DNase I Digestion of Chromatin from Avian Liver Nuclei Liberates DNA-dependent RNA Polymerase II*

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In an effort to develop mild conditions for the isolation of DNA-dependent RNA polymerase II, we have used DNase I covalently coupled to Sepharose 4B to digest chromatin from hypotenically lysed nuclei from rooster liver. The RNA polymerase II released was at least 2 times more active in in vitro transcription than was RNA polymerase II prepared by the conventional method of sonication of chromatin in buffers containing high salt. The numbers of RNA polymerase II molecules in RNA polymerase preparations prepared both by DNase I-Sepharose digestion and by sonication were determined by titration with $[^{3}H]$amanitin and were similar in both preparations, indicating that the two methods were equally efficient at liberating RNA polymerase from the chromatin but that treatment with DNase I-Sepharose yielded higher levels of enzymatic activity.

In order to identify RNA polymerase II in complex mixtures of proteins, we have utilized the technique of binding $[^{3}H]$amanitin to RNA polymerase II followed by electrophoresis on gradient polyacrylamide gels under non-denaturing conditions. We have identified two forms of RNA polymerase II molecules in RNA polymerase preparations prepared both by the very gentle treatment of chromatin from rooster liver and by isolation from nuclei with DNase I-Sepharose which could have an important role in the template specificity and activity of the polymerase.

The release of highly active eukaryotic RNA polymerases by the very gentle treatment of chromatin from lysed nuclei with DNase I-Sepharose may facilitate the reconstitution of an in vitro transcription system using RNA polymerase II.

The mechanisms involved in DNA-dependent RNA synthesis in eukaryotes are largely unknown. Three major classes of DNA-dependent RNA polymerases have been isolated and purified from various eukaryotic organisms (see reviews by Chambon, 1975; Roeder, 1976), but very little is known about their interaction with DNA and various control elements to initiate specific and accurate transcription of eukaryotic genes.

In order to study eukaryotic gene regulation it would be advantageous to develop an in vitro transcription system utilizing eukaryotic RNA polymerase. Recent evidence with Xenopus germinal vesicle extracts has demonstrated correct initiation and termination of cloned 5 S rDNA transcripts as well as the almost complete asymmetry of transcription (Birkenmeier et al., 1978). This indicates that the nuclear extract of Xenopus contained all the components necessary for authentic 5 S ribosomal RNA transcription with RNA polymerase III. However, in studies using RNA polymerase II, the enzyme responsible for messenger RNA synthesis in eukaryotes, attempts to demonstrate specificity of initiation and selective transcription have, thus far, been unsuccessful.

Another important consideration in the development of an in vitro transcription system is whether to utilize the most rigorously purified polymerase and the risk of having lost some aspect of its physiological function or to utilize crude polymerase and be prepared to cope with ambiguities that may result from working with complex mixtures of proteins.

We have endeavored to develop the gentlest possible procedures for preparing RNA polymerase that is dependent on exogenous DNA for enzymatic activity. As part of a series of experiments designed to isolate RNA polymerase that is dependent on exogenous DNA for activity and that has not been subjected to sonication or high salts, we have prepared DNase I coupled to Sepharose 4B by cyanogen bromide activation of the resin. Part of this work has been presented in preliminary form (Kastern et al., 1978).

EXPERIMENTAL PROCEDURES

Experimental Animals and Chemicals—Four-week-old cockerels were obtained from Truslow Farms, Inc. The DNase II and orcinol were provided by Sigma Chemical Co. [5,6-$^{3}H$]UTP (specific activity, 40 Ci/mmol) was prepared by ICN Radiochemicals. Cyanogen bromide was from Fluka. Calf thymus DNA was prepared by P-L Biochemical, and a-amanitin was obtained from Boehringer-Mannheim. DNase I (electrophoretically pure) was from Worthington. Diphenylamine was from the G. Frederick Smith Chemical Co. 1 H-labeled sea urchin DNA (specific activity, 1 x 10$^{6}$ cpm/μg) was a gift from H. Kastern.

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E. coli RNA polymerase (with factor A) was provided by<br>Boehringer-Manhein. High molecular weight protein standards and<br>the precast gradient polyacrylamide gels were obtained from<br>Pharmacia. Purified wheat germ RNA polymerase II was obtained from<br>Miles.

The CM-dextran was furnished by Drs. A. Peterson and A. R.<br>Torres, Laboratory of Biochemistry, National Cancer Institute; the<br>dimethyl [O-methyl-3H]ethylamamine was a gracious gift of Dr. Roger<br>Younas, of the Biological Laboratories, Harvard University.

DNAse Coupling to Sepharose 4B—DNase I, or DNase II, was coupled to cyanogen bromide-activated Sepharose 4B according to the procedure described by Cuatrecassas and Anfinsen (1971). Approximately 100 mg of DNase I was coupled to 10 ml of Sepharose 4B and then stored at 20°C in 0.1 M Tris HCl, pH 7.6, containing 50% glycerol. The preparation stored in this manner was stable for several months with no detectable loss of DNAase activity.

Assay for DNAse Activity—To assay DNase I coupled to Sepharose 4B, approximately 0.1 ml of packed beads was placed in 20 mm magnesium acetate, 22 mm NaCl, and 10 mm Tris-HCl, pH 7.4. Approximately 4 × 10^7 cpm of nick-translated `H-labeled sea urchin DNA (specific activity, 10^7 cpn/µg) were mixed with 0.1 mg of homogenous unlabelled DNA and added to the activated mixture containing the DNase I-Sepharose (1 ml total). At various times during incubation at 37°C, the reaction mixture was centrifuged and 0.1-ml aliquots of supernatant fluid were removed and precipitated in cold 10% trichloroacetic acid. The precipitates were collected on glass fiber filters (Whatman GF-C), dried, and assayed for radioactivity using a scintillation counter.

DNase II-Sepharose 4B was prepared and assayed in essentially the same manner except that 10 mM sodium acetate, pH 6.0, was substituted for the Tris-HCl, pH 7.4.

Supernatant fractions from incubated DNase I-Sepharose were assayed for DNase activity similarly with a few modifications. DNase I-Sepharose was incubated as described above for 1 h except that DNA was not added. The DNase I-Sepharose then was removed from this mixture by low speed centrifugation and approximately 4 × 10^7 cpm of `H-labeled sea urchin sperm DNA (specific activity, 10^7 cpn/µg) were added to the supernatant. To increase the sensitivity of the assay, unlabeled DNA was not added.

Isolation of Nuclei—Nuclei were isolated from the livers of 4-week-old cockerels by a modification of the technique of Marshall and Huang (1975). Approximately 15 g of tissue were minced in cold homogenization buffer (0.01 M Tris-HCl (pH 8.0), 0.3 M sucrose, 5 mM magnesium acetate, and 1 mM dithiothreitol). The tissue then was homogenized in 10 volumes (w/v) of homogenization buffer using a Dounce or polytron. After centrifugation at 500 × g for 10 min at 0°C, the pellet was resuspended in 4 volumes of homogenization buffer containing 0.1% Triton X-100 and centrifuged at 500 × g for 10 min. The nuclear pellet then was resuspended in 3 volumes of homogenization buffer containing 0.1% Triton X-100 and made 1.9 M in sucrose using 2.3 volumes of homogenization buffer containing 0.1% Triton X-100. The nuclear suspension was stored in liquid N, until use.

Isolation of RNA Polymerase by DNase-Sepharose Treatment—For these experiments, the homogenization buffer was made 1.9 M in sucrose using 2.3 volumes of homogenization buffer containing 0.1% Triton X-100 and made 1.9 M in sucrose using 2.3 volumes of homogenization buffer containing 0.1% Triton X-100. The nuclear suspension was stored in liquid N, until use.

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Measurements of the Concentrations of DNA and Protein—The concentration of DNA in nuclear suspensions or nuclear lysates was measured using the diphenylamine reaction according to Burton (1956). Protein concentrations were determined using the biuret method as described previously (Zamenhoff, 1957).

Gel Filtration on Sepharose 6B-CL—A column (2.5 × 100 cm) was packed at 4°C with Sepharose 6B-CL equilibrated in TPD 30 buffer containing 1 mM ammonium sulfate. The column was eluted at 15 ml/h and 5-ml fractions were collected. The fractions containing the RNA polymerase activity were pooled and stored under liquid N₂ until use.

RNA Polymerase Chromatography on DEAE-Septaphex—A column (1.0 × 10 cm) was packed at 4°C with DEAE-Septaphex A-25 equilibrated in TPD 20 buffer containing 0.07 M ammonium sulfate. The column was eluted with a linear gradient of from 0.075 to 0.6 M ammonium sulfate in TPD 20 buffer. The conductivity of every fifth fraction was determined using a Radiometer model CDM-3 conductivity meter. The pooled fractions of RNA polymerase activity were stored under liquid N₂ until use.

Displacement Chromatography—A column (0.2 × 20 cm) was packed at 4°C with a total volume of 100 ml of DEAE-cellulose (Whatman DE52) equilibrated in DC buffer. The dilute mixture of RNA polymerase to be concentrated was adjusted to the salt concentrations of DC buffer and then applied to the column at a flow rate of 4 ml/h. The column was washed with 1 ml of DC buffer, then eluted with a 1% solution of CM-dextran in DC buffer. Fractions of 3 drops (0.06 ml) were collected.
tion were added and the mixture was incubated at 37°C for 15 min. Upon completion of the incubation, the mixture was applied to a column (0.7 x 30 cm) packed with Sephadex G-50 which had been equilibrated with TPD-20 buffer containing 0.1 M ammonium sulfate at 4°C. The column was washed with the same buffer at a flow rate of approximately 15 ml/h. Fractions (0.5 ml each) were collected, and the radioactivity in each fraction then was determined.

Gradient Polyacrylamide Gel Electrophoresis under Nondenaturing Conditions—Precast slab gels containing either 2 to 16% or 4 to 30% linear gradients of polyacrylamide were mounted in a Pharmacia electrophoresis apparatus model GE-4. The electrode buffer (pH 8.3) consisted of 0.09 M Tris, 0.08 M boric acid, 1 mM thioglycolic acid, and 3 mM disodium EDTA. The buffer was continuously recirculated and maintained at 5°C with a Lauda K-2/RD circulating water bath. After electrophoresis, the gels were stained in a solution of 10% methanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250.

RESULTS

Pancreatin DNase I was coupled to cyanogen bromide-activated Sepharose 4B according to the method of Cuatrecasas and Anfinsen (1971) and activity was measured by incubation of the immobilized enzyme with [3H]-labeled DNA. The activity of the DNase Sepharose is shown in Fig. 1. Since it is essential that all of the DNase be removed by pelleting the Sepharose beads, we tested the supernatant fluid for residual DNase activity by incubating it with nick-translated sea urchin [3H]DNA. Since there was no substantial decrease in acid-precipitable radioactivity (Fig. 1), we concluded that there was no appreciable DNase activity remaining in the supernatant fluid after the DNase I-Sepharose was removed.

We then tested the capacity of DNase I-Sepharose to liberate RNA polymerase II from chicken liver nuclei. These preparations were assayed for RNA polymerase II activity using excess denatured calf thymus DNA as template and [3H]UTP as the labeled precursor in an in vitro transcription assay. From the assay of a typical preparation of DNase I-Sepharose-treated nuclear lysate, it is apparent that a transcriptionally active enzyme activity could be obtained (Table I). To demonstrate that it is the DNase I-Sepharose digestion of chromatin that liberated this RNA polymerase activity, an identical nuclear lysate that had been incubated in the presence of magnesium ions alone (without DNase I-Sepharose) was tested. The treatment of the nuclear lysate with DNase I-Sepharose resulted in at least a 2-fold increase in the RNA polymerase activity obtained (Table I). Since incubation of the nuclear lysate with EDTA in the absence of DNase I-Sepharose (Table I) did not alter the basal activity, we conclude that this activity is not a function of endogenous nucleases and may represent a pool of unbound, or loosely bound, RNA polymerases. Moreover, it is unlikely that incubation with DNase I-Sepharose yielded higher RNA polymerase activity due to the adsorption of inhibitory substances to the Sepharose resin since incubation with Sepharose 4B alone resulted in no increase in activity over the basal level seen without DNase I-Sepharose (data not shown).

A comparison of the kinetics of RNA polymerase release from lysed nuclei revealed that the time of incubation with DNase I-Sepharose was an important factor in the amount of RNA polymerase activity obtained (Table I). There was a basal level of activity obtained without incubation of the nuclear lysate. This activity was relatively constant regardless of the presence or absence of DNase I-Sepharose or other treatments. In the presence of DNase Sepharose, the release of RNA polymerase increased with time and was essentially complete by 30 min of incubation.

We also characterized the salt requirements of the RNA polymerase in our preparations of DNase I-Sepharose-treated, crude, nuclear lysate. The enzyme displayed an optimum of activity at 7 mM manganous ions and an optimum for ammonium sulfate concentration at 80 mM (data not shown). These optima were typical of RNA polymerase II isolated from other organisms (see review by Roeder, 1976).

Eukaryotic RNA polymerase of the three major classes display differing sensitivities or resistance to the fungal toxin a-amanitin (see reviews by Chambon, 1975; Roeder, 1976). We used these criteria, along with their optimal monovalent cation requirements, to assay for each of the three eukaryotic RNA polymerases in our crude nuclear extracts. The amount of RNA polymerase of each of the three types was compared with the amount of polymerase of each type resulting from the conventional method of sonication of an identical amount.

### Table I

**Effect of DNase I-Sepharose 4B on the solubilization of nuclear RNA polymerase II**

| Incubation mixture | Incubation time (min) | RNA Polymerase II activity (units) | Per cent of DNase I-Sepharose at 30 min |
|-------------------|-----------------------|---------------------------------|---------------------------------------|
| Lysozyme plus Mg²⁺ and DNase | 0 | 21.4 | 100 |
| I-Sepharose | 15 | 64.3 | |
| | 30 | 138.0 | |
| | 45 | 199.3 | |
| Lysozyme plus Mg²⁺ | 0 | 22.8 | |
| | 15 | 35.0 | |
| | 30 | 61.2 | |
| | 45 | 60.3 | |
| Lysate plus 10 mM EDTA | 0 | 19.1 | |
| | 30 | 53.7 | 38.9 |
| Nuclear lysate | 0 | 21.7 | |
| | 30 | 43.4 | 31.4 |
of nuclei in buffers containing high concentrations of ammonium sulfate.

The results from DNase-treated nuclear lysate (Table II) indicated that greater than 90% of the in vitro transcriptional activity was due to RNA polymerase II, as seen by its sensitivity to very low amounts of α-amanitin. There was a low level of polymerase I activity as seen by the insensitivity of RNA synthesis under polymerase I conditions to high concentrations (i.e. 100 μg/ml) of α-amanitin. In contrast, at least 30% of the in vitro transcriptional activity in the sonicated preparation was accounted for by RNA polymerase I. In addition, the RNA polymerase II activity/mg of nuclear DNA in the DNase I-Sepharose-treated preparation was almost 3-fold greater than that obtained by sonication. The low level of incorporation in the presence of high levels (100 μg/ml) of α-amanitin under polymerase III conditions was also insensitive to actinomycin D and continued in the absence of one of the four nucleotide triphosphates (data not shown). Therefore, it was due to an enzyme activity other than RNA polymerase and has not been further characterized. Thus, there was no detectable RNA polymerase III in our preparations. From these results, we conclude that the DNase I-Sepharose method of RNA polymerase isolation was almost completely specific for RNA polymerase II from avian liver nuclei.

At the completion of the incubation with DNase I-Sepharose, the remaining chromatin was pelleted along with the DNase resin and other nuclear debris during the low speed centrifugation. We tested these chromatin fragments for the presence of residual RNA polymerase II activity by removing the DNase I-Sepharose beads and then subjecting the debris to sonication in buffer containing 0.3 M (NH₄)₂SO₄. An RNA polymerase II assay of the partially fractionated sonicate revealed that not more than 10 to 15% of the total RNA polymerase II activity remained in the residual chromatin (data not shown).

A particular difficulty with most conventional methods of RNA polymerase isolation concerns the removal of the fragments of endogenous DNA which may be present in the preparation. Since most studies of RNA polymerase require the enzyme to be completely dependent upon exogenous DNA for activity, various methods such as ammonium sulfate fractionation (e.g. Roeder, 1974) or protamine sulfate precipitation (see Kedinger et al., 1972) are necessary to eliminate the residual DNA. The presence of endogenous DNA was assayed in the DNase I-Sepharose-treated crude nuclear lysate by two independent methods. Both an in vitro transcription assay without exogenous DNA (Table III) and a sensitive assay for the presence of DNA using diphenylamine (data not shown) revealed that there was no endogenous DNA present in the RNA polymerase preparations. Thus, it was not necessary to include purification methods designed specifically to remove DNA from the RNA polymerase II preparation.

We also tested the ability of another readily available DNase (which we coupled to Sepharose), acid DNase II, to release RNA polymerase II from lysed nuclei. When incubated with [³H]DNA in an assay of DNase activity, DNase II-Sepharose solubilized the [³H]DNA much faster than did DNase I-Sepharose (Fig. 1), suggesting that a more efficient release of RNA polymerase II from the nuclear lysate might be possible.

The in vitro transcription activity of the RNA polymerase prepared with DNase II-Sepharose was compared with an identical preparation that had been treated with DNase I-Sepharose. The RNA polymerase activity was essentially the same in both preparations except that DNase I-Sepharose yielded more activity (Table III) even though the chromatin appeared to be more completely digested with the DNase II-Sepharose treatment. The low RNA polymerase activity obtained through the use of DNase II-Sepharose may reflect a relative instability of RNA polymerase in the acidic conditions necessary for DNase II activity rather than differential release from the chromatin. In all other respects, both DNase I and DNase II yielded identical products. The RNA polymerase preparations had the same template activities with regard to denatured and native DNA, they had the same degree of DNA dependence for transcriptional activity, and they had the same low level of RNA polymerase I activity (Table III).

Thus, a different DNase yielded an RNA polymerase preparation which was by several criteria the same as that derived from DNase I treatment.

To further characterize the RNA polymerase II obtained by DNase I-Sepharose digestion of hypotonically lysed chicken liver nuclei, we have subjected it to various procedures including gel filtration and ion exchange chromatography in order to effect some purification. For our purposes, a useful first step in purification was the precipitation of the enzyme by bringing the solution to 70% saturation with ammonium sulfate. The major advantage of this step was to concentrate

| TABLE II |

Comparison of the RNA polymerase activity obtained by the DNase I-Sepharose and sonication methods of RNA polymerase solubilization

RNA polymerase was isolated from purified rooster liver nuclei by either the DNase I-Sepharose or sonication methods as described under "Experimental Procedures." The unpurified enzyme preparations were assayed for each of the three RNA polymerase activities using an in vitro transcription assay as outlined under "Experimental Procedures," in the presence of high or low concentration of α-amanitin as indicated. Activity is expressed as the total amount of RNA polymerase activity obtained from each preparation.

| Assay conditions | RNA polymerase activity |
|------------------|------------------------|
|                  | [α-Amanitin] | DNase I-Sepharose-treated | Sonicated |
|                  | μg/ml      | units                | units    |
| RNA polymerase I | None       | 63                  | 240      |
|                  | 1          | 60                  | 224      |
|                  | 100        | 58                  | 231      |
| RNA polymerase II| None       | 2120                | 756      |
|                  | 1          | 171                 | 125      |
|                  | 100        | 183                 | 127      |
| RNA polymerase III| 1         | 167                 | 121      |
|                  | 100        | 155                 | 114      |

| TABLE III |

A comparison of the RNA polymerase activities obtained with either DNase I or DNase II coupled to Sepharose 4B

Equal amounts of purified liver nuclei were lysed and incubated with either DNase I-Sepharose or DNase II-Sepharose as described under "Experimental Procedures." The RNA polymerase activity in each lysate was assayed using an in vitro transcription assay under either RNA polymerase I or II conditions as under "Experimental Procedures," using either native or denatured calf thymus DNA. RNA polymerase activity is expressed as the total amount of RNA polymerase activity obtained from each preparation.

| Treatment of nuclear lysate | Exogenous template used | DNA polymerase II activity |
|----------------------------|-------------------------|---------------------------|
|                            | Denatured DNA    | Native DNA   | Denatured/native ratio | RNA polymerase I activity |
|                            | units          | units        | units                  | units        |
| DNase I-Sepharose          | 0.14           | 15.14        | 0.08                   | 6.44         |
| DNase II-Sepharose         | 0.08           | 9.29         | 0.08                   | 3.85         |

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the enzyme to make further analyses more manageable. Following successive dialysis of the dissolved ammonium sulfate precipitate against TPD-30 buffer containing 0.1 M ammonium sulfate, there was no loss of enzyme activity by ammonium sulfate precipitation (Table IV).

Alternatively, we chose gel filtration of the DNase I-Sepharose-treated crude nuclear lysate on Sepharose 6B-CL to avoid the high salt conditions necessary in ammonium sulfate precipitation. When the crude nuclear lysate was chromatographed on a column of Sepharose 6B-CL, RNA polymerase II eluted in a broad peak within the included volume of the column (Fig. 2). The large amount of UV-absorbing material that eluted in the void volume was mostly RNA, and RNA was undetectable in the RNA polymerase fraction. There was a substantial loss of total protein during this step of purification, and this was reflected in the approximately 95% increase in specific activity over the unpurified nuclear lysate (Table IV).

The most efficient purification step for purifying RNA polymerase II was ion exchange chromatography on DEAE-Sephadex. The profile of RNA polymerase activity that was eluted from the column (Fig. 3) demonstrated that only RNA polymerase II was present and that this enzyme was eluted from the column in a position of from 0.2 to 0.3 M ammonium sulfate. These data indicate that continued purification of RNA polymerase II results in the loss of large amounts of enzyme activity. In just two purification steps (i.e., Sepharose 6B-CL gel filtration and DEAE-Sephadex A-25 ion exchange chromatography) nearly 83% of the initial enzymatic activity was lost.

In order to estimate the numbers of molecules of RNA polymerase II in our preparations, independent of the enzymatic activity, we have utilized the binding of amanitin to RNA polymerase II. The procedure, a modification of that previously described by Cochet-Meilhac and Chambon (1974) involved the use of the fungal toxin amanitin. We obtained amanitin which had been labeled with tritium (demethyl-[O- methyl-3H]-amanitin). This compound forms a complex with RNA polymerase II in an equimolar ratio. Thus, we were able to titrate the binding of amanitin to RNA polymerase II and estimate the moles of enzyme present.

After incubation of the RNA polymerase preparation with [3H]amanitin, we employed chromatography on Sephadex G-50 to separate the [3H]amanitin-RNA polymerase complexes from the free [3H]amanitin. When an excess of unlabeled α-amanitin was incubated with the RNA polymerase II prior to

Table IV
Recovery of RNA polymerase activity and [3H]Amanitin binding activity through a limited purification as compared to the sonication method of RNA polymerase preparation

| Isolation or purification step | Transcriptional activity | [3H]Amanitin binding activity |
|-------------------------------|--------------------------|-----------------------------|
|                               | Units | Recovery | Specific activity | Specific bound/ mg protein | Recovery |
| Diaized nuclear lysate        | 9684  | 1.00     | 365 | 4.4 | 1.00 |
| Ammonium sulfate precipitate  | 9835  | 1.00     | 408 | 6.9 | 0.00 |
| Sepharose 6B-CL fraction      | 3923  | 0.40     | 709 | —    | —    |
| DEAE-Sephadex fraction        | 1765  | 0.18     | 1765 | 20.8 | 0.18 |
| Sonicated nuclear lysate      | 7594  | 0.78     | 255 | 5.17 | 0.00 |

a —, amanitin binding assay could not be performed on these fractions.
the addition of the [3H]amanitin, the unlabeled α-amanitin effectively competed with the [3H]amanitin, and no radioactivity was recovered in the void volume of the column (data not shown).

We have used the technique of [3H]amanitin binding to follow the recovery of RNA polymerase II through the steps of purification described above. A comparison of the results obtained by in vitro transcription assays with those obtained through [3H]amanitin titration (Table IV) demonstrated that the values obtained through these two independent techniques were in close agreement. In the case of DEAE-Sephadex chromatography, there was an apparent 4.8-fold purification with an 18% yield as seen by transcriptional activity and a 4.7-fold purification with an 18% yield calculated using [3H]amanitin binding. Indeed, for all stages of purification, the results of [3H]amanitin binding were in close agreement with those obtained by in vitro transcription, thus verifying and supporting the transcriptional results. However, a discrepancy between the two assays was observed when material prepared from an equal number of nuclei (as determined by the DNA content) by sonication in buffer containing high salt (Krebs and Chambon, 1976) was compared with that obtained through the DNase I-Sepharose method. The preparation of enzyme isolated by sonication had an equal or slightly greater capacity for binding [3H]amanitin than did the crude extract prepared by the DNase I-Sepharose method, but it had a significantly decreased ability to transcribe in the in vitro assay (Table IV). One possible explanation for this result is that sonication damages a large number of polymerase molecules in such a way as to inactivate their enzymatic activity but not their ability to bind amanitin.

Many studies have examined the multiple forms of eukaryotic RNA polymerase II following electrophoresis of the polymerase on polyacrylamide gels under nondenaturing conditions (see review by Roeder, 1976). However, since it is difficult to recover and assay RNA polymerase activity from acrylamide gels (Kedinger et al., 1974; Roeder, 1976), electrophoresis under nondenaturing conditions has, in the past, required the highly purified, homogeneous preparations of RNA polymerase. Thus, an estimate of the size of RNA polymerase II throughout the various stages of purification was not feasible. We have overcome the requirement for applying homogeneous preparations of RNA polymerase II to non-denaturing polyacrylamide gels by utilizing [3H]amanitin binding. With the ability to form stable complexes with [3H]amanitin, the RNA polymerase II could be subjected to electrophoresis on 2 to 16% gradient polyacrylamide gels under nondenaturing conditions and subsequently located by the radioactive label. When commercially prepared purified wheat germ RNA polymerase II was treated in this manner, the profile of radioactivity shown in Fig. 4 was obtained. Since wheat germ RNA polymerase can form a dimer under the conditions of nondenaturing electrophoresis, two peaks of radioactivity are seen. RNA polymerase from E. coli, which is not affected by and does not bind α-amanitin, did not bind radioactive amanitin (Fig. 4).

The 2 to 16% gradient polyacrylamide slab gels had several advantages for the study of RNA polymerase II, including the ability to visualize very small amounts of protein after electrophoresis since the proteins in the mixture formed discrete protein bands as a function of molecular weight when the time of electrophoresis took place over extended periods of time (e.g. 15 h). Thus, we could detect very small amounts of protein (approximately 10 to 50 ng/band). Moreover, the extended time of electrophoresis combined with the shallow gradient of acrylamide concentrations in the gel had the advantage of eliminating all proteins with a molecular weight less than approximately 100,000. Thus, it was possible to detect RNA polymerase in even the most crude preparations with a very low protein background in the gel.

When [3H]amanitin was bound to avian liver RNA polymerase II and the resulting complex was subjected to electrophoresis under nondenaturing conditions, the profile of radioactivity from the sliced gel revealed two peaks of radioactivity (Fig. 5). These peaks of radioactivity corresponded to two stained protein bands with molecular weights of 640,000 and 550,000, respectively. When a similar procedure was followed utilizing a 4 to 30% gradient acrylamide slab gel instead of a 2 to 16% gradient as described above, the peaks of radioactivity still migrated with the same two protein bands even though their positions had changed substantially (data not shown).

Although analysis of the nuclear lysates on a 4 to 30% gradient acrylamide gel resulted in the retention of several low molecular weight proteins (i.e. 50,000 to 100,000), there were no new [3H]amanitin-binding bands.

![Gradient polyacrylamide gel electrophoresis of [3H]amanitin in a complex with avian liver RNA polymerase II under nondenaturing conditions](image-url)
increasing the protein concentration without the addition of permitted stabilization of the RNA polymerase activity by mixture polymerase II by displacement chromatography. The technique difficulty, we have concentrated dilute mixtures of RNA polymerase II by displacement chromatography in DC buffer. Fractions of 0.06 ml were collected and the RNA polymerase II activity in each fraction was determined using the in vitro transcription assay. The activity represents the total acid-precipitable incorporation of [3H]UMP in a 10-ml aliquot of each fraction. The CM-dextran eluted in Fraction 6.

In some of the isolation and purification steps such as Sepharose 6B-CL gel filtration, the RNA polymerase II enzyme was present in a rather dilute mixture of proteins. This made gel electrophoretic analysis of the polymerase under non-denaturing conditions difficult. Moreover, conventional concentration procedures, such as ammonium sulfate precipitation or ion exchange chromatography, may have resulted in inactivation or loss of the enzyme, or both. To avoid this difficulty, we have concentrated dilute mixtures of RNA polymerase II by displacement chromatography. The technique permitted stabilization of the RNA polymerase activity by increasing the protein concentration without the addition of exogenous protein.

A 100-ml column of DEAE-cellulose was loaded with a dilute mixture of RNA polymerase II obtained by Sepharose 6B-CL gel filtration. The protein was eluted with a 1% solution of carboxymethylated dextran (CM-dextran). CM-dextran had a sufficient charge density to displace all proteins from the DEAE-cellulose, resulting in their eluting in a very small volume just ahead of the CM-dextran front. By collecting fractions of small volume, RNA polymerase preparations that were free from the CM-dextran could be obtained. A typical profile of transcriptional activity obtained by this procedure (Fig. 6) demonstrated that a sample of approximately 10.0 ml had been concentrated to approximately 0.2 ml (50-fold) without loss of activity. Moreover, we observed similar recoveries of RNA polymerase II activity when preparations from various stages of purification were applied to the displacement column (data not shown).

Using CM-dextran displacement chromatography to concentrate dilute RNA polymerase II preparations from Sepharose 6B-CL and DEAE-Sephadex chromatography, we have been able to obtain samples of RNA polymerase II from each step of purification that are sufficiently concentrated for analysis on gradient acrylamide slab gel electrophoresis under non-denaturing conditions. A typical profile of the stained 2 to 16% gradient acrylamide slab gel (Fig. 7) demonstrated that there were no gross differences in the molecular weights of the two forms of RNA polymerase II throughout all stages of purification. Thus, at least at this level of resolution, the RNA polymerase II molecule appeared to remain intact throughout the purification steps we have employed.

**DISCUSSION**

We have developed a procedure that utilizes the observation that DNase I preferentially digests actively transcribing regions of chromatin (Weintraub and Groudine, 1976). Since RNA polymerase is expected to be associated with actively transcribed genes, digestion of the chromatin with DNase I appears to be a method for specifically releasing actively transcribing RNA polymerase. Treatment with soluble DNase I had been employed as a means for isolating DNA-dependent RNA polymerase from *E. coli* (Berg et al., 1971). However,
the method was unacceptable because of the difficulty in completely removing the DNase from the preparation (Mangel, 1974). Since RNA polymerase can initiate transcription at single-strand nicks and gaps (see reviews by Chambon, 1975, and Roeder, 1976), the presence of contaminating DNase in the RNA polymerase preparation would introduce ambiguity into any study involving the specificity of initiation by RNA polymerase. By coupling DNase I to a solid support (Sepharose 4B), we have eliminated the difficulties in removing exogenous DNase from the preparation. This has allowed us to exploit the advantage of DNase treatment of chromatin for the isolation of eukaryotic RNA polymerases.

Treatment of a nuclear lysate with DNase I-Sepharose resulted in a preparation that was almost entirely RNA polymerase II (Table I). We are unsure of the explanation for this observation. It may indicate that the genes transcribed by RNA polymerases I and III were somehow inaccessible to digestion by DNase I-Sepharose, at least in chicken liver nuclei. However, a similar observation by Sugden and Keller (1973) demonstrated that a simple incubation of the nuclear lysate at 35°C for 30 min resulted in a 2- to 3-fold increase in RNA polymerase II activity, while RNA polymerase I activity remained unchanged. In our system, it is also possible that the RNA polymerase I and III are more unstable during the isolation than is RNA polymerase II. This possibility was supported by our observation that DNase I-Sepharose treatment of purified nuclei from BSC-1 cells yielded eukaryotic RNA polymerase of all three classes.

The DNase I-Sepharose appeared to be very efficient in releasing RNA polymerase II from the nuclear lysate, leaving less than 15% of the total activity behind in the nuclear debris pellet. This residual activity could possibly be adventitiously associated with the pellet of debris and DNase I-Sepharose present after centrifugation. Thus, the residual RNA polymerase II activity in the pellet probably did not represent a very tightly chromatin-bound or protected class of RNA polymerase II molecules.

While DNase I-Sepharose treatment of the nuclear lysate was necessary for optimum yield of RNA polymerase II, we found that a small amount of RNA polymerase II could be obtained by lysis of the nuclei and that this level of activity was the same whether or not DNase I-Sepharose was present. This fraction probably represented the free or loosely bound RNA polymerase II described by others (Ramas et al., 1965; Strain et al., 1971; Kedinger et al., 1972; Guilfoyle et al., 1975; Jendrisak and Burgess, 1975; Gupta and Taylor, 1977; Jendrisak and Guilfoyle, 1978). It should be noted that, while the procedure described in this report results in the extraction of almost all of the RNA polymerase II molecules, it may be possible to use this method to fractionate the enzyme based on the kinetics of its release by DNase I. A comparison of the free (or loosely bound) RNA polymerase II with the more tightly bound fraction released by DNase I-Sepharose would be possible.

While the procedure was devised to maintain mild conditions for RNA polymerase II isolation, we found that it had other important advantages. The removal of virtually all of the endogenous DNA from the preparation eliminated the need for an additional purification step specifically for that purpose. The absence of endogenous DNA also dispensed with the requirement for high ionic strength conditions during RNA polymerase release from the chromatin, conditions that had been used to prevent reaggregation of the RNA polymerase with DNA fragments during subsequent purification (Kedinger et al., 1972).

While the activities reported here for RNA polymerase II using the DNase I-Sepharose treatment of a nuclear lysate showed increases over non-DNase I-Sepharose-treated lysates and over the high salt sonication method, we emphasize that all of the studies were performed on crude, or minimally purified, preparations of enzyme. These preparations may have contained a variety of inhibitors or stimulatory factors, or both, which would grossly affect the observed activity of the RNA polymerase II and, therefore, affect our estimate of yield. Although we do not intend to further purify RNA polymerase II for use in studies on specific transcription in vitro, we have employed several purification procedures simply to characterize this enzyme with respect to its chromatographic behavior, size, and specific activity.

Furthermore, in the in vitro transcription assays for RNA polymerase activity, commercial calf thymus DNA, either native or denatured, was used as a template. Since this DNA contains single-strand nicks, gaps, and breaks (Vogt, 1969), it is useful only for obtaining a very rough estimate of RNA polymerase activity. Experiments studying the specific transcription of eukaryotic genes using well defined templates are currently in progress.

The RNA polymerase activity that was recovered from the Sepharose 6B-CL gel filtration column or the DEAE-Sepharose ion exchange column was quite dilute when compared with the concentration of RNA polymerase in the original nuclear lysate. RNA polymerase is known to be unstable in dilute protein solutions (Roeder, 1976), and some investigators have found that the addition of protein, usually bovine serum albumin, stabilized the enzyme activity and increased recovery of RNA polymerase activity (e.g. Schwartz and Roeder, 1974). We have found that crystallized bovine serum albumin and most other commercially available proteins contain high levels of ribonuclease and introduce artifacts into in vitro transcription assays (data not shown) and, therefore, were not added to the dilute eluates from column chromatography. This may explain the low recovery of RNA polymerase II activity during these purification steps.

A functional estimate of the yield of RNA polymerase II, such as an in vitro transcription assay, is dependent on many variables which may affect enzyme activity but which may not be related to the actual yield of the polymerase. For this reason, we have chosen to employ the [3H]amaminit binding assay devised by Chambon and co-workers (Cochet-Meilhac and Chambon, 1974; Cochet-Meilhac et al., 1974) to quantify the numbers of RNA polymerase II, molecules throughout the purification. The results, which indicated that there was no difference between the enzyme activity recovered and the [3H]amaminit binding activity recovered, were similar to previous results concerning the purification of hen liver RNA polymerase by different methods (Krebs and Chambon, 1976). However, our observation that nearly 25% of the activity of RNA polymerase molecules was lost after they were exposed to sonication supported the suggestion that sonication in the presence of high salt was an unnecessarily harsh treatment of the RNA polymerase. This result agreed with observations that the sonication of nuclear lysates in high salt resulted in a loss of from 20 to 50% of RNA polymerase II activity (Sugden and Keller, 1973).

It is apparent that the eukaryotic DNA-dependent RNA polymerases are a major component in a highly complex system concerned with regulation of gene expression. The difficulties encountered in the study of these enzymes are a tribute to the complexity of eukaryotic transcription. The inability to reconstitute an in vitro transcription system that shows specificity of gene expression (see review, Roeder, 1976) with highly purified RNA polymerase II suggests that perhaps
purification of this enzyme is detrimental. Possibly, a closer examination of other nuclear components will uncover critical components that are necessary for the specificity of the type required during eukaryotic transcription. The transcriptional specificity on well defined DNA templates of unpurified or minimally purified RNA polymerase II prepared with the DNase I-Sepharose method is currently being examined in this laboratory.

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