (NH₄)₂SO₄, are 1.3 times more active with native DNA and are inhibited only 12% by α-amanitin (0.2 µg/ml). The fractions of the 2nd peak, similarly combined and concentrated, are twice as active with denatured DNA and are inhibited 85% by α-amanitin. A shoulder is sometimes observed in the trailing edge of Form II activity. When active fractions from this area are combined and concentrated, a small amount of activity is observed that by template preference, salt preference, and α-amanitin sensitivity appears to be a mixture of Forms II and III.

These results confirm the existence of Forms I and II in bovine lymphosarcoma when extraction is performed in the...
RNA Polymerase Obtained by Precipitation of the Low-Salt Extract with Protamine Sulfate, Elution with Sodium Succinate, and Chromatography on Phosphocellulose. When purification of the low-salt extract is performed as described by Furth et al. (10), an enzyme of unusual properties is obtained. Native DNA is twice as effective a template as denatured DNA, Mn$^{2+}$ is twice as effective a cofactor as Mg$^{2+}$, the salt optimum of the reaction is 0.05 M (NH$_4$)$_2$SO$_4$, and the enzyme is inhibited by high (40 μg/ml) levels of α-amanitin but not by low levels (7, 10). These studies suggested that it did not correspond to any of the RNA polymerase forms described by Roeder and Rutter.

Chart 1. DEAE-Sephadex chromatography of high-salt extract. Bovine lymphosarcoma tissue was minced in 10 mM Tris-HCl (pH 7.9), 50 mM MgCl$_2$, 1.0 M sucrose, and 5 mM 2-mercaptoethanol adjusted to 0.32 M (NH$_4$)$_2$SO$_4$; sonically extracted; and centrifuged (see “Materials and Methods”). The supernatant was concentrated with (NH$_4$)$_2$SO$_4$ (43.6 g/100 ml), resuspended in Buffer A, and dialyzed overnight against Buffer A. The supernatant was centrifuged at 78,000 × g for 120 min; the supernatant was adjusted to 0.025 M (NH$_4$)$_2$SO$_4$ by dilution with Buffer A and applied to a DEAE-Sephadex column (5 sq cm x 10 cm) at a concentration of 1 mg protein per ml column volume. The column was washed with Buffer A containing 0.025 M (NH$_4$)$_2$SO$_4$, and protein was eluted with a 300-ml linear gradient extending from 0.025 to 0.65 M (NH$_4$)$_2$SO$_4$. Fractions of 10 ml were collected and 0.2-ml aliquots were assayed under standard conditions, except that the reaction was allowed to proceed for 20 min.

RNA Polymerase Isolated by Sequential Low- and High-Salt Extraction. Forms I and II can be obtained if tissue is extracted in buffer that does not contain (NH$_4$)$_2$SO$_4$. This crude extract can be directly applied to DEAE-Sephadex (Chart 2). The elution pattern of RNA polymerase activity indicates that both Form I and Form II are present. Enzyme activity that elutes at 0.08 M (NH$_4$)$_2$SO$_4$ prefers native DNA and is not inhibited significantly by α-amanitin. The predominant peak is at 0.2 M (NH$_4$)$_2$SO$_4$ and shows 2-fold greater activity with denatured DNA and 85% inhibition by α-amanitin (0.2 μg/ml). The proportions of Forms I and II vary from 1:4 to 1:2 in different experiments (using the preferred template for each).

Approximately one-third as much enzyme is obtained by low-salt extraction as by high-salt extraction, and very little Form III activity is observed. Extraction from lymphosarcoma of considerable α-amanitin-sensitive RNA polymerase by low salt, as well as α-amanitin-resistant enzyme, differs from the results obtained with rat liver by Chesterton and Butterworth (4). These investigators found that low-salt extraction of rat liver nuclei yielded only α-amanitin-resistant Form I.

When the residue remaining after the low-salt extraction is extracted with 0.3 M (NH$_4$)$_2$SO$_4$ and the extract is purified by chromatography on DEAE-Sephadex, additional RNA polymerase is obtained. The properties of this enzyme indicate that it is Form II. It prefers denatured DNA as a template and is sensitive to low concentrations of α-amanitin (Chart 3).
RNA Polymerase of Bovine Lymphosarcoma

In order to gain more insight into the nature of the enzyme of neoplastic tissue, the relative amounts of this enzyme, termed RNAP$_L$, in lymphosarcoma and calf thymus were compared, together with levels of Form II obtained by sequential high-salt extraction (Table I). The amount of RNAP$_L$ isolated from lymphosarcoma, although varying from preparation to preparation, is consistently greater than the amount of RNAP$_L$ isolated from calf thymus; in contrast, somewhat more Form II is isolated from calf thymus than from lymphosarcoma.

RNAP$_L$ shows some properties of Form I, such as a low-salt optimum (7), but there are important differences between the 2 enzymes. Two properties of RNAP$_L$, considered previously in a preliminary manner (7), are explored more fully here.

Chart 3 compares the α-amanitin sensitivity of RNAP$_L$, E. coli RNA polymerase, Form II obtained from sequential high salt extraction of lymphosarcoma, Form I RNA polymerase. RNAP$_L$ has an intermediate sensitivity to α-amanitin. It is not inhibited by the low concentrations of α-amanitin, which completely inhibit Form II. At high concentrations RNAP$_L$ is significantly more inhibited than are Form II or E. coli RNA polymerase. The requirement of high levels of α-amanitin for inhibition of RNAP$_L$ is not due to an impurity in the enzyme that complexes with or inactivates α-amanitin, since Form II retains its sensitivity to low levels of α-amanitin when RNAP$_L$ is also present in the reaction mixture.

Chart 4 shows the elution pattern of lymphosarcoma RNAP$_L$ upon DEAE-Sephadex chromatography. Most of the RNA polymerase activity elutes in the broad peak at 0.2 M (NH$_4$)$_2$SO$_4$, in a position characteristic of Form II enzyme. Some activity is occasionally observed in the pass-through fraction. In the experiment shown, fractions were assayed with denatured DNA, since all known mammalian RNA polymerases can transcribe denatured DNA, but Form II does not transcribe native DNA well (15). In other experiments, assaying the fractions with native DNA gave a similar peak. All fractions retain their resistance to low levels of α-amanitin and prefer native DNA as template. Thus, although RNAP$_L$ elutes from DEAE-Sephadex at a position characteristic of Form II, the eluted enzyme differs from Form II in template preference and resistance to α-amanitin. To investigate the possibility that aggregation with protein impurities may influence the elution pattern of RNAP$_L$, DEAE-Sephadex chromatography was also performed in the presence of a detergent (0.1% Triton X-100). The elution pattern of the enzyme was almost identical to that shown in Chart 4.

Transcription of Chromatin. In order to characterize further RNAP$_L$ and Form II, we have examined the transcription of calf thymus DNA and chromatin by these enzymes. Table 2 shows kinetic parameters of transcription by RNAP$_L$ under conditions of low and high ionic strength. The analysis of such data for mammalian RNA polymerase has been described previously (19). At high ionic strength the $K_m$ for chromatin is greater than the $K_m$ for DNA, but the maximal velocity attained is similar. This indicates that more chromatin than DNA is needed for saturation, suggesting that there are fewer binding sites for RNAP$_L$ in chromatin. The similar $V_{max}$ for DNA and chromatin suggests that all enzyme molecules that do bind are active in transcription. At low ionic strength binding of enzyme to chromatin relative to DNA may be even more restricted than at high ionic strength. The lower $V_{max}$ for chromatin indicates that not all enzyme molecules bound are active under these conditions or, alternatively, are functioning inefficiently. Similar results are obtained with calf thymus

| Enzyme | Units$^a$ | Specific activity$^b$ | Units$^a$ | Specific activity$^b$
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</thead>
<tbody>
<tr>
<td>RNAP$_L$</td>
<td>147</td>
<td>2.61</td>
<td>55</td>
<td>1.49</td>
</tr>
<tr>
<td>Form II</td>
<td>258</td>
<td>0.18</td>
<td>300</td>
<td>0.46</td>
</tr>
</tbody>
</table>

* Expressed as yield from 1000 g tissue.
* Defined in "Materials and Methods."
Table 2

Transcription of DNA and chromatin by RNAP

Experiments were performed and kinetic constants obtained by double reciprocal plots as described previously (19). RNA synthesis was measured at saturating substrate levels at 5 concentrations of calf thymus DNA or chromatin ranging from 10 to 120 µM deoxynucleotides. The amounts of enzyme used were 0.7 and 1.7 units in low- and high-ionic-strength assays, respectively. The enzyme preparations contained (NH₄)₂SO₄, giving a concentration in the reaction vessels of 0.05 M. Double reciprocal (Lineweaver-Burk) plots of 1/velocity versus 1/substrate concentration were drawn to give the V˳max at saturating substrate levels and the K˳m of the template.

<table>
<thead>
<tr>
<th>Template</th>
<th>Low ionic strength</th>
<th>High ionic strength</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>K˳m (µM)</td>
<td>V˳max</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>15.4</td>
<td>0.69</td>
</tr>
<tr>
<td>Calf thymus chromatin</td>
<td>126</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* No added KCl.
* 0.2 M KCl.
* K˳m is expressed as µM deoxynucleotides.
* V˳max is expressed as nmoles of [¹H]JUMP incorporated.

RNAP (19) and imply that bovine lymphosarcoma RNAP possesses specificity in its ability to transcribe chromatin.

In contrast to these results, Form II transcribes native DNA and chromatin at only 14 and 5%, respectively, of the rate obtained with denatured DNA as template.

DISCUSSION

RNA polymerase can be isolated from bovine lymphosarcoma by a number of procedures. High-salt extraction and DEAE-Sephadex chromatography yield Form I and Form II enzymes. Low-salt extraction and DEAE-Sephadex chromatography also yield Forms I and II, although in lesser amounts. Subsequent extraction of the residue remaining after low-salt extraction followed by DEAE-Sephadex chromatography yields additional Form II enzyme. Most probably, the Form I remaining after low-salt extraction is inactivated upon storage or freezing and thawing of the residue.

Purification of the low-salt extract by protamine precipitation, sucinate elution, and phosphocelulose chromatography yields an enzyme (RNAP) with hybrid properties in comparison with the properties of Forms I, II, and III. It has an intermediate sensitivity to α-amanitin, possibly like that of Form III (28); elutes from DEAE-Sephadex, mostly at a position characteristic of Form II (23); and has a salt optimum most like that of Form I (3, 23). While the possibility of a mixture or Forms I, II, and III has not been rigidly excluded, the fact that RNAP possesses a distinct property of each form makes the possibility of a mixture less probable. The hybrid properties of RNAP could be explained by some combination of the subunits of the various forms. Interconvertibility of Forms I and II, although of a different type, has been suggested by Chesterton and Butterworth (5).

No differences have been observed in the properties of bovine lymphosarcoma RNAP, and Form II as compared with the corresponding enzymes of calf thymus (7, 10), and the levels of Form II are similar in the 2 tissues. The level of RNAP appears to be higher in the neoplastic tissue, but the significance of this observation is not clear in the absence of an understanding of the in vivo role of this polymerase. Conceivably, the increased levels could be related to the altered gene expression observed in neoplasia or the synthesis of an RNA primer for DNA polymerase in the more rapidly dividing tissue.

Despite the uncertainty as to the classification of RNAP, it transcribes chromatin relatively efficiently and can be used as an in vitro probe of chromatin to elucidate the factors that regulate transcription in normal and neoplastic cells. Such studies are in progress.

Finally, the procedure used to obtain RNAP results in its separation from the high-molecular-weight DNA polymerase (12). Polyrriboadenylate polymerase is also separated; it is found in the supernatant after protamine precipitation (A. A. Keshgegian, unpublished observations). We have not assayed yet for RNA-dependent DNA polymerase. We plan to look for this enzyme, as its presence or absence in lymphosarcoma and calf thymus may be relevant to the possible viral etiology of bovine lymphosarcoma (6).

REFERENCES

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