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A Factor in Sea Urchin Eggs Inhibits Transcription in Isolated Nuclei by Sea Urchin RNA Polymerase III†

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ABSTRACT: Isolated nuclei from sea urchin embryos synthesize RNA at a rate comparable to other animal cell nuclei. All three RNA polymerases are active as judged by α-amanitin sensitivity and hybridization to specific cloned DNAs. Extracts were prepared from sea urchin eggs and embryos by extraction with 0.35 M KCl. None of the crude extracts had a large effect on total RNA synthesis. However, extracts from sea urchin eggs inhibited RNA polymerase III activity in nuclei from blastula and gastrula embryos. There was no effect on the synthesis of ribosomal RNA by RNA polymerase I or on the synthesis of two RNA polymerase II products, histone mRNA and the sea urchin analogue of U1 RNA. The inhibitor is present in two different species of sea urchin and has been 50-fold purified by diethylaminoethylcellulose and hydroxylapatite chromatography. The inhibitor is not present in extracts prepared from sea urchin blastula embryos.

During oogenesis, the sea urchin synthesizes and stores a large portion of the RNA molecules that will be used subsequently in the developing embryo [reviewed by Davidson (1976)]. In addition, the products of RNA polymerase II, messenger RNA and heterogeneous nuclear RNA, continue to be synthesized at high rates in the mature sea urchin egg (Levner, 1974; Brandhorst, 1980; Dworkin & Infante, 1978). The structural RNAs which are synthesized by RNA polymerases I and III are probably not synthesized in the egg (Levner, 1974). The synthesis of these RNAs is activated early in embryonic development (Nijhawan, 1978; O'Melia, 1979a,b; Nijhawan & Marzluff, 1979; Griffith et al., 1981). The differences in expression of these major classes of RNA molecules might arise from independent regulation of the three RNA polymerases involved in their synthesis [reviewed by Roeder (1976)].

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The onset of synthesis of the small structural RNAs, 5S and tRNAs, by RNA polymerase III occurs early in sea urchin development (O'Melia, 1979a,b; Nijhawan & Marzluff, 1979). Since the 18S and 26S rRNAs are transcribed as a large precursor (Blin et al., 1979; Bieber & Stafford, 1979; Griffith & Humphreys, 1979), the period of development when their synthesis is initiated has been more difficult to identify. Consequently, various results concerning the beginning of large rRNA synthesis have been reported (Sconzo & Guidice, 1971; Kijima & Wilt, 1969; Emerson & Humphreys, 1970). Refined techniques have led to the detection of the precursor to the large rRNAs as early as the 32–64-cell stage (Griffith et al., 1981). Despite the difficulty in detection of the rRNA precursor, it has been generally reported that RNA polymerase I and RNA polymerase III are not coordinately regulated (Ford, 1971; Miller, 1974; O'Melia, 1979b; Roeder, 1974) in developing sea urchin or developing frog embryos.

In order to study the mechanism of developmental regulation of RNA synthesis in the sea urchin, we have established a cell-free transcription system using isolated nuclei. Cellular extracts from heterologous or homologous stages of development are combined with the isolated nuclei in order to identify factors present in the extracts which exert control over gene expression. We report here a factor(s) in unfertilized sea urchin eggs inhibit(s) synthesis of RNA polymerase III products. The factors cannot be detected in extracts from the morula or later stages of development. Activation of RNA polymerase III could be the result of rapid inactivation of an inhibitor of the enzyme stored in the sea urchin egg.

Experimental Procedures

Materials. Lytechinus variegatus were collected at the FSU marine lab and maintained at room temperature. *Strongylocentrotus purpuratus* were obtained from Pacific Bio-marine and maintained at 16 °C. Restriction enzymes were purchased from BRL and used as directed. α-Amanitin was purchased from Boehringer Mannheim. Omnifluor was purchased from New England Nuclear. Nitrocellulose (0.45 μm) was purchased from Schleicher & Schuell. [α-^{32}P]GTP (20–50 Ci/mmol) was synthesized by the method of Reeve & Huang (1979) or purchased from ICN. Plasmids pLv4, pLv1334 (Blin et al., 1979), and pLu305 (Lu et al., 1981) were gifts of Dr. Darrel Stafford. Plasmids pSp102 and pSp117 (Levy et al., 1978) were gifts of Dr. Larry Kedes.

Preparation of Nuclei. Fertilization of eggs and growth of *L. variegatus* and *S. purpuratus* embryos have been described (Nijhawan & Marzluff, 1979). At the desired stage of development, the embryos were collected by filtration of the culture through a 35-μm nylon mesh (Small Parts, Inc., Miami, FL). After collection of the embryos, all operations were at 0–4 °C. The embryos were washed 2 times with 0.55 M KCl and once with 2–3 volumes of 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris),[1] pH 8.0, and 0.1 mM EDTA. They were then suspended in 5–10 volumes of 0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris, pH 8.0, 1 mM EGTA, 1 mM DTT, 1 mM spermidine, and 0.1 mM PMSF (buffer I) and homogenized by 10–20 strokes in a Dounce homogenizer with a B pestle. Cell breakage was monitored by microscopy. Two volumes of buffer I with 2 M sucrose (buffer II) were added to the homogenate. This was layered over a pad of buffer II and centrifuged at 50000*g* for 45 min. The pelleted nuclei were resuspended by homogenization in glycerol storage buffer (25% glycerol, 50 mM Tris, pH 8.0, 1 mM EGTA, 1 mM spermidine, 1 mM DTT, 5 mM $MgCl_2$, and 0.1 mM PMSF) at (5–10) × 10^8 nuclei/mL and stored in liquid nitrogen.

Preparation of Whole Cell Extracts. Embryos were collected and washed with potassium chloride and sucrose solutions as for nuclei preparation. Following the sucrose wash, the embryos were resuspended in 1 mL/3 mL of packed embryos in 25 mM KCl, 1 mM DTT, 10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mM PMSF, and 1 mM spermidine. The suspension was homogenized with 10 strokes in a Dounce homogenizer with a B pestle. Then KCl was added to a final concentration of 0.35 M. Following five more strokes with the B pestle, the homogenate was centrifuged at 10000*g*. The supernatant was removed and centrifuged for 1 h at 100000*g*. Care was taken to remove only the clear supernatant following the high-speed centrifugation. This extract was used in subsequent fractionation procedures described below.

Fractionation of Whole Cell Extracts. The crude whole cell extract was dialyzed for 8 h against buffer A (10% glycerol, 10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM PMSF, and 1 mM DTT). The dialyzed extract (6 mg of protein/mL bed volume) was then applied to a DEAE-cellulose column equilibrated in buffer A. The column was step eluted with buffer A plus 0.1, 0.2, and 0.35 M KCl in sequential washes. Fractions (1–2 mL) were collected and monitored for protein by the absorbance at 280 nm. After the fractions containing protein in each wash were pooled, the protein was precipitated by the addition of 0.42 g/mL ammonium sulfate. Following centrifugation at 65000*g* for 30 min, the recovered protein from each step elution was resuspended in buffer A and dialyzed for 8 h. The dialyzed material was centrifuged at 10000*g* for 5 min and frozen in liquid nitrogen.

The DEAE-cellulose fraction was further fractionated by hydroxylapatite chromatography. The sample was dialyzed for 6 h against buffer B (10% glycerol, 50 mM NaH_2PO_4, pH 7.0, 1 mM DTT, and 0.1 mM PMSF) and applied to a hydroxylapatite column (8 mg of protein/mL bed volume) equilibrated in buffer B. The column was step eluted with buffer B containing 0.1, 0.2, and 0.35 M NaH_2PO_4, pH 7.0, in succession. One-milliliter fractions were collected and monitored for the absorbance at 280 nm. Peak fractions were pooled and precipitated with ammonium sulfate as described above.

Gel filtration chromatography was on a Sephadex G-25 column equilibrated in buffer A. The absorbance at 280 nm was monitored, and the peak fractions were pooled and frozen as droplets by dropping directly into liquid nitrogen.

RNA Synthesis. The standard 0.2-mL reaction contained 90 mM KCl, 7.5 mM $MgCl_2$, 500 μM each of ATP, CTP, and UTP, and 50 μM [α-^{32}P]GTP (1–10 Ci/mmol). The reaction was initiated by the addition of 100 μL of nuclei in the glycerol storage buffer. *L. variegatus* nuclei and *S. purpuratus* nuclei were incubated at 22 and 18 °C, respectively. Two-microliter aliquots were removed at 10-min intervals and assayed for trichloroacetic acid insoluble material (Marzluff et al., 1973). After 30 min, 0.1 mL of cold 0.1 M KCl was added to the transcription reaction, and it was placed on ice. Then 0.2 mL of cold glycerol storage buffer was layered under the reaction. The nuclei were pelleted by centrifugation at 1500*g* for 5 min. The supernatant was removed and RNA prepared from both the released and nuclear fraction by phenol extraction as

[1] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

previously described (Marzluff et al., 1973). The recovered RNA was dissolved in sterile water, and an equal proportion of each sample was applied to a 10% polyacrylamide gel in 7 M urea (Peattie, 1979) or aqueous buffer (Marzluff et al., 1975). Electrophoresis was continued until the bromophenol blue marker reached the bottom of the gel (aqueous gel) or the xylene cyanol marker reached the bottom of the gel (urea gel). The gel was stained for 15 min with 1 μg/mL ethidium bromide in water. The stained gel was photographed by using a red filter with UV illumination. The gel was then soaked in succession in water for 10–20 min and 70% (v/v) methanol for 10 min. Then the gel was dried in vacuo on Whatman 3MM paper. The dried gel was exposed to Kodak X-Omat AR X-ray film with a Du Pont Lightning Plus intensifying screen at −80 °C.

Hybridization of RNA to Immobilized DNA. The DNA blots [prepared according to Southern 1975)] were preincubated in a sealed plastic bag for 1 h at 52 °C in 5–10 mL of 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$ 0.1% Ficoll, 0.1% poly(vinylpyrrolidone), 2 mM EDTA, and 25 μg/mL poly(adenylic acid). This solution was removed and replaced by 1–2 mL of the same solution containing the radioactive RNA. Hybridization was for 2–3 days at 52 °C. The filters were washed with three changes of 0.15 M NaCl, 15 mM sodium citrate, 10 mM Tris, pH 7.5, 0.1% $NaDodSO_4$, and 2 mM EDTA for 30 min each at 52 °C. The filters were removed from the plastic bags, rinsed at room temperature in a solution of 0.15 M NaCl and 15 mM sodium citrate, and air-dried, and the hybridized RNA was detected by radioautography using a Lightning-Plus (Du Pont) intensifying screen.

Plasmid DNA for the dot hybridization assay (Kafatos et al., 1979) was digested with a restriction enzyme followed by phenol extraction and ethanol precipitation. The linear DNA was resuspended in 2 M sodium chloride and 0.2 M ammonium hydroxide and denatured by heating at 100 °C for 2 min. Five micrograms per dot of the denatured DNA was applied to nitrocellulose in a BRL Hybridot manifold as directed by the manufacturer. Hybridization to the DNA dots was performed as described above.

Results

Sea Urchin Nuclei Active in RNA Synthesis. Nuclei were routinely isolated from the swimming blastula stage of development. The addition of a protease inhibitor and the exclusion of Ca^{2+} were necessary for the isolation of nuclei with high molecular weight DNA. The incorporation of GMP into RNA in these nuclei [2–10 pmol of GMP (μg of DNA)$^{-1}$ (30 min)$^{-1}$] is similar to that in other nuclear transcription systems (Marzluff et al., 1973; Stallcup et al., 1978; Ernest et al., 1976; Jelinek, 1974). Although this incorporation is significantly reduced from the in vivo rate, all three polymerases remain active. As expected, most of the activity (about 80–90%) was due to RNA polymerase II, which is sensitive to low levels of α-amanitin (Roeder, 1976) (Figure 1). The relative proportion of the three polymerase activities reflected in Figure 1 was not independent of the developmental stage. Nuclei isolated from gastrula or later stages of development had a higher proportion of activity insensitive to low concentrations of α-amanitin (not shown). These data correlate with a reported decline in RNA polymerase II activity as development continues (Kijima & Wilt, 1969; Emerson & Humphreys, 1970).

Detection of Specific RNA Products. The activity of the three RNA polymerases was distinguished with varying concentrations of α-amanitin. Specific transcripts synthesized by each polymerase were detected as well. More than 95% of the small RNA polymerase III transcripts were released from nuclei when the nuclei were removed from the reaction by centrifugation (see below). Large transcripts, such as the rRNAs, remained with the nuclear pellet. RNAs of an intermediate size such as histone mRNA were partitioned into both the pellet and supernatant fractions. These conclusions are supported by electrophoretic analysis (see Figure 7C) and hybridization to cloned DNA probes (not shown). The small RNAs were isolated from the supernatant fraction and analyzed by gel electrophoresis in 7 M urea (Figure 2A). The RNA polymerase III transcripts were identified by their sensitivity to high concentrations of α-amanitin (Roeder, 1976) (250–500 μg/mL) (Figure 2B).

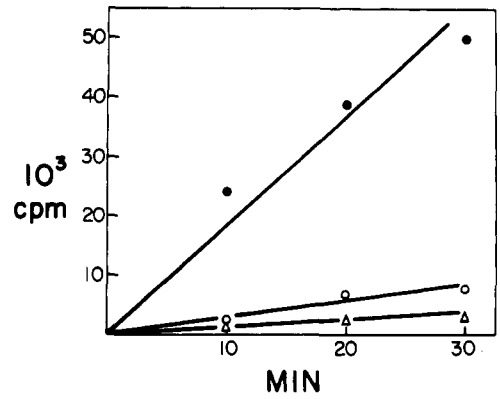

FIGURE 1: Effect of α-amanitin on the incorporation of [α-^{32}P]GTP. *S. purpuratus* nuclei (hatching blastula) were incubated in buffer in the transcription assay (see Experimental Procedures) with variable amounts of α-amanitin added. At the times indicated, aliquots were removed from the transcription reaction and assayed for trichloroacetic acid insoluble radioactivity (Marzluff et al., 1973). (●) No α-amanitin; (○) 1.0 μg/mL α-amanitin; (△) 500 μg/mL α-amanitin. The zero time measurement (1000 cpm) has been subtracted from all points.

Several of the RNA polymerase III transcripts evident in the autoradiograph (Figure 2B) have electrophoretic mobilities identical with those of the RNAs present in the nuclei. There is a doublet present in the 5S rRNA region in the RNA synthesized in vitro, the lower member of which coelectrophoreses with 5S rRNA. No RNA is found in long-term in vivo labels of RNA with the mobility of the upper member of the doublet. A large number of RNAs in the region between 4S and 5S RNA presumably represent precursor tRNAs. RNA with this mobility is made in short pulses in vivo with [^{3}H]uridine but is not present in longer labels (Nijhawan & Marzluff, 1979). RNAs with similar mobilities have been identified as precursor tRNAs in mammalian systems (Marzluff et al., 1974, Figure 9). There are three RNA polymerase III products with electrophoretic mobilities faster than tRNA that are made in the isolated nuclei (labeled A, B, and C in Figure 2B). Bands A and B have mobilities slightly faster than tRNA and would be difficult to detect in vivo. Band C is about 55 nucleotides long, and we have not detected any RNA of this mobility in long-term labels of *S. purpuratus* or *L. variegatus* embryos (G. F. Morris, unpublished results).

There are two RNA polymerase III transcripts larger than 5S rRNA. One migrates slightly faster than a major nuclear RNA species designated N3 in Figure 2A,B. The other major polymerase III product larger than 5S rRNA is a cytoplasmic RNA designated C3 in Figure 2B (Nijhawan, 1978; Nijhawan & Marzluff, 1979).

One of the small RNAs (labeled N1) was synthesized by RNA polymerase II as judged by its sensitivity to a low

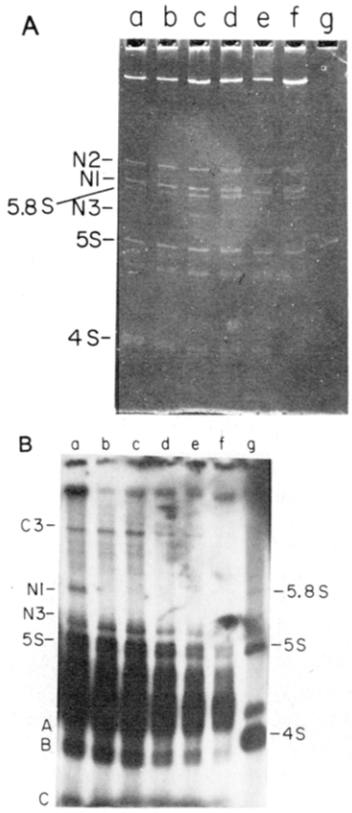

FIGURE 2: Effect of α-amanitin on the synthesis of the low molecular weight RNA. *S. purpuratus* hatching blastula nuclei were incubated in buffer with increasing concentrations of α-amanitin under conditions for RNA synthesis. The RNA was prepared from the released (supernatant) portion of the reaction as described. The released RNA was analyzed on a 10% polyacrylamide–7 M urea vertical slab gel. Identification of the released RNA species was made from the ethidium bromide stained gel shown in (A). The lane designations are the same for (A) and (B). 5.8S is the 5.8S rRNA; A, B, and C are RNAs synthesized in vitro which do not have stained counterparts observed in vivo. (B) Autoradiograph of the same gel shown in (A): (a) no α-amanitin; (b) 1 μg/mL α-amanitin; (c) 10 μg/mL α-amanitin; (d) 100 μg/mL α-amanitin; (e) 250 μg/mL α-amanitin; (f) 500 μg/mL α-amanitin; (g) in vivo labeled *S. purpuratus* small cytoplasmic RNA.

concentration (1 μg/mL) of α-amanitin (Figure 2B, lanes a and b). The N1 RNA is the sea urchin homologue of the U1 RNA in higher eucaryotes. Another specific polymerase II product, histone mRNA, was also synthesized by RNA polymerase II (Levy et al., 1978; see below) in these nuclei.

RNA polymerase I activity was detected by resistance to high concentrations of α-amanitin (Figure 1) and hybridization to the cloned sea urchin rDNA plasmids pLv4 and pLv1334 (Blin et al., 1979). These two plasmids contained the entire sea urchin ribosomal RNA repeat. The transcribed region as well as the coding region is indicated in Figure 3A,B. The *Eco*RI fragment labeled D in Figure 3B from pLv1334 contains the start of the 40S ribosomal RNA precursor (Bieber et al., 1981). There was hybridization to all of the fragments containing transcribed portions of the ribosomal RNA repeat. There was no hybridization to the plasmid vector or to the nontranscribed spacer (post 26 S) region. Synthesis of these RNAs was not affected by high concentrations (200 μg/mL) of α-amanitin (Figure 3C). No mature 18S and 26S rRNAs were detected in these experiments, when the RNA was analyzed by agarose gel electrophoresis (data not shown).

Detection of an Inhibitor of RNA Polymerase III in Sea Urchin Eggs. Whole cell extracts were prepared and added back to the isolated nuclei (Figure 4) in an attempt to modulate the RNA synthesis in the isolated nuclei. These extracts

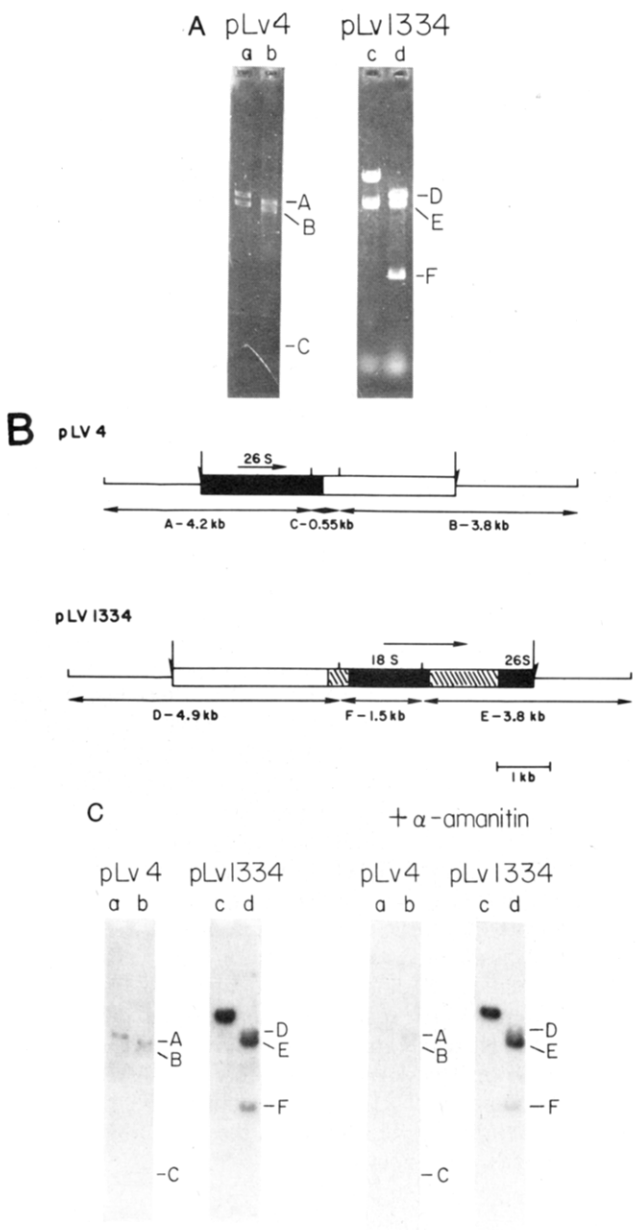

FIGURE 3: Synthesis of ribosomal RNA in isolated nuclei. Recombinant DNA plasmids containing *L. variegatus* ribosomal RNA genes were cleaved with the restriction enzyme *Eco*RI or *Sal*I. (A) The DNA fragments were separated on a 0.7% (w/v) agarose gel, denatured, and blotted onto nitrocellulose (Southern, 1975). (a) pLv4 digested with *Sal*I. The bottom band is plasmid DNA, and the top band is the 4.8-kilobase pairs (kbp) insert sea urchin DNA. (b) pLv4 digested with *Eco*RI. The bands are labeled corresponding to the map in Figure 3B (A, B, and C). (c) pLv1334 digested with *Sal*I. The top band is the 6.6-kbp insert DNA, and the bottom band is the plasmid DNA. (d) pLv1334 digested with *Eco*RI. The bands are labeled corresponding to the map in Figure 3B (D, E, and F). (B) A schematic diagram of the recombinant ribosomal RNA plasmid is shown [from Blin et al. (1979)]. Plasmid DNA is denoted as a solid horizontal line, the *L. varigatus* DNA as open boxes, the regions corresponding to the mature RNA species as closed boxes, and the transcribed spacer region as hatched boxes. The fragments generated by digestion of the plasmids with *Eco*RI are labeled A–F. The sizes are in kilobase pairs: (↓) *Sal*I site; (|) *Eco*RI site. (C) [α-^{32}P]-GTP-labeled RNA was synthesized in *L. variegatus* mesenchyme blastula nuclei in the absence (two left panels) and presence (two right panels) of 200 μg/mL α-amanitin. This RNA preparation was hybridized (see Experimental Procedures) to the rDNA blot. The hybridized RNA was detected by radioautography. Exposure time was 8 h. (a) pLv4 digested with *Sal*I; hybridization was to the insert DNA. (b) pLv4 digested with *Eco*RI; hybridization was to fragment A and fragment C in longer exposures. (c) pLv1334 digested with *Sal*I; hybridization was to the insert DNA. (d) pLv1334 digested with *Eco*RI; hybridization was to fragments D–F. *Eco*RI cleavage products are labeled as in part B.

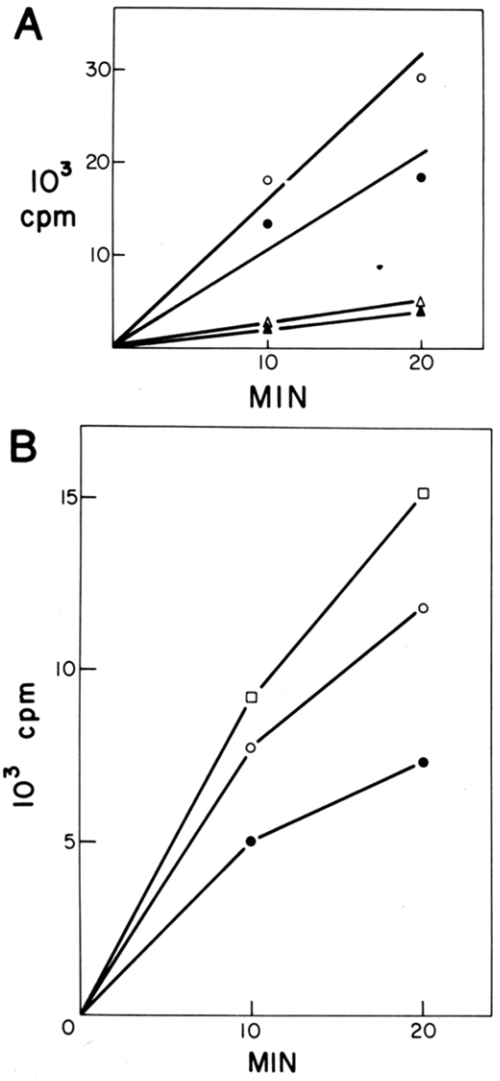

FIGURE 4: Effect of a crude egg extract on RNA synthesis. (A) A *S. purpuratus* egg extract was prepared as described under Experimental Procedures. The excess salt and triphosphates were removed by chromatography on a Sephadex G-25 column in buffer A. Isolated hatching blastula nuclei were incubated with the egg extract as described under Experimental Procedures. At the indicated time, aliquots from the reactions were assayed for incorporation of [α-^{32}P]GTP into trichloroacetic acid insoluble material (Marzluff et al., 1973). (○) Nuclei in crude egg extract; (●) nuclei in buffer; (△) nuclei in crude egg extract plus 1 μg/mL α-amanitin; (▲) nuclei in buffer plus 1 μg/mL α-amanitin. The zero time incorporation (1000 cpm) was subtracted from all points. (B) Extracts were prepared from *L. variegatus* eggs and blastula embryos as in (A). The extracts were incubated with *L. variegatus* gastrula nuclei as described in part A. At the indicated times, aliquots of the reactions were assayed for trichloroacetic acid insoluble incorporation of [α-^{32}P]GTP as in part A. The zero time value was subtracted from all points. (●) Nuclei in buffer; (○) nuclei + crude egg extract; (□) nuclei + crude blastula extract.

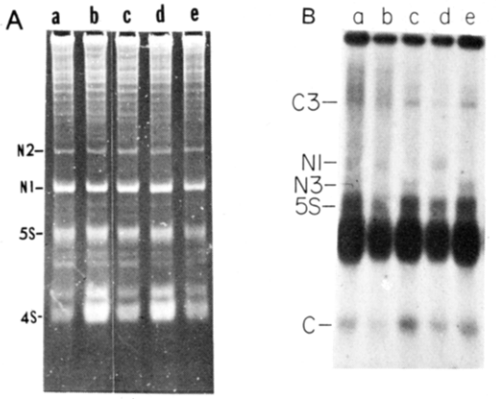

FIGURE 5: Inhibition of RNA polymerase III by extracts from sea urchin eggs. Crude whole cell extracts were prepared from *L. variegatus* embryos or eggs as described in Figure 4. Nuceli from hatching blastula or gastrula *L. variegatus* embryos were incubated with the crude extracts for 30 min, and RNA was prepared from the released (supernatant) portion of the reaction and fractionated on a 10% polyacrylamide gel under aqueous conditions (see Experimental Procedures). The ethidium bromide stained gel is shown in (A). The ethidium bromide stained bands near the top of the gel represent small DNA fragments present in this nuclear preparation. (B) The autoradiograph of the gel shown in (A) shows the RNA synthesized and released in *L. variegatus* gastrula or blastula nuclei assayed for RNA synthesis as designated below: (a) blastula nuclei in blastula extract; (b) blastula nuclei in egg extract; (c) gastrula nuclei in blastula extract; (d) gastrula nuclei in egg extract; (e) gastrula nuclei in buffer. The RNAs are labeled as in Figure 2B. The RNA components A and B were not detected in this experiment, presumably due to their migrating differently in this gel system (without urea) compared to the gel system used in Figures 2, 6, and 7.

Table I

extract	sp act.[a]	x-fold purification
crude egg extract	6.5[b]	
DEAE-purified factor	0.93[b]	7
hydroxylapatite-purified factor	0.14	46

[a] The specific activity was determined from the ratio of protein added to the amount of DNA in the reaction (determined by counting nuclei). The values given are the protein to DNA ratios at which pre-tRNA synthesis was inhibited 50%. [b] Values given were obtained by densitometric scanning of autoradiographs. Percent inhibition is relative to the amount of pre-tRNA synthesized in nuclei incubated in buffer only.

were prepared from eggs or various stages of embryonic development by homogenization at moderate ionic strength, 0.35 M KCl. Crude extracts prepared from eggs as described in the legend to Figure 4 stimulated total RNA synthesis 20–50%. This stimulation was seen in 80% of the crude extracts tested from eggs or embryos and predominantly represents stimulation of RNA polymerase II activity. This is true even for the egg extracts which reproducibly inhibited RNA polymerase III (Figure 5). The extracts lost the stimulatory activity upon further fractionation.

The effect of the extracts on the level of specific RNA products was determined. Extracts from either *S. purpuratus* or *L. variegatus* eggs reproducibly reduced synthesis of the RNA polymerase III products while extracts from several other embryonic stages had no effect on the RNA polymerase III products (Figure 5). The synthesis of all RNA polymerase III products was inhibited by the addition of an egg extract to either blastula or gastrula nuclei. The synthesis of the RNA which comigrated with N1 was not affected by the egg extract (see below). The maximum degree of inhibition of RNA polymerase III in these experiments with crude extract was 50%. More complete inhibition was obtained with partially purified factors (see below).

Partial Purification and Specificity of the Inhibitor. We believe the inhibitor is a protein. It is heat sensitive and nondialyzable. The inhibitor has been partially purified by chromatography on DEAE-cellulose and hydroxylapatite. It binds tightly to DEAE-cellulose, eluting between 0.25 and 0.35 M NaCl (Figure 6A,B), and to hydroxylapatite, eluting between 0.2 and 0.35 M NaH_2PO_4, pH 7.0 (Figure 6B). The concentrated inhibitor prepared by hydroxylapatite chromatography inhibited RNA polymerase III in a concentration-dependent manner (Figure 6D). It was enriched about 50-fold

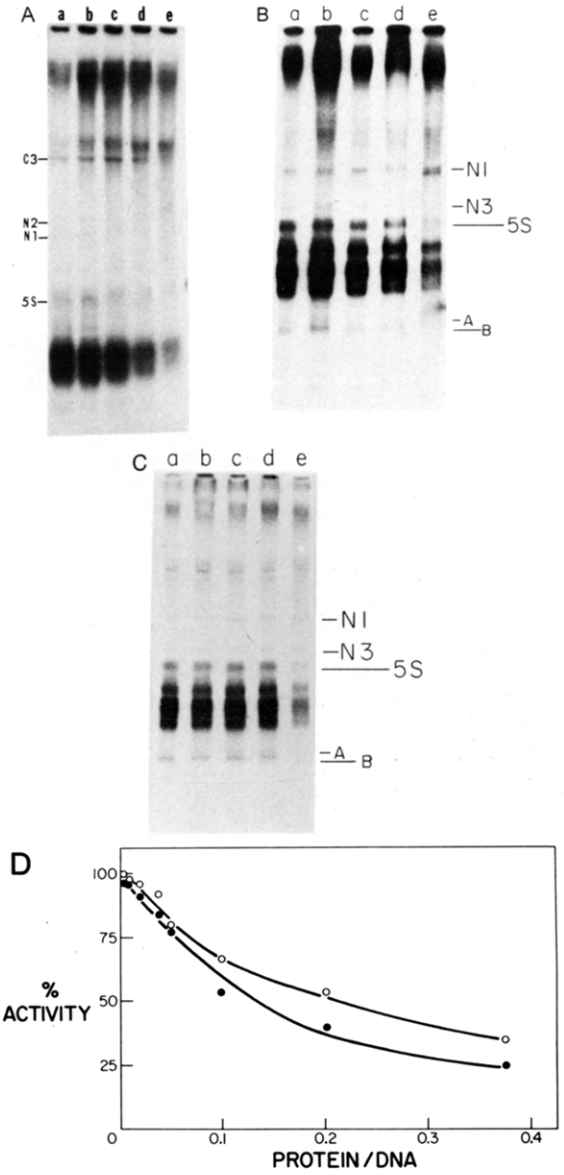

FIGURE 6: Partial purification of the RNA polymerase III inhibitor. (A) Extracts were prepared from *L. variegatus* eggs and fractionated on DEAE-cellulose by step elution as described under Experimental Procedures. The fractions were assayed with *L. variegatus* blastula nuclei. The RNAs released from the nuclei were analyzed by polyacrylamide gel electrophoresis in 7 M urea. (a) No extract; (b) unbound protein; (c) 0.1 M step; (d) 0.2 M step; (e) 0.3 M step. (B) A whole cell extract prepared from *S. purpuratus* eggs was fractionated on DEAE-cellulose by step elution as described under Experimental Procedures. Aliquots of each fraction were incubated with *S. purpuratus* nuclei in the transcription assay. The RNA released from the nuclei was analyzed on a 10% polyacrylamide–7 M urea gel (see Experimental Procedures) and detected by radioautography. (a) No extract; (b) unbound protein; (c) 0.1 M step; (d) 0.2 M step; (e) 0.35 M step. (C) Hydroxylapatite chromatography. The material eluting from the DEAE-cellulose column with 0.35 M KCl (Figure 6B) was recovered by precipitation with ammonium sulfate and applied to a hydroxylapatite column. The protein was eluted with a stepwise increase in sodium phosphate buffer, pH 7.0, as described under Experimental Procedures. Each fraction was tested for its affect on the synthesis of RNA in isolated *S. purpuratus* blastula nuclei as in Figure 6B. (a) No protein added; (b) unbound protein; (c) 0.1 M phosphate; (d) 0.20 M phosphate; (e) 0.35 M phosphate. The RNA bands are identified as in Figure 2. (D) Concentration dependence of the inhibitor. Equal amounts of nuclei were incubated with varying amounts of inhibitor prepared by hydroxylapatite chromatography (Figure 6C, lane e). The released RNA was prepared and analyzed by gel electrophoresis. The gel was stained with ethidium bromide and the region corresponding to 5S RNA and 4.5S pre-tRNA excised and counted. The results are expressed as the percent inhibition relative to the control with no inhibitor added: (○) 5S rRNA (control = 3100 cpm); (●) 4.5S RNA (control = 29 700 cpm).

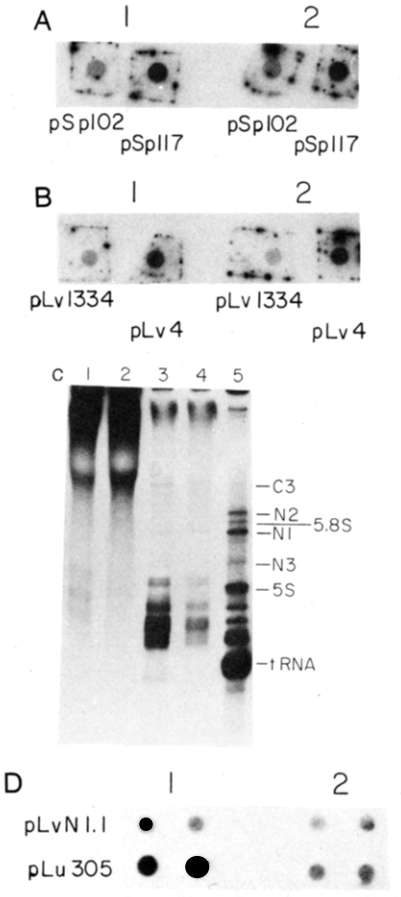

FIGURE 7: Effect of the inhibitor on ribosomal RNA and histone RNA synthesis. Isolated blastula nuclei (*S. purpuratus*) were incubated with and without the inhibitor purified on hydroxylapatite (see Experimental Procedures). Total RNA was prepared and hybridized to plasmids containing the sea urchin histone genes pSp102 and pSp117 (A) or the ribosomal genes pLv4 and pLv1334 (B). An autoradiogram of the filter is shown. The protein to DNA ratio for Figure 7A–D was 0.3 (see Figure 6D). (A) Histone RNA synthesis: (1) absence of inhibitor; (2) presence of inhibitor. (B) Ribosomal RNA synthesis: (1) absence of inhibitor; (2) presence of inhibitor. (C) In a separate experiment, the inhibitor purified as described in Figure 6C was incubated with blastula nuclei (*S. purpuratus*) under conditions for RNA synthesis. After 30 min, RNA was prepared from the released and pellet fractions (see Experimental Procedures). Equal amounts of the recovered RNA were run on a 10% polyacrylamide gel containing 7 M urea: (lanes 1 and 3) RNA in the pellet and supernatant fractions, respectively, from nuclei incubated in buffer; (lanes 2 and 4) RNA in the pellet and supernatant fractions, respectively, from nuclei incubated with the partially purified inhibitor; (lane 5) in vivo labeled *L. variegatus* small nuclear RNA marker. (D) The RNA in the released (supernatant) fraction (shown in Figure 7C, lanes 3 and 4) was hybridized to plasmid pLu305 containing the sea urchin 5S rRNA gene and plasmid pLvN1.1 containing the sea urchin N1 RNA gene (D. T. Brown, G. F. Morris, and W. F. Marzluff, unpublished results). Duplicate DNA dots are shown for each sample. (1) RNA synthesized in the absence of inhibitor; (2) RNA synthesized in the presence of inhibitor.

as judged by the amount of protein required to inhibit RNA polymerase III 50% (Table I).

The inhibitor has several properties which argue that it is not a nonspecific inhibitor but may be physiologically relevant. It does not inhibit either RNA polymerase I or RNA polymerase II. The synthesis of specific gene products of all three polymerases was analyzed by RNA–DNA hybridization (Figure 7). Plasmid DNAs containing the ribosomal RNA genes (RNA polymerase I), the histone genes, an N1 RNA gene (RNA polymerase II), and a 5S rRNA gene (RNA polymerase III) were hybridized with RNA synthesized in the presence or absence of the inhibitor. The synthesis of neither

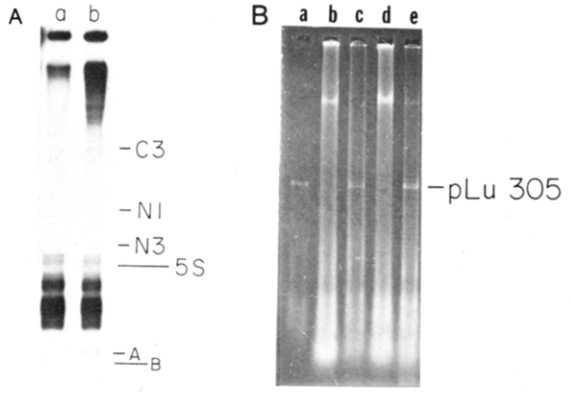

FIGURE 8: Nuclease activity of the partially purified RNA polymerase III inhibitor. (A) RNase assay. *S. purpuratus* nuclei were incubated in a 0.2-mL reaction under conditions for RNA synthesis. After 30 min, the released RNA was prepared from 0.1 mL of the reaction (lane a). 0.1 mL of solution containing a large excess of nonradioactive GTP and 75 μL of the 0.35 M KCl DEAE-cellulose step fraction (see Figure 6A) from *S. purpuratus* eggs was added to the remaining 0.1 mL. This reaction was incubated for an additional 30 min, and the released RNA was prepared (lane b). The RNAs were analyzed by gel electrophoresis on a 10% polyacrylamide–7 M urea gel as described under Experimental Procedures. (B) DNase assay. Two micrograms of the recombinant plasmid pLu305, containing the *L. variegatus* 5S rRNA gene, was incubated in a transcription reaction with *S. purpuratus* nuclei plus the 0.35 M KCl DEAE-cellulose step fraction (see Figure 6A) from a *S. purpuratus* egg extract. The nucleic acids were recovered from the supernatant and pellet portions of the reaction as described under Experimental Procedures. One-fourth of the recovered nucleic acids was analyzed by electrophoresis on a 0.7% (w/v) agarose gel. The gel was stained with ethidium bromide. Lane a shows 0.5 μg of the input plasmid DNA. Lanes b–e show the recovered nucleic acids from the following: (b) the retained (pellet) fraction of nuclei incubated in buffer, (c) the released (supernatant) fraction of nuclei incubated in buffer, (d) the retained fraction form nuclei in extract, and (e) the released fraction from nuclei in extract.

histone RNA nor ribosomal RNA was affected by the partially purified inhibitor (after chromatography on hydroxylapatite) (Figure 7A,B). In addition, the inhibitor did not have any effect on the synthesis of the small RNA that coelectrophoresed with N1 RNA which was synthesized by RNA polymerase II (Figure 6C and 7D). This RNA is in the same size range as the RNA polymerase III transcripts. These results strongly suggest that there is little alteration of the activity of the other RNA polymerases while synthesis of all RNA polymerase III transcripts is inhibited. The inhibitor is not present in extracts prepared from several embryonic stages (Figure 5B) even after fractionation of the extracts on DEAE-cellulose (our unpublished results). Thus, it is destroyed at some time after fertilization, possibly leading to activation of RNA polymerase III in vivo.

We show here that the activity is not due to contaminating nuclease activity. RNase activity was assayed as described in the legend to Figure 8. Once the RNA polymerase III transcripts are synthesized, they are not degraded upon addition of the inhibitor to the transcription assay (Figure 8A). In addition, the RNAs in the nuclei, detected by ethidium bromide staining, were not degraded by addition of the inhibitor (Figure 5A). To assay for DNase activity, we carried out the reaction (nuclei plus inhibitor) in the presence of plasmid DNA. The plasmid DNA was recovered intact primarily in the supernatant portion of the reaction (Figure 8B). In a separate assay, the factor did not relax supercoiled plasmid DNA (data not shown). Thus, there was not substantial DNase activity in the inhibitor preparation.

An inhibitor with similar properties was detected in both *S. purpuratus* and *L. variegatus* eggs (Figure 6A,B), and the

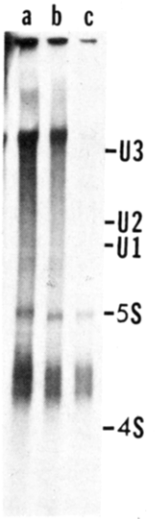

FIGURE 9: Effect of the sea urchin RNA polymerase III inhibitors on mouse myeloma nuclei. Mouse myeloma nuclei [prepared as described by Marzluff (1978)] were assayed as described under Experimental Procedures in the presence of a partially purified inhibitor from a DEAE-cellulose column of a *S. purpuratus* egg extract (see Figure 6A). The released RNA was prepared and analyzed on a 10% polyacrylamide–7 M urea gel as described under Experimental Procedures. The autoradiograph shows the gel analysis of the released RNA synthesized by myeloma nuclei in (a) buffer, (b) buffer plus inhibitor, and (c) buffer plus inhibitor plus 1 μg/mL α-amanitin.

inhibitor from either species was equally effective when incubated with nuclei from the other species of sea urchin (data not shown). However, the inhibitor had little effect on RNA polymerase III activity in mouse myeloma nuclei. Incorporation of [α-^{32}P]GTP into total RNA was reduced slightly in mouse myeloma nuclei by addition of the inhibitor. The slight inhibition affected all three RNA polymerases. Less than 50% inhibition of RNA polymerase III could be detected in all the fractions from the DEAE column of the sea urchin egg extract (Figure 9). The same inhibitor preparation inhibited RNA polymerase III activity in sea urchin nuclei 75% at a similar protein to DNA ratio.

Discussion

We have established an isolated nuclei transcription system from the sea urchin which faithfully reproduces in vitro many of the in vivo phenomena associated with RNA synthesis in sea urchin embryos. The proportion of activity due to each of the three polymerases is similar to that reported in vivo. The synthesis of specific products of all three polymerases was also demonstrated in this system.

The RNA polymerase III transcripts (identified by their sensitivity to high concentrations of α-amanitin) were present in the released (supernatant) portion of our transcription reaction. The release of these small RNAs is probably nonspecific and merely a result of their small size. Similar observations were previously made with mouse myeloma nuclei (Marzluff, 1978). The discrete size of the RNA polymerase III transcripts makes them amenable to direct analysis by gel electrophoresis. The combination of the mobility of these RNAs on denaturing gels as well as the fact that they are synthesized by RNA polymerase III provided the basis for the tentative identification of several of the released RNA species.

The results presented here show that RNA polymerase III can be specifically inhibited in isolated nuclei from sea urchin embryos by a factor from sea urchin eggs. While most genes are probably regulated by gene-specific factors which have specificity for the DNA or chromatin structure, the activity

of genes coding for structural RNAs could be efficiently regulated by regulating the activity of RNA polymerase. RNA polymerases I and III transcribe a small number of genes and produce RNA products which are found in all cells. In this case, regulation could be primarily at the level of the RNA polymerase. It is possible that the inhibitor which we are detecting affects the activity of the RNA polymerase III, since it inhibits the synthesis of all RNA polymerase III transcripts equally well. It is also possible that it interacts with the DNA or chromatin directly since some regions of homology have been attributed to putative promoter regions of many genes transcribed by RNA polymerase III (Koski et al., 1980). In addition, distinct gene-specific factors are required for the transcription of 5S and tRNA genes, both RNA polymerase III templates. At least one of these factors plays a positive role in regulating 5S rRNA transcription in the *Xenopus* oocyte (Segall et al., 1980; Pelham et al., 1981). Other factors (of a negative nature) have been implicated in the developmental control of *Xenopus* 5S rRNA genes (Korn & Gurdon, 1981; Bogenhagen et al., 1982; Gottesfeld & Bloomer, 1982). Similar factors to these are probably present in the isolated nuclei. The regulation of the RNA polymerase III activity would be an additional control on the synthesis of these RNAs. The selection of specific genes the polymerase transcribes is accomplished by other proteins.

Although these data do not prove that this inhibitor functions to regulate RNA polymerase III activity in the sea urchin embryo, several facts indicate that this may be the case. Regulation of RNA polymerase III activity is a logical mechanism in the special case of sea urchin development, when the embryo presumably uses RNA polymerase III synthesized during oogenesis. Early in development, the RNA polymerase III products are not detectable (Nijhawan & Marzluff, 1979) even though mRNA is being synthesized. The inhibitor of RNA polymerase III could be synthesized late in oogenesis to shut off synthesis of small structural RNA. At some time early in development, the inhibitor is inactivated or destroyed. We do not find any detectable inhibitor in the extracts of morula, blastula, or gastrula embryos when RNA polymerase III is active in vivo. The mechanism of this rapid inactivation of the inhibitor is not known.

The inhibitor had no effect on RNA polymerase II or RNA polymerase I activity in isolated nuclei. The synthesis of the main, if not the only, product of RNA polymerase I, ribosomal RNA, was not affected as assayed by RNA–DNA hybridization. Similarly, the synthesis of two RNA polymerase II products, histone RNA and a small nuclear RNA, N1, the analogue of the mammalian U1 RNA, was not affected by the inhibitor. This RNA is also synthesized by RNA polymerase II in chicken (Roop et al., 1981) and in human (Murphy et al., 1982). The RNA polymerase II products measured here, particularly the N1 RNA, are small transcripts. Their size is similar to that of the RNA polymerase III products.

The inhibitor is found in two sea urchin species, and the inhibitor from one species inactivates RNA polymerase III in nuclei from the other species. However, the sea urchin inhibitor had no selective effect on the RNA polymerase III activity in mouse myeloma nuclei, although there was nonselective depression (about 30%) of all RNA synthesis. The inhibitor does show some species specificity in its interaction with RNA polymerase III.

There are undoubtedly other factors involved in regulating gene activity which one might detect in isolated nuclei. We see a stimulation of total RNA polymerase II activity in extracts from eggs as well as other stages (Figure 4). This stimulatory activity is lost during purification of the RNA polymerase III inhibitor. In addition, alterations in transcription are occurring during development, particularly with the histone genes (Maxson & Wilt, 1981; Hieter et al., 1979) and small nuclear RNA genes (Nijhawan & Marzluff, 1979). It may be possible to detect gene-specific factors as well in this system.

Registry No. RNA polymerase, 9014-24-8.

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Monoclonal Antibodies to Rhodopsin: Characterization, Cross-Reactivity, and Application as Structural Probes†

R. S. Molday* and D. MacKenzie

ABSTRACT: Two monoclonal antibodies designated as rhodopsin (rho) 1D4 and rho 4A2 were obtained from hybridoma cells cloned after the fusion of mouse myeloma cells with spleen cells of a mouse immunized with bleached bovine rod outer segment disk membranes. These antibodies were specific for rhodopsin as determined by radioimmune labeling of bovine rod outer segment disk membrane proteins electrophoretically transferred from sodium dodecyl sulfate gels to CNBr-activated paper. Limited proteolytic digestion of rhodopsin in sealed disk membranes in conjunction with radioimmune assays indicated that the rho 1D4 antibody bound to the carboxyl-terminal segment of rhodopsin on the cytoplasmic side of disk membranes, whereas the rho 4A2 antibody bound to a determinant along the amino-terminal third of the rhodopsin polypeptide chain. Binding of the rho 4A2 antibody was sensitive to solubilization and photobleaching of rhodopsin. The rho 4A2 antibody did not bind to rhodopsin in sealed membrane disks but did bind to detergent-solubilized rhodopsin. Detergent-solubilized bleached rhodopsin was 13 times more antigenic than unbleached rhodopsin. Rhodopsin solubilized in Triton X-100 was more antigenic than rhodopsin solubilized in cholate. These results indicate that the 4A2 antibody serves as a sensitive immunological probe for structural changes of rhodopsin caused by solubilization and photobleaching. Both the rho 1D4 and 4A2 antibodies were also found to cross-react with frog rhodopsin but not *Halobacterium halobium* bacteriorhodopsin. The rho 4A2 antibody bound to the three forms of frog rhodopsin resolved by sodium dodecyl sulfate gel electrophoresis whereas rho 1D4 bound to only the two higher molecular weight frog rhodopsins. Finally, lectin inhibition studies using ^{125}I-labeled succinyl-Con A and antibody inhibition studies confirmed previous results indicating freshly prepared bovine disks were sealed with the lectin binding sites oriented toward the inside of the disk, whereas frozen–thawed disks were predominantly unsealed with both membrane surfaces exposed. Frog disk membrane vesicles were shown to have the same orientation.

Rhodopsin is the major membrane glycoprotein in rod outer segment (ROS)[1] disk membranes of vertebrate retinal rod photoreceptor cells. Biochemical and electron microscopic studies indicate that rhodopsin is a transmembrane protein (Jan & Revel, 1974; Fung & Hubbell, 1978; Molday & Molday, 1979) with the amino-terminal segment containing two Con A specific and WGA-specific carbohydrate chains (Steineman & Stryer, 1973; Hargrave, 1977; Fukuda et al., 1979) oriented toward the interior of the disks (Röhlich, 1976; Clark & Molday, 1979) and a protease-sensitive carboxyl-terminal segment and internal segments of the polypeptide chain exposed on the interdisk or cytoplasmic side (Pober & Stryer, 1975; Sale et al., 1978; Molday & Molday, 1979). Although the detailed organization of rhodopsin in the disk membrane is not yet known, it has been proposed that the rhodopsin polypeptide traverses the membrane an odd number of times, possibly as many as 7 times, in α-helical conformations (Fung & Hubbell, 1978; Albert & Litman, 1978; Michel-Villaz et al., 1979). Rhodopsin, therefore, would have structural features similar to those of bacteriorhodopsin (Henderson & Unwin, 1975; Engelman & Zaccai, 1980).

In order to obtain further insight into the structure and function of ROS disk membrane proteins, we have recently prepared and characterized several monoclonal antibodies against rhodopsin and the M_r 220 000 glycoprotein (MacKenzie & Molday, 1982). These antibodies were used in conjunction with radioimmune assays and immunoferritin electron microscopic techniques as probes to study the organization of these proteins in ROS disk membranes. We have extended these initial studies, and in this paper, we report on the binding properties and cross-reactivity of two other mo-

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[1] Abbreviations: ROS, rod outer segment(s); Con A, concanavalin A; FCS, fetal calf serum; BSA, bovine serum albumin; RIA, radioimmune assay; PBS, phosphate-buffered saline; Ig, immunoglobulin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; rho, rhodopsin; WGA, wheat germ agglutinin.

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