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Characterization of a Crude Selective *Pol*I Transcription System from *Tetrahymena pyriformis*†

Joyce Sutiphong, Cynthia Matzura, and Edward G. Niles*

ABSTRACT: A crude in vitro transcription system which selectively transcribes DNA fragments containing the promoter region of the *Tetrahymena pyriformis* rRNA gene has been prepared from *T. pyriformis*. The system requires both an S100 fraction of lysed isolated macronuclei and an S100 extract of whole cells. When a *Hha*I–*Hin*dIII fragment of the promoter containing plasmid pEN 19-1 is employed as a template, transcription yields two major products of about 560 (A) and 510 (B) bases in length. The analysis of the transcription products of truncated templates showed that RNA A is a runoff transcript and RNA B is produced by nucleolytic cleavage of RNA A at a site about 50 nucleotides to the left of the *Hin*dIII cleavage site. S1 nuclease mapping was used to demonstrate that the 5′ end of RNA A is identical with that predicted for a transcript which was initiated at the same site on the gene as the in vivo 35S rRNA precursor. Transcription is dependent upon the addition of promoter containing DNA, is inhibited by 1 μg/mL actinomycin D, and is insensitive to 200 μg/mL α-amanitin. Transcription is dependent upon the salt levels in the assay exhibiting activity peaks at 58 mM KCl, 28 mM $(NH_4)_2SO_4$, and 3 mM $MgCl_2$. Several minor transcription start sites to the left of the major initiation site become active at high salt, yielding several minor longer transcripts. High salt also inhibits the RNA cleavage activity, reducing the levels of RNA B produced.

In eukaryotes there are three forms of RNA polymerase, I, II, and III, which catalyze the synthesis of precursors to rRNA, mRNA, and tRNA and 5S rRNA, respectively. Since transcription initiation is a likely site for the regulation of gene expression, a major effort has been expended in order to identify the functional regions of eukaryotic promoters. DNA sequence analysis and in vitro transcription studies of several genes transcribed by form II RNA polymerases have revealed that high levels of specific initiation require an A-T-rich region (TATA box) centered at about −25 from the 5′ end of the mRNA plus, in some cases, additional upstream sequences (Breathnack & Chambon, 1981). The form III RNA polymerase requires an intragenic region which binds an activator protein plus additional sequences to the left of the transcription start site (Hall et al., 1982).

Less is known about transcription initiation mediated by the form I RNA polymerase. Transcription initiation sites have been mapped and their base sequences determined for rRNA genes cloned from *X. laevis* (Sollner-Webb & Reeder, 1979; Bakken et al., 1982), yeast (Bayev et al., 1980; Swanson & Holland, 1983), mouse (Urano et al., 1980; Bach et al., 1981; Miller & Sollner-Webb, 1981), *D. melanogaster* (Long et al., 1981), human (Finanscsek et al., 1982), *Tetrahymena pyriformis* (Niles & Jain, 1981; Niles et al., 1981a,b; Higashinakagawa et al., 1981; Saiga et al., 1982), *Dictyostelium discoideum* (Hoshikawa et al., 1983), and *Physarum polycephalum* (Blum et al., 1983). Contrary to the genes transcribed by RNA polymerase *Pol*II and *Pol*III, only limited sequence conservation is noted in the rRNA gene transcription

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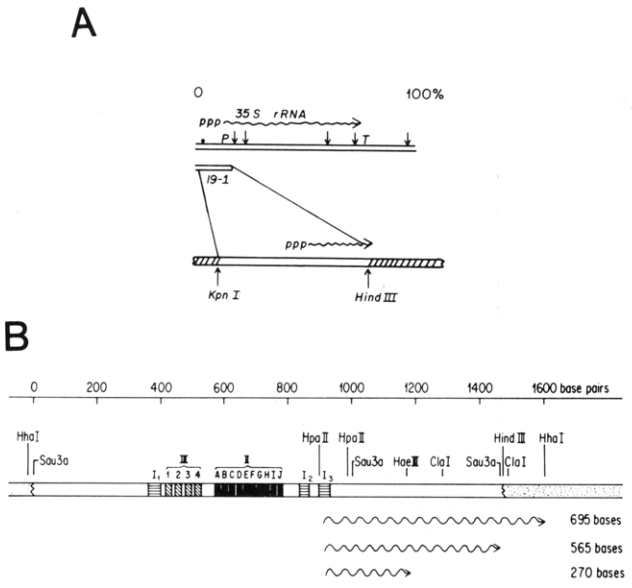

FIGURE 1: Physical map of the *T. pyriformis* rRNA gene. (A) Half of the *T. pyriformis* rRNA gene palindrome is drawn with the positions of the transcription promoter (P) and terminator (T), and the *Hin*dIII (↓) and Kpn I (•) cleavage sites are indicated (Niles & Jain, 1981; Niles et al., 1981a,b). Below is drawn the portion of the rDNA inserted in the recombinant DNA plasmid pEN 19-1. (B) A physical map of the rDNA portion of pEN 19-1 (Niles et al., 1981b; Niles, 1984). The positions of the *Hha*I, *Hin*dIII, *Hae*III, and *Hpa*II cleavage sites are presented along with the lengths of the expected runoff transcripts.

initiation regions. Selective in vitro transcription systems have recently been developed for several rRNA genes (Miller & Sollner-Webb, 1981; Grummt, 1981, 1982; Mishima et al., 1981; Grummt et al., 1982; Kohorn & Rae, 1982; Wilkinson & Sollner-Webb, 1982) in an effort to identify promoter regions required for transcription initiation. Results to date demonstrate that the region from about −158 to +20 are required for high level of accurate transcription in vitro (Learned et al., 1983; Kohorn & Rae, 1983). In addition, Bushy & Reeder (1983) have presented data that an additional site far up stream of the transcription start site of the *Xenopus* rRNA gene influences the efficiency of transcription of plasmids injected into eggs.

Recently we have identified several families of highly conserved, tandemly repeated, sequences upstream from the transcription start site in the *T. pyriformis* rRNA gene (Niles et al., 1981b; Niles, 1984). In order to access the functional significance of these repeat sequences, we have developed a selective in vitro transcription system from *T. pyriformis*. In this report we describe the specific initiation of rRNA gene transcription in vitro and cleavage of the product rRNA by a nuclease activity.

Materials and Methods

Materials. Restriction endonucleases, nuclease S1, and T4 polynucleotide kinase were obtained from BRL or New England Biolabs. RNAse-free DNase I was obtained from Worthington Chemical Co. Radioactive nucleotides were purchased either from New England Nuclear or ICN. The recombinant plasmid pEN 19-1 containing the transcription initiation region from 3% to 16.9% on the rDNA physical map has been described (Niles et al., 1981b) (Figure 1A). The templates used in the transcription studies were obtained as follows: pEN 19-1 was cleaved with *Hha*I, *Hha*I plus *Hin*dIII, or *Hha*I plus *Hae*III. The largest fragment produced by each digestion was separated from the smaller products by chromatography on a 2.5 cm × 45 cm Sepharose 4B column, equilibrated with 0.15 M $NaCH_3COO^-$, pH 4.8, plus 0.1% NaN_3. The purity of each fragment preparation was determined by gel electrophoresis. The 890 base pair *Hha*I/*Hpa*II fragment was prepared by digestion of the large 19-1 *Hha*I/*Hin*dIII fragment with *Hpa*II followed by preparative electrophoresis in a horizontal 1.6% agarose gel. The appropriate DNA fragment was isolated by electroelution (Galibert et al, 1974).

The DNA sequence reported (Niles et al., 1981b) lacks two small *Hinf*I fragments derived from the repeat family II region. The DNA sequence has been redetermined and will be reported elsewhere (Niles, 1984).

Cell Growth. Growth and maintenance of *T. pyriformis* (formerly strain GL) were described previously (Niles, 1977).

Lysate Preparation. Macronuclear lysate (ML) was prepared from macronuclei, isolated as described (Niles & Jain, 1981) and stored at −70 °C. (1.5–2) × 10^8 nuclei were suspended in 800 μL of buffer I (0.2 mM EDTA,[1] 10% glycerol, 5 mM $MgCl_2$, 50 mM Tris-Cl, pH 8.0, 1 mM β-mercaptoethanol) and transferred to a 10-mL polycarbonate centrifuge tube. A total of 200 μL of buffer I containing 1 M $(NH_4)_2SO_4$ was added, and macronuclear lysis was allowed to proceed for 10 min on ice. The macronuclear lysate was sonicated for 2 s at no. 3 setting with a Branson sonifier, Model 185, using the microtip, and a supernatant fraction was prepared by centrifugation in a Beckman 40 rotor at 35K rpm, 4 °C, for 60 min. The supernatant was aliquoted and stored at −70 °C. The lysate loses 50% of its activity over a 2-week period. The total cellular extract (CE) was prepared by a modification of the procedures described by Miller & Sollner-Webb (1981) and Weil et al. (1979). A total of 1500 mL of cells was grown to (1–2) × 10^5/mL in proteose peptone medium and collected by centrifugation at 3K rpm in a Sorvall GSA rotor for 5 min at 4 °C. Cells were resuspended in 200 mL of 120 mM NaCl, 30 mM Tris-HCl, pH 8.0 and 10 mM β-mercaptoethanol and recentrifuged as above. This wash step was repeated 1 time. The pellet was resuspended in 8 volumes of 10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.1 mM EDTA and recentrifuged (10^8 cells equals 2 mL packed cell volume). The cells were finally resuspended in 3 volumes of the same buffer and lysed by using a hand-driven glass homogenizer by 15–20 strokes at 4 °C. Typically 75% of the cells lyse. The volume was measured, and $^1/_5$ volume of 200 mM Tris-HCl, pH 7.9, 17.5 mM $MgCl_2$, 50% glycerol, 0.5 mM EDTA, 50 mM β-mercaptoethanol, and 0.75 M KCl was added. The supernatant fraction was prepared by sedimentation in a Beckman 50.2 rotor at 4 °C, 38K rpm, for 60 min. The supernatant was stored in aliquots at −70 °C and retained its activity for several months. The protein concentration, as determined by Lowry et al. (1951), was 0.7–2.5 mg/mL in ML sample and 2.0–7.0 mg/mL in the CE fraction.

Transcription Assays. Standard assays were carried out at 30 °C in 25 μL final volume. Each assay contained 3 mM $MgCl_2$, 15 mM Tris, pH 8.0, 5 mM NaCl, 25 mM KCl, 10% glycerol, 0.5 mM of each unlabeled nucleoside triphosphate, and 15 μM for the one which was radioactive: [^{3}H]UTP, 3.3 μCi/nmol; [α-^{32}P]ATP or -GTP, 8 μCi/nmol. One unit of RNA polymerase activity is defined as 1 nmol of UTP incorporated per 30-min incubation at 30 °C. Various ML preparations exhibited from 0.05 to 0.314 unit/mL at a specific activity range of 0.08–0.5 unit/mg. The specific activity of

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

the CE preparations was at least 10-fold lower. In later experiments the NaCl was eliminated from the assay mix. Each assay contained between 1 and 5 μL (0.7–2.5 mg/mL) of macronuclear lysate (ML) and 2.5–10 μL (2–7.0 mg/mL) of cytoplasmic extract (CE). KCl and $(NH_4)_2SO_4$ optima were determined by eliminating NaCl from the assay mix and varying the final levels of KCl and $(NH_4)_2SO_4$. ML was 200 mM $(NH_4)_2SO_4$, and CE was 150 mM KCl, respectively. DNA concentration dependencies in the in vitro transcription assay were carried out, and maximum activities were obtained with 3–5 μg of a single DNA fragment or 5 μg of a linearized plasmid template. Four micrograms (3–5 pmol) of DNA fragment and 5 μg (2 pmol) of plasmid DNA were employed per assay. To determine the level of radioactivity incorporated into RNA, aliquots of the reaction mixture were dried onto Whatman 3MM paper squares and the squares washed for 15 min on ice, 4 times in cold 5% trichloroacetic acid–0.1% $Na_4P_2O_7$, followed by one wash in 95% ethanol and one wash in ether. For RNA samples prepared for gel electrophoresis 10 μL of carrier RNA (1 mg/mL) and 10 μL of pancreatic DNase I 25 μg/mL) was added to the reaction mixture, and the mixture was incubated for 5 min at 30 °C. The reaction mixture was diluted with 400 μL of 150 mM $NaCH_3COO^-$, pH 5.5, and 0.05% sodium dodecyl sulfate, and 10 mM EDTA, extracted 2 times with phenol–chloroform, 1:1, and precipitated 2 times with 3 volumes of 95% ethanol. The dried RNA pellet was resuspended in 25 μL of 90% deionized formamide–0.015% xylene cyanol and 0.01% bromophenol blue–10 mM Tris-HCl, pH 8.0. Prior to electrophoresis the RNA was heated at 90 °C for 5 min and cooled to 0 °C before application to the gel.

S1 nuclease mapping experiments were carried out as described previously (Niles et al., 1981a,b). The *Hha*I–*Hin*dIII fragment of pEN 19-1, labeled at the *Hin*dIII cleavage site with [α-^{32}P]ATP by T4 polynucleotide kinase, was used as the S1 nuclease probe. The 35S rRNA prepared from isolated macronuclei (Niles & Jain, 1981) was used as a standard, and a hybridization reaction lacking RNA was used as a control.

Gel Electrophoresis. Agarose gel electrophoresis was previously described (Niles & Jain, 1981; Niles et al., 1981a,b). Analytical separation of [^{32}P]RNA was carried out in 14 × 17 in. by 0.5 mm gels prepared with 4% acrylamide and 7 M urea in 75 mM Tris–borate, pH 8.3, and 1.5 mM EDTA. Electrophoresis was carried out at 200 V for 18 h, and the migration positions were determined by autoradiography of the wet gel covered with Glad Wrap, at −70 °C with the aid of a Du Pont Cronex screen. Preparative separation of the A and B bands was carried out in a 7 × 16 in. × 1.5 mm gel prepared as above. RNA products were identified by autoradiography and removed from the gel by electroelution. Incorporation into RNA products was quantitated either by densitometry of autoradiographs or by cutting out the appropriate region of the gel, incubating the gel slice in 1 mL of H_2O overnight, and counting the radioactivity by a liquid scintillation counting. Nearby regions of the gel channel were also cut out, counted, and subtracted as background.

Results

Crude Transcription System. The selective in vitro transcription system from *T. pyriformis* requires both an S100 fraction from isolated macronuclei (ML) which provides polymerizing activity and an S100 fraction of whole cells (CE) which provides both undefined "specificity factors" and an RNA cleavage activity. Figure 2 (lanes 1–3) shows that transcription employing only the ML fraction yields many RNA products, both large and small, and exhibits little specificity, although at high levels of ML, low amounts of RNA products of the appropriate length can be observed. Transcription using only CE (Figure 2, lanes 4–6) yields few larger RNA products but high levels of incorporation of radioactivity into tRNA-sized molecules. This latter incorporation is much greater when [α-^{32}P]ATP is used as a substrate, is independent of exogenous DNA, and is insensitive to 10 μg/mL actinomycin D. This incorporation is likely to be due to a tRNA CCA terminal transferase modifying enzyme in *T. pyriformis*. When the ML fraction is combined with the CE fraction, substantial synthesis of the expected RNA product can be observed (Figure 2, lanes 7–12).

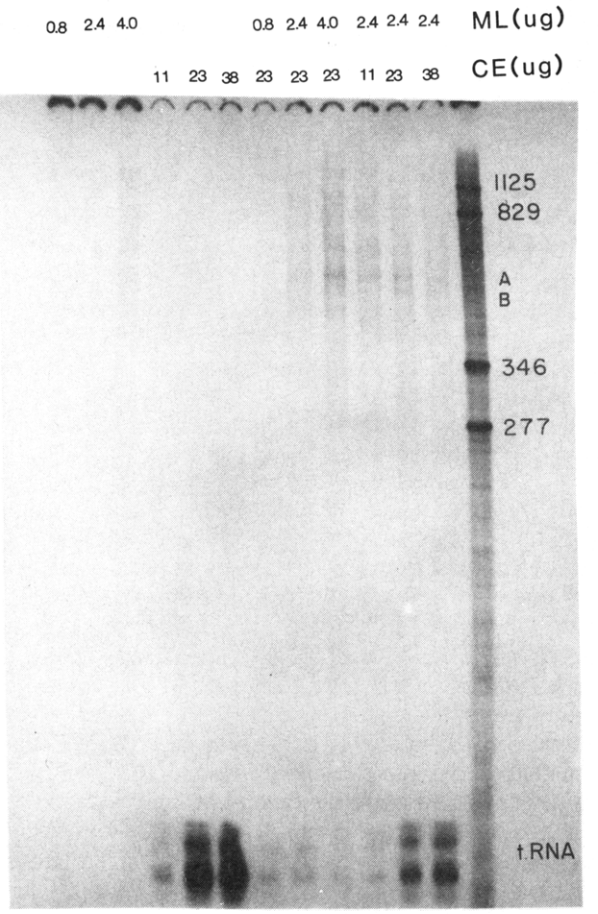

FIGURE 2: ML and CE dependence of in vitro transcription. RNA products synthesized in vitro under a variety of conditions were observed after electrophoresis in a 4% polyacrylamide gel followed by autoradiography. M, 5′-end-labeled single-stranded DNA markers. Reactions were 25 μL in volume and carried out for 30 min at 30 °C. The ML specific activity is 0.5. The *Hha*I/*Hin*dIII template was employed (Figure 1B).

Selective transcription is completely inhibited by 1 μg/mL actinomycin D (Figure 3, lanes 7 and 8), dependent upon exogenous plasmid DNA, linear for 30–60 min, and insensitive to 200 μg/mL amanitin (Figure 3, lane 4). These facts demonstrate that the incorporation is not due to elongation of preexisting RNA chains and is catalyzed by a form I type RNA polymerase. The extent of requirement for CE is somewhat variable, and this variability is likely to be due to the purity of the macronuclei used to produce the ML fraction. The cleanest preparations of macronuclei exhibit the greatest dependence upon added CE, although the activity of all ML preps are enhanced by added CE.

Both the ML and CE S100 fractions were prepared from *T. pyriformis* grown to different points in the expodential growth curve, and their activities were compared. The polymerizing activity in the ML fraction from cells grown to

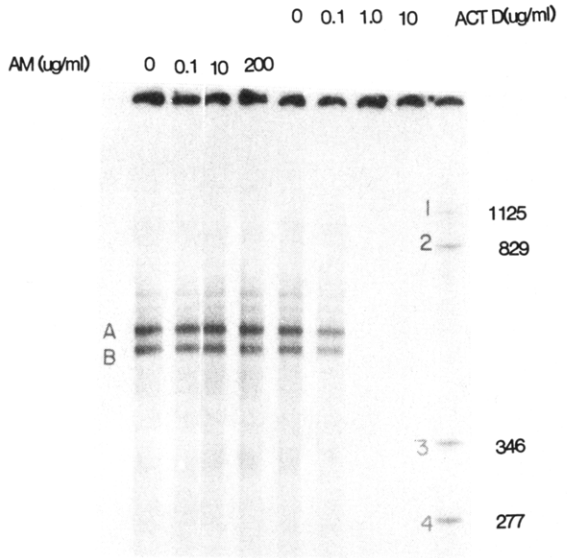

FIGURE 3: Effect of α-amanitin (AM) and actinomycin D (Act D) on in vitro transcription. Each reaction contained the following: ML, 2.4 μg/mreaction; CE, 23 μg/reaction; 43 mM KCl; 3 μg of *Hha*I/*Hind*III template. Markers 1–4 are the same as in Figure 2. ML specific activity is 0.2.

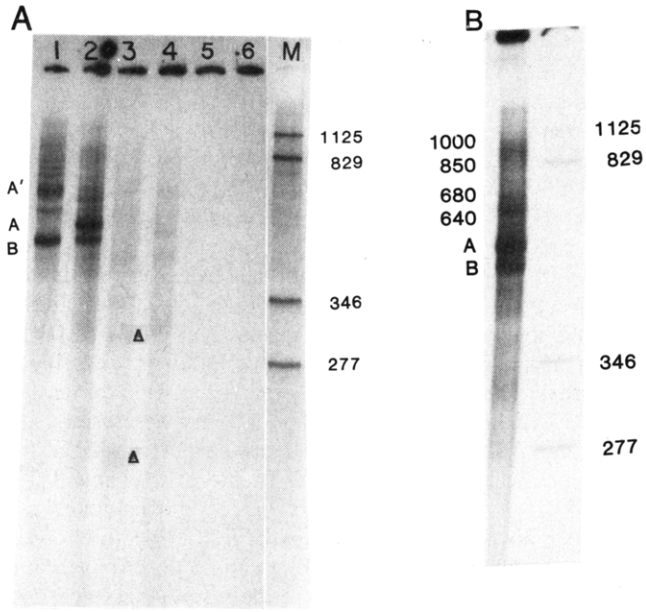

FIGURE 4: Transcription of truncated templates. (A) The products of in vitro transcription of the following templates, *Hha*I/*Hha*I (lane 1), *Hha*I/*Hin*dIII (lane 2), *Hha*I/*Hae*III (lane 3), and *Hha*I/*Hpa*II (lane 4), *Hpa*II/*Hpa*II (lane 5), and *Hpa*II/*Hin*dIII (lane 6), were separated in a 4% acrylamide gel and autoradiographed. Each reaction contained 6.7 μg of ML, 5 μg of CE, and a final level of 43 mM KCl. The marker DNA fragments are the same as in Figure 2. MP specific activity is 0.2. (B) The products of a single, 60-min in vitro transcription reaction, containing 2.0 μg of ML, 5 μg of CE, and 78 mM KCl, is presented in order to show the larger minor RNA products. The marker DNA fragments are the same as in Figure 2. Numbers to the left are approximate molecular weights of selected RNA products. ML specific activity is 0.5. The *Hha*I/*Hin*dIII template was used for this reaction.

between 5×10^4/mL and 2×10^5/mL was 2–3 times greater than cells grown into stationary phase, 5×10^5/mL. ML mixing experiments have shown that the lower activity found in stationary cells is not due to a soluble inhibitor present in the cells but due to less active RNA polymerase. The products produced by all ML reactions were identical when compared by gel electrophoresis. However, the nuclear density is of critical importance in preparing an active ML fraction. Macronuclei must be at least (1–2) $\times 10^8$/mL. All CE fractions prepared from cells grown to different densities exhibited identical in vitro activities. Large-scale preprations of similar CE activity have been prepared by freeze–thaw, detergent (1% NP40), or sonication (Branson, 7 power, macrotip, 15 s) methods of cell breakage.

Transcript Mapping. In the initial experiments, the RNA products of pEN 19-1 linearized at the *Hin*dIII or *Pst*1 cleavage sites were compared to products of pBR 322 linearized at these same cut sites. Substantial levels of transcription of all templates were observed. However, within a background of RNA products, the expected runoff transcripts from pEN 19-1/*Hin*dIII (565 bases) and pEN 19-1/*Pst*1 (1344 bases) templates were identified. In addition, a smaller pEN 19-1-specific RNA product (510 bases) was found which was produced from all conformations of pEN 19-1, linear or circular. In order to remove the background transcription of the pBR 322 portion of pEN 19-1, a single large *Hha*I fragment of pEN 19-1 which contains the entire rDNA insert can be obtained. Truncated versions of this fragment can be produced by cleavage at the *Hin*dIII, *Hae*III, or *Hpa*II cleavage sites (Niles et al., 1981a) (Figure 1B).

In vitro transcription of the promoter containing the *Hha*I/*Hin*dIII fragment yields two major products of about 560 and 510 bases in length, respectively (Figures 2–4A, lane 2). In order to map the origin of these transcripts and to determine their polarity, additional promoter containing DNA fragments were prepared, which possess the same left end and different right ends. The in vitro products generated by transcription of these templates are presented in Figure 4A. If the *Hha*I/*Hha*I template is employed (Figure 4A, lane 1), the RNA A product observed in lane 2 is replaced by a larger species, A′, while the RNA B continues to be synthesized. Since the RNA A is elongated by the addition of 130 base pairs to the right end of the template, this demonstrates that RNA A is a rightward transcript which is produced by initiation in the promoter region followed by runoff. When a *Hha*I/*Hae*III template is employed, both the A and B RNA products are eliminated (Figure 4A, lane 3), demonstrating that the RNA B product is also derived from the right side of the template fragment but its polarity is yet undetermined. The runoff transcript from the *Hha*I/*Hae*III template should be 270 bases in length. There is no prominent transcript of this molecular weight apparent in the gel, but this may be due to either the instability of this runoff transcript in the crude system or to its unusual mobility in the gel. There are two possible candidates for this runoff transcript, labeled with triangles in Figure 4A (lane 3), of about 310 and 190 bases in length, respectively. The right-hand *Hpa*II/*Hin*dIII fragment does not serve as a template in vitro, demonstrating that there are no initiation sites for rightward or leftward transcription to the right of the major start site (Figure 4A, lane 5); therefore, the initiation site for RNA B must be to the left of the rightmost *Hpa*II cleavage site and transcription must be rightward like RNA A. Finally, if the *Hha*I/*Hpa*II template is used, minor RNA products can be observed (Figure 4A, lane 4).

From all templates, minor larger RNA products can be observed (Figure 4A). If the *Hha*I/*Hin*dIII template is used as the standard, they range from approximately 1000 to 580 bases in length (Figure 4B). Although these larger RNAs form a complex collection, the data in Figure 4A indicate that each of these minor RNAs appears to be elongated from left to right, since as the length of the template is reduced, the length of the collection of RNA products is shortened.

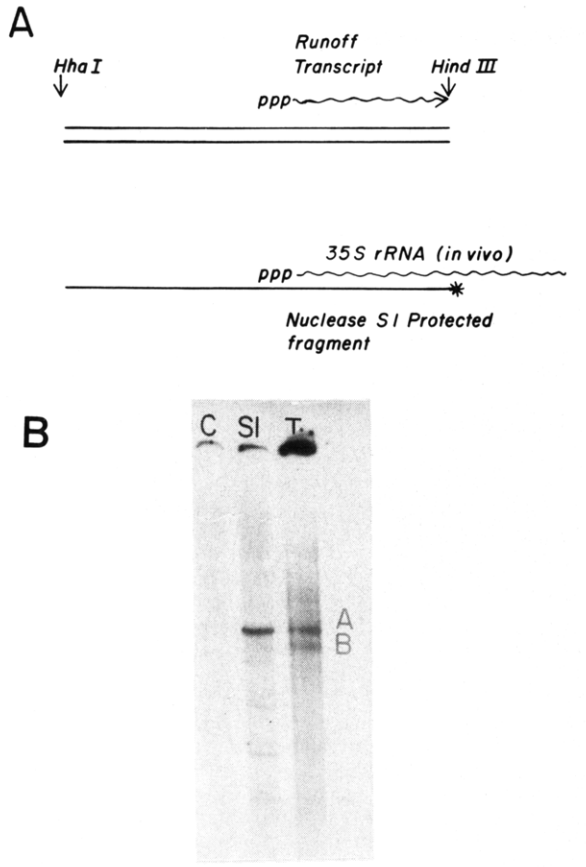

FIGURE 5: S1 nuclease protection experiment. (A) A physical map of the rDNA transcription initiation region in which the size of the RNA product expected from runoff transcription of the *Hha*I/*Hin*dIII template is compared to the size of the rDNA 5′ end labeled at the *Hin*dIII cleavage site which is protected from S1 nuclease digestion by the in vivo 35S rRNA. (B) Gel electrophoretic separation of the following: C, S1 nuclease control lacking added 35S rRNA; S1, DNA fragment protected from S1 nuclease digestion by hybridization to in vivo 35S rRNA; T, in vitro transcripts synthesized in a 30-min reaction which contained 4 μg ofg *Hha*I/*Hin*dIII template, 2.4 μg of ML, and 23 μg of CE.

Leftward transcripts less than 1000 bases in length would be unaffected by templates cleaved at the *Hin*dIII, *Hae*III, or *Hpa*II sites. These observations indicate that several potential transcription initiation sites exist to the left of the major start site.

Identity of RNA A with the 35S rRNA Precursor. It has been shown (Niles, 1978) that the in vivo 35S rRNA is a primary transcript since high yields of pppAp have been isolated from pulse-labeled 35S rRNA. In order to test if either RNA A or RNA B shares a common 5′ terminus with the in vivo 35S rRNA, we compared the size of the in vitro transcripts from the *Hha*I/*Hin*dIII template with the size of the DNA, end labeled at the 5′ end of the *Hin*dIII cut site, which can be protected from digestion with nuclease S1 by hybridization with the in vivo 35S rRNA (Figure 5A). In Figure 5B, it can be seen that the RNA A is identical in size with the DNA fragment protected by the in vivo 35S rRNA, demonstrating that the 5′ end of RNA A is the same as that of the in vivo 35S rRNA. There is no protected DNA fragment that comigrates with RNA B, consistent with the notion that RNA B is not produced by runoff of the *Hin*dIII terminated template and therefore is unable to hybridize to the 5′ end of the template strand and protect it from S1 nuclease digestion.

Salt Dependence of RNA Synthesis. The effects of various salt concentrations on in vitro transcription were observed by

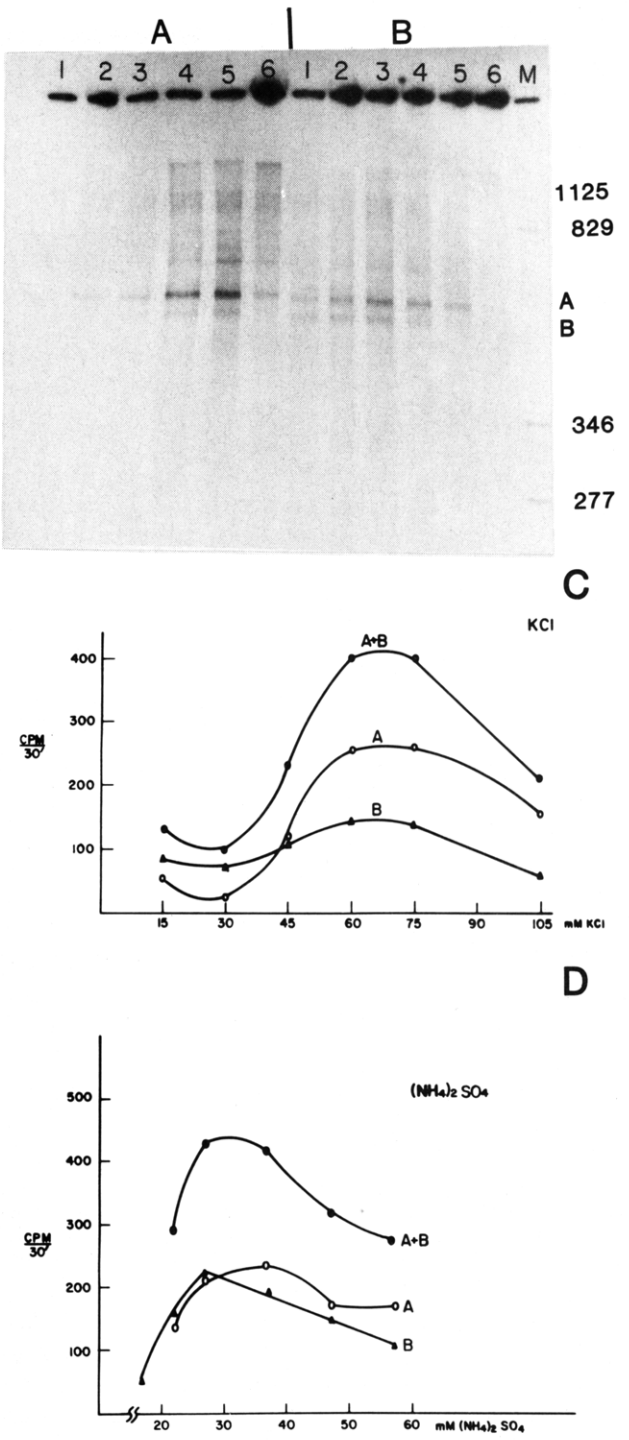

FIGURE 6: Salt dependence of the in vitro transcription reaction. The in vitro reaction contained 2.0 μg/reaction ML and 9.5 μg/reaction CE and was carried out at 30 °C for 30 min. (A) 20 mM $(NH_4)_2SO_4$; KCl levels are 15 (lane 1), 30 (lane 2), 45 (lane 3), 60 (lane 4), 75 (lane 5), and 105 mM (lane 6). (B) 15 mM KCl; $(NH_4)_2SO_4$ levels are 20 (lane 1), 25 (lane 2), 30 (lane 3), 40 (lane 4), 50 (lane 5), and 60 mM (lane 6). Marker DNA fragments are the same as in Figure 2. ML specific activity is 0.2. The *Hha*I/*Hin*dIII fragment was used as the template. Synthesis of RNA A and B at various salt levels was quantitated by excising from a gel the region corresponding to RNA A and B in an autoradiograph and determining the radioactivity by scintillation counting. (C) KCl; (D) $(NH_4)_2SO_4$.

gel electrophoresis of the in vitro RNA products (Figure 6A,B). When KCl concentrations are increased from 15 to 105 mM, the extent of incorporation into RNA A and RNA B increases with a maximum at about 58 mM (Figure 6A,C, lanes 1–6). $(NH_4)_2SO_4$ exhibits an activity maximum at 28

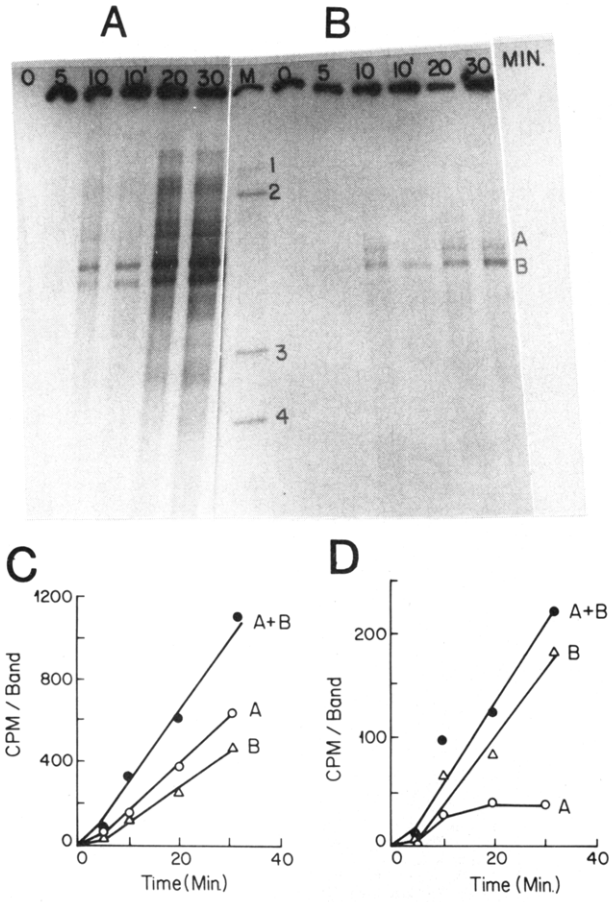

FIGURE 7: Time course of RNA synthesis in vitro. Two reaction mixtures of 150 μL were prepared, and at the times indicated 25-μL aliquots were removed and the RNA products separated by electrophoresis in a 4% acrylamide gel and located by autoradiography. In the samples marked 10′, a 20-fold excess of cold GTP was added to dilute the specific activity, and the incubation was continued for an additional 20 min. (A) The reaction contained 1.6 μg/reaction ML, 9.5 μg/reaction CE, and a final level of 58 mM KCl. (B) The reaction contained 1.6 μg/reaction ML, 29 μg/reaction CE, and a final level of 54 mM KCl. The extent of synthesis of RNA A and B was determined by cutting out the radioactive bands and, after incubating overnight in 1 mL of H_2O, counting the radioactivity. (C) Rate of synthesis of A and B in reaction A. (D) Rate of synthesis of A and B in reaction B. The marker DNA fragments are the same as in Figure 2. ML specific activity is 0.5. The *Hha*I/*Hin*dIII fragment of pEN 19-1 was used as the template.

mM (Figure 6B, lanes 1–6). In addition, two other salt dependent phenomena can be observed. The first, most apparent in Figure 6A, is that as the KCl levels are elevated, the extent of synthesis of the minor larger RNA products observed in Figure 4A,B is raised. The second observation relates to the ratio of RNA A to RNA B produced. As the salt concentration is increased, the ratio of A to B is also increased (Figure 6C,D), e.g., from 0.3 at 30 mM KCl to 1.8 at 60 mM KCl.

Relationship of RNA A and B. We have determined that RNA A is a rightward transcript that has a left end at about 560 bases to the left of the *Hin*dIII cleavage site. Furthermore, we have shown that RNA B is also derived from the right-hand end of the template but not produced by runoff. How are RNA A and B related? Is RNA B produced by cleavage of RNA A or by an independent transcription termination event? This question was examined in several ways. The rate of RNA A and B synthesis was measured at the optimal KCl concentration and both low and high CE levels (Figure 7A,B). In Figure 7A,C, it can be seen that the rate of synthesis of both RNA A and RNA B is linear with time for at least 30 min, suggesting that RNA A and B synthesis is independent.

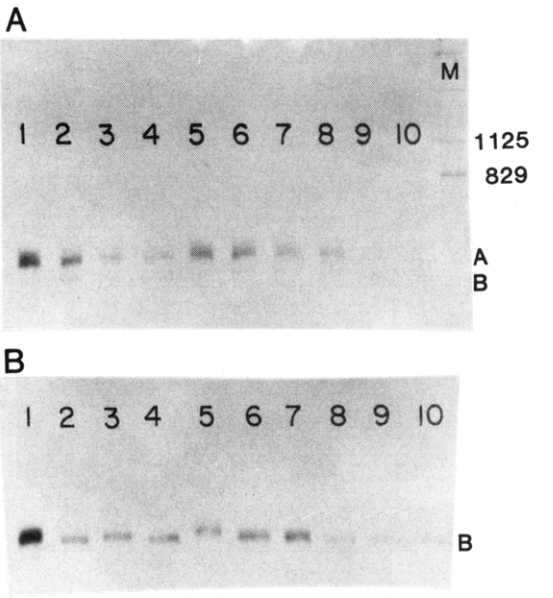

FIGURE 8: Conversion of RNA A into RNA B. [^{32}P]RNA A and [^{32}P]RNA B from a 1-mL reaction were isolated by preparative gel electrophoresis, and following reincubation in reaction mixtures prepared in a variety of ways, the RNAs were reisolated and separated by electrophoresis in a 4% polyacrylamide gel. (A) RNA A; (B) RNA B. Lane 1, no reincubation; lanes 2–4, 27 μg/reaction ML, 20 μg/reaction CE, and 18 mM KCl at times of 10, 20, and 30 min; lanes 5–7, 27 μg/reaction ML, 20 μg/reaction CE, and 78 mM KCl at times of 10, 20, and 30 min; lanes 8–10, 27 μg/reaction ML, 106 μg/reaction CE, and 72 mM KCl at times of 10, 20, and 30 min. Reaction mixtures of 100 μL were prepared, and 33-μL aliquots were removed at the times indicated. Marker DNA fragments are the same as in Figure 2. ML specific activity is 0.5.

At higher CE levels, however (Figure 7B,D), the production of RNA A plateaus after 20 min while the synthesis of RNA B continues for at least 30 min. Under these latter conditions RNA B is relatively stable while RNA A is unstable, which suggests that RNA A may be a precursor to RNA B. This inference was first tested by lowering the specific activity of the [α-^{32}P]GTP 20 times, by adding cold GTP, 10 min after the transcription assay was begun, and by continuing the incubation for an additional 20 min. In Figure 7A (in the lane marked 10′), at lower CE levels, it can be seen that the ratio of RNA A and B is lowered slightly during the low specific activity incubation. In Figure 7B, however, it can be seen that the ratio of A to B is lowered substantially, which supports the contention that RNA A is unstable in a high CE incubation and consistent with the conversion of RNA A to RNA B. Finally, in order to resolve this question, the stability of RNA A and B was measured directly by reincubation of isolated RNA A and RNA B with an in vitro transcription mixture lacking template DNA and radioactive substrates. It can be seen in Figure 8A (lanes 2–4) that RNA A can be converted into RNA B in a time-dependent way. Thus, the rate of conversion is substantially inhibited by 78 mM KCl (Figure 8A, lanes 5–7) and is increased at 78 mM KCl by elevating the CE level (Figure 8A, lanes 8–10). This KCl inhibition of the RNA A to RNA B conversion explains the increase in the RNA A/RNA B ratio at higher KCl levels observed in Figure 6. The increased rate of the RNA A to B conversion observed in Figure 8A at high CE levels accounts for the low RNA A/RNA B ratio and instability of RNA A observed in Figure 7B,D. Although there is significant loss of both RNA A and RNA B during reincubation in the transcription mixtures, RNA B appears to be relatively stable under all conditions (Figure 8B). These data demonstrate that

RNA B is produced by cleavage of RNA A, that, from the size of the transcripts (560 vs. 510 bases), the cleavage must occur at a site about 50 nucleotides to the left of the *Hin*dIII cleavage site, that this cleavage activity is highly salt dependent, being inhibited by 78 mM KCl, and finally that the cleavage activity is enhanced at high CE levels.

Discussion

We have developed a crude in vitro transcription system from *T. pyriformis* that selectively transcribes cloned fragments of the rRNA gene which contain the promoter region (Figures 2 and 3). Selectivity has been determined by identification of a specific runoff product from a series of truncated templates (Figure 4). The major product RNA A was shown to possess the identical 5′ region as the in vitro 35S rRNA, by S1 nuclease mapping (Figure 5).

In other RNA polymerase *Pol*I transcription systems (Miller & Sollner-Webb, 1981; Grummt, 1981; Mishima et al., 1981; Grummt et al., 1981; Kohorn & Rae, 1982) a crude cellular lystate is sufficient to support selective transcription. In our hands, the S100 extract from whole *T. pyriformis* is inactive and requires the addition of the S100 fraction from isolated macronuclei (Figure 2). Transcription is dependent upon exogenous template DNA, is linear for 30–60 min, is inhibited by 1 μg/mL actinomycin D, and is insensitive to 200 μg/mL α-amanitin (Figure 3). This demonstrates that transcription is catalyzed by the form I RNA polymerase and involves initiation on the DNA template and not merely elongation of preexisting RNA chains.

The rate of transcription is dependent upon the level of monovalent and divalent cations (Figure 6) exhibiting maxima for KCl, $(NH_4)_2SO_4$, and $MgCl_2$ of 58, 28, and 3 mM, respectively. At low salt levels two RNA products predominate, RNA A and B, when the standard *Hha*I/*Hin*dIII template is employed. As the salt level is raised, two phenomena can be observed. One is the appearance of the series of minor RNA products larger than the major A and B RNA species. Since the sizes of these minor species decrease when the truncated templates are employed, they are likely to be the product of rightward transcription in which initiation occurs to the left of the major RNA A start site. From the molecular weights of these RNA products, their initiation sites can be mapped to the regions of the families of tandemly repeated sequences found upstream from the major start site (Niles et al., 1981a,b). A second observation which can be made is that the ratio of the two major RNA products, A/B, increases at elevated salt levels.

In addition to the major RNA A product, a second RNA species B is produced. The synthesis of RNA B is not generated by transcription runoff of the *Hha*/*Hin*dIII template; therefore, its 3′ end must be to the left of *Hin*dIII cleavage site. Reincubation of isolated RNA A in a transcription mixture results in the production of RNA B. The extent of conversion of RNA A into B is dependent upon time, increased by raising cytoplasmic extract levels, and inhibited by elevated levels of KCl. RNA B must be generated from RNA A by nucleolytic cleavage of RNA A by one or more ribonuclease activities. From the molecular weight of the RNA B product we can localize the cleavage site on the DNA map at about 50 base pairs to the left of the *Hin*dIII cleavage site. Under these reincubation conditions, RNA B is relatively stable; however, minor stable digestion products can be observed upon long exposure of the film. There is no evidence to suggest that this nuclease activity can be found in vivo (Niles, 1978; Kister et al., 1983) and at this time must be presumed to be an in vitro artifact.

An interesting oddity can be observed between 43 and 58 mM KCl (Figure 7C). The rate of transcription of RNA A is balanced by the rate of cleavage of RNA A into B. This coincidence yields data that were initially interpreted in this lab as indicating that the syntheses of A and B are independent and that RNA B is produced by termination of transcription at a site about 50 base pairs to the left of the *Hin*dIII site. This interpretation was shown to be incorrect by the data in Figures 6–8 in which the salt and CE concentration dependence of the conversion of RNA A into RNA B was documented.

Since a portion of the in vitro transcripts is being cleaved after runoff, the minor larger RNA products observed at higher salt must actually be in pairs in which one member is a runoff product and the other is a cleavage product (Figure 4B). Since there are so many of these minor RNA products produced in low yield, it is impossible at this point to determine which are cleaved and which are not. As a result we cannot identify the location of the 5′ ends of these RNA products precisely on the DNA physical map.

In other RNA polymerase *Pol*I transcription systems studied to date, only a single major RNA product resulting from initiation at a single start site is observed (Miller & Sollner-Webb, 1981; Grummt, 1981; Mishima et al., 1981; Grummt et al., 1981; Kohorn & Rae, 1982). Since each of these studies was carried out at 75–100 mM KCl, one would expect to have observed the production of larger, minor products such as we see at high salt in this system, if they exist in the other systems. Therefore, these multiple initiations to the left of the major start site appear to be unique to this system.

Is the production of these minor RNA species significant in vivo? We have not observed 35S rRNA products which possess 5′ termini corresponding to these in vitro products (Niles et al., 1981a,b). However, since they are minor products and heterogeneous in size, their presence might have been missed. Since the rate of rRNA synthesis varies over a wide range, in vivo, it makes biological sense to have auxilliary start sites upstream from the major initiation point, which can be used in times of high rates of ribosome biosynthesis. The *T. pyiformis* rRNA transcription initiation region possesses several families of AT-rich repeat sequences upstream from the major start site (Niles et al., 1981a,b). One function of these upstream repeat families may be to direct transcription under growth conditions which require more rRNA than can be provided by the major start site. Alternatively, the synthesis of these larger transcripts may simply be an in vitro artifact. The repeat families that apparently serve as the initiation sites for these transcripts are highly A-T rich (Niles et al., 1981b; Niles, 1984), and these transcripts may be the result of nonselective initiation at these A-T-rich blocks. There are, however, A-T rich segments both to the left of the longest minor start point and to the right of the major start point which are not utilized in vitro.

In vivo these repeat families may be bound to regulatory factors which modulate transcription. It has recently been observed (Niles, 1984) that the repeat family II region of the gene is a 10-member set of sequences capable of binding the *Escherichia coli* catabolite regulatory protein in vitro. It is tempting to speculate that, in *Tetrahymena*, an analogous factor modulates transcription through binding to the repeat family II sequences, perhaps responsive to cellular cylic AMP levels. Support for this speculation can be found in the *Xenopus laevis* system in which multiple 80/61 base pair repeats lie upstream from the transcription start site (Bosley et al., 1979). Recent experiments demonstrate a requirement for

these repeat sequences to achieve high levels of transcription in injected oocytes (Moss, 1983; Busby & Reeder, 1983; Sollner-Webb et al., 1983; Reeder et al., 1983). The favored interpretation of these data suggests that a soluble factor regulates transcription initiation by binding to these repeat sequences.

The role of the upstream repeated sequences in the regulation of the expression of the *Tetrahymena* rRNA gene will be deduced by analyzing the effects of in vitro generated mutations on transcription in vitro.

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

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