Faithful Initiation of Ribosomal RNA Transcription from Cloned DNA by Purified RNA Polymerase I†

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ABSTRACT: A faithful transcription system for ribosomal RNA genes has been developed by using components from the small free-living amoeba *Acanthamoeba castellanii*. The system utilizes protein-free recombinant DNA as a template and in addition requires a crude cell-free extract containing RNA polymerase I and a transcription initiation factor (TIF-I). The transcript is initiated at the same position as the in vivo precursor ribosomal RNA: templates truncated at various sites downstream of the transcription start site give rise to only the predicted runoff RNA transcripts, and the runoff transcript produced has a 5′-terminus identical with the 5′-terminus of the isolated ribosomal RNA precursor. Faithful initiation can be elicited by the DNA sequence extending from −55 to +19 in the template. Subclones containing this sequence yield only the predicted runoff RNAs regardless of the orientation of this fragment in the cloning vector DNA; thus, only the in vivo sense strand of the template is specifically transcribed in the in vitro system. The system is specific for the RNA polymerase responsible for the transcription of ribosomal RNA genes in vivo. Faithful transcription, like RNA polymerase I from *Acanthamoeba*, is insensitive to α-amanitin inhibition, and transcription is greatly stimulated by highly purified RNA polymerase I but not by RNA polymerases II or III. Conditions for optimal transcription were determined.

In eukaryotic cells, transcription is carried out by three DNA-dependent RNA polymerases. Each is responsible for the transcription of a different set of cellular genes [reviewed in Roeder (1976), Chambon (1974, 1975), and Lewis & Burgess (1982)]: RNA polymerase I synthesizes ribosomal RNA precursor, RNA polymerase II transcribes heterogeneous nuclear RNA, and RNA polymerase III makes pretransfer RNA, 5S RNA, and some other cellular RNAs (Schultz, 1978). Though very few complete sets of all three polymerases have been purified and carefully characterized, studies of yeast, *Acanthamoeba*, and plant enzymes have shown that each polymerase consists of a complex set of polypeptides; a "core" of common subunits associated with a number of polypeptides unique to the particular polymerase class [D'Alessio et al., 1979; reviewed in Paule (1981)]. This finding has recently been extended to higher animal cells (Engelke et al., 1983).

A large number of studies have shown that despite their complexity of structure, the purified RNA polymerase(s) show(s) no transcriptional specificity when presented with deproteinzed DNAs. A third element is necessary. RNA polymerase III mediated transcription was the first to yield to a search for this component: Roeder and his colleagues (Parker, 1976, 1977) showed that when natural chromatin templates were used, 5S RNA, tRNA, and adenovirus virus-associated (VA) RNA genes could be transcribed by purified RNA polymerase III. Using similar chromatin templates, Rutter and colleagues (Tekamp et al., 1979) found selective RNA polymerase I transcription of rRNA. Apparently the additional components were associated with the chromatin template preparations. These preparations were not readily amenable to fractionation, however, The breakthrough came when Wu (1978) developed the first soluble cell-free preparation which utilized endogenous RNA polymerase III plus protein factors to transcribe VA RNA from deproteinized adenovirus DNA. This was soon followed by the development of similar soluble systems capable of accurately transcribing 5S RNA and tRNA genes from protein-free, cloned DNAs (Birkenmeyer et al., 1978; Schmidt et al., 1978; Ng et al., 1979). Subsequently, Weil et al. (1979) and Manley et al. (1980) developed cell-free systems for RNA polymerase II transcribed genes. These methods have been extended to the selective transcription of a number of different class II and III genes to date, and the extracts have been shown to be functional with exogenous RNA polymerase(s).

Development of RNA polymerase I systems has lagged behind the other two enzyme classes. This is in part due to the difficulty in defining the exact transcription start site for the large and rapidly processed preribosomal RNA transcription units. In addition, we have recently shown than, in contrast to class II and III transcription systems, transcription of ribosomal RNA genes is much more species specific; only homologous assemblies of RNA polymerase I, DNA, and cell-free extracts result in efficient specific initiation (Grummt et al., 1982).

Despite these difficulties, transcriptional systems for ribosomal RNA genes were developed from mouse (Grummt, 1981; Miller & Sollner-Webb, 1981), from human cells (Learned & Tjian, 1982), from *Drosophila* (Kohorn & Rae, 1982), from *Xenopus* (Reeder et al., 1983), and from rat (Mishima et al., 1982). We report here the development of a sixth system from the protozoan *Acanthamoeba castellanii* which selectively and accurately initiates rRNA transcription from deproteinized DNA. The system requires, in addition to the cloned rDNA fragments and purified RNA polymerase I, a cell-free extract containing transcription factor(s) similar to that (those) found for the class II and III enzymes.

Materials and Methods

Growth of Cells. A. castellanii (Neff strain) were grown in the yeast extract–proteose peptone–glucose medium of Neff as previously described (Detke & Paule, 1975).

Preparation of S100 Extracts. The S100 extracts were prepared from mid- to late-log-phase *Acanthamoeba* cells as described by Weil et al. (1979). Extracts were frozen and stored in liquid nitrogen for up to 5 months without noticeable loss of activity.

† From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523. *Received January 5, 1984.* This work was supported by Grants GM 22580 and GM 26059 from the U.S. Public Health Service to M.R.P. and by Grants ATP 006520/50 from the Centre National de la Recherche Scientifique and PRC Genie Genetique 124026 from the Institut National de la Sante et de la Recherche Medicale to P. Chambon. M.R.P. was supported in part by Senior International Fellowship TW 00524 from the Fogarty International Center of the National Institutes of Health.

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Cell-Free Transcription System. The standard reaction mixture contained in 25 μL 600 μM each of ATP, GTP, and UTP, 25 μM CTP, 5 μCi of [α-^{32}P]CTP (specific activity 3000 Ci/mmol), 150 mM KCl, 8 mM $MgCl_2$, 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.9), 10% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol, and 0.5 μg of DNA. The reactions were started by the addition of 2–8 μL of S100 and 30–100 milliunits of heparin–Sepharose-purified RNA polymerase I (Spindler et al., 1978a). Incubation was for 20 min at 25 °C. The reaction was terminated by the addition of 475 μL of 50 mM ammonium acetate, 0.5% sodium dodecyl sulfate, and 50 μg/mL *Escherichia coli* tRNA. Samples were extracted once with phenol and once with chloroform–isoamyl alcohol (100:1 v/v). To each aqueous phase were added 15 μL of 5 M ammonium acetate and 1 mL of ethanol. The RNAs were precipitated at −70 °C for 30 min and centrifuged. Lyophilized RNA pellets were dissolved in 10 μL of 80% formamide and 0.5× Tris–borate–EDTA sample buffer (1× = 0.10 M Tris, 0.12 M boric acid, and 2 mM ethylenediaminetetraacetic acid) and heated at 65 °C for 7 min. RNAs were separated by electrophoresis on 31 cm × 31 cm × 0.4 mm 5% T (5% C) polyacrylamide gels in the presence of 8 M urea. Autoradiography was carried out for 3–24 h at −70 °C using Du Pont Lightning-Plus intensification screens and Kodak AR X-ray film.

S1 Nuclease Protection Experiments. S1 nuclease protection experiments were carried out as described previously (Berk & Sharp, 1977).

Results and Discussion

DNA Template. A complete tandem repeat unit of *Acanthamoeba castellanii* ribosomal DNA was cloned as a recombinant in λ Charon 9 and characterized (D'Alessio et al., 1981). A 2.3 kilobase pair (kbp) fragment of the rDNA repeat containing the 5′-end of the 18S RNA coding region and the transcription start site (which is approximately 1.6 kbp upstream of the 18S RNA coding region) was subcloned into the *Hin*dIII–*Eco*RI sites of pBR322 to produce the recombinant plasmid pAr4 (Figure 1A). Subsequent mapping experiments using the 39S pre-rRNA from *Acanthamoeba* have shown that the in vivo transcription start site is located 495 base pairs (bp) upstream of an *Hha*I site in pAr4 (Figure 1B) (Perna, 1981; M. R. Paule et al., unpublished results). A 915 bp DNA fragment of pAr4 (pAr4/*Hha*I, extending from −420 to +495) encompasses the transcription start site (Figure 1B). This DNA fragment was isolated and used in most of the in vitro transcription experiments described below.

Runoff Assay. Specific initiation of transcription was examined by using an RNA runoff assay. In this assay, the DNA template is cut with a restriction endonuclease at a known distance downstream of the transcription initiation site. When initiation occurs correctly, an RNA product whose length is equal to the distance between the initiating base (+1) and the restriction enzyme site is produced, 495 bases in the pAr4/*Hha*I case. After transcription in the presence of labeled precursors, the RNA products are isolated and analyzed by polyacrylamide gel electrophoresis and autoradiography.

Components in Addition to DNA and RNA Polymerase I Are Needed for Correct in Vitro Initiation. Transcription of pAr4/*Hha*I by heparin–Sepharose-purified RNA polymerase I results in very little transcription (Figure 2A, lane 4). We have found previously that purified polymerase I from *Acanthamoeba* is very inefficient at transcribing double-stranded templates which do not contain nicks or single-stranded gaps. In some experiments, a single RNA is transcribed from the DNA template (not shown). This labeled band is always the same length as the template DNA (915 bases in the pAr4/*Hha*I case) and results from preferential initiation by the polymerase at the ends of the DNA followed by "end to end" transcription. No RNA representing faithful initiation at the correct in vivo start site for the rRNA transcription unit is seen, however, with the highly purified RNA polymerase I. When a crude, postnuclear supernatant (S100) of *A. castellanii* cells is added, there is a dramatic change in the pattern of labeled products (Figure 2A, lane 2). The major transcribed product is 495 bases in length (arrow) which corresponds to the length predicted for an RNA runoff from the in vivo transcription initiation site. In addition, a significant amount of label is found in the region corresponding to 4S and 5S RNA (which is run off the lower end of the standard gels used in the studies reported here). This appears to result from CCA addition to the termini of endogenous transfer RNAs and perhaps from other reactions, since upon removal of nucleic acids from the S100 during purification of the transcription components, these labeled bands are no longer observed. That the labeled products are indeed RNA was demonstrated by digesting them with pancreatic ribonuclease (Figure 2A, lane 7). All of the products are digested by the enzyme. Further, all of the bands were stable to pancreatic DNase treatment (Figure 2A, lane 8). There is increased background in lane 8 since this experiment is carried out by the addition of DNase to the incubation mixture followed by an additional 10-min incubation period. The DNase nicks the DNA template and allows increased nonspecific initiation to occur during the extended incubation. The products are not synthesized when actinomycin D is included in the incubation mixture (Figure 2A, lane 6) or when the pAr4/*Hha*I DNA template is omitted (Figure 2A, lane 3). The labeled products are, therefore, RNA and arise from DNA-dependent transcription, not by end labeling of preexisting RNA or DNA molecules. Further, these experiments show that the runoff RNA is a transcript of the input rDNA and does not arise because of transcription of a contaminating DNA from the S100.

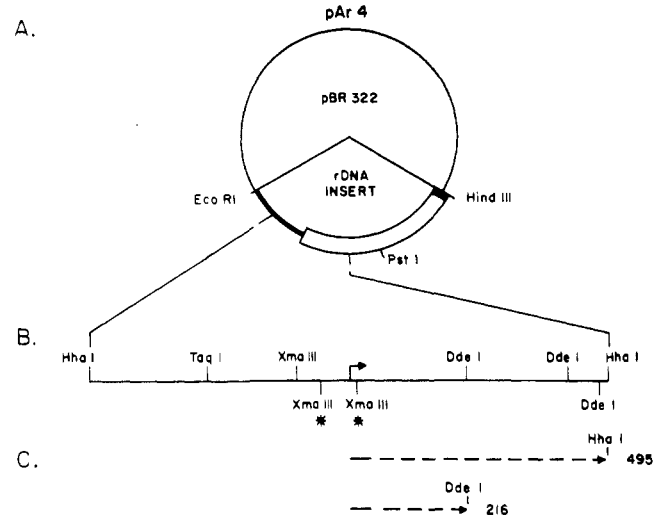

FIGURE 1: Structure of the recombinant DNA plasmid pAr4 and runoff transcripts. (A) pAr4 showing the pBR322 plasmid, the rDNA nontranscribed spacer region (heavy line), the external transcribed spacer (open box), and part of the 18S RNA coding region (filled box). (B) Detail of the *Hha*I-bounded region containing the transcription initiation site (bent arrow). The two *Xma*III sites indicated with asterisks are those which bound the fragment subcloned into pBR322 (see text). (C) Map and lengths of the runoff transcripts resulting from transcription of DNA fragments terminating at the restriction endonuclease sites indicated.

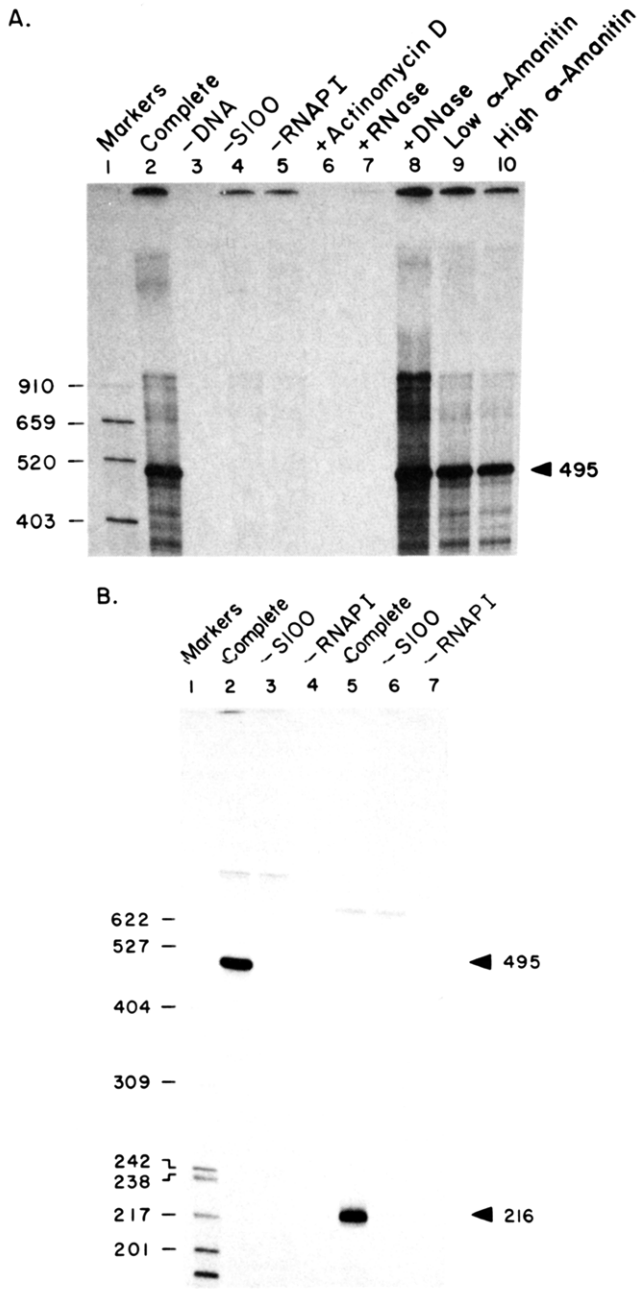

FIGURE 2: In vitro transcription of pAr4 DNA fragments under a variety of conditions. (A) pAr4/*Hha*I (−420 to +495) was transcribed and analyzed as described under Materials and Methods unless otherwise indicated. (Lane 1) pBR322/*Alu*I markers; (lane 2) complete; (lane 3) complete minus DNA; (lane 4) complete minus S100; (lane 5) complete minus exogenous RNA polymerase I; (lane 6) complete plus 40 μg/mL actinomycin D; (lane 7) complete, treated with 40 μg/mL ribonuclease A for 5 min following incubation; (lane 8) complete, treated with 4 μg/mL deoxyribonuclease I for 10 min following incubation; (lane 9) complete plus 1 μg/mL α-amanitin; (lane 10) complete plus 600 μg/mL α-amanitin. (B) pAr4/*Hha*I (lanes 2–4) or pAr4/*Hha*I cut with *Dde*I (−420 to +216) (lanes 5–7) was transcribed and analyzed as described under Materials and Methods unless otherwise indicated. (Lane 1) pBR322/*Msp*I markers; (lane 2) pAr4/*Hha*I complete; (lane 3) pAr4/*Hha*I complete minus S100; (lane 4) pAr4/*Hha*I complete minus RNA polymerase I; (lane 5) pAr4/*Hha*I cut with *Dde*I complete; (lane 6) pAr4/*Hha*I cut with *Dde*I complete minus S100; (lane 7) pAr4/*Hha*I cut with *Dde*I complete minus RNA polymerase I.

A Different Length Runoff RNA Resulted When the Template Was Truncated. Adventitious initiation and termination at physiologically nonspecific sequences in the DNA could give rise to RNA products equal in length to the predicted runoff RNA. To test this possibility, the pAr4/*Hha*I DNA fragment was digested with *Dde*I and utilized as an in vitro template. Transcription of this shortened template (−420 to +216) yields a major RNA runoff of 216 bases, exactly as predicted (Figure 2B, lane 5). The 495-base runoff RNA obtained from transcription of the pAr4/*Hha*I DNA template disappears. (Transcription of this template plus controls is shown in Figure 2B, lanes 2–4.) As above, omission of the S100 (lane 6) or of exogenous RNA polymerase I (lane 7) results in disappearance of the 216-base runoff RNA. Similar results were obtained when pAr4 was cut with *Pst*I, yielding a 920-base runoff (data not shown).

A Small Segment of pAr4 Promotes Specific Transcription When Subcloned into a Plasmid Vector. Further evidence that specific transcription occurs in this system was obtained by subcloning a 74 bp *Xma*III fragment surrounding the transcription start site (from −55 to +19; see *Xma*III sites marked with asterisks in Figure 1B) into the single *Xma*III site (at 938) of pBR322. Two recombinant plasmids that contain this 74 bp fragment inserted in opposite orientations were obtained, as determined first by restriction enzyme mapping and then by primary sequencing. In pSBX60, the insert is oriented in such a way that faithful transcription should occur in a clockwise direction around pBR322 (Sutcliff, 1979). pSBX60i contains an inverted insert, and transcription, therefore, is predicted to proceed counterclockwise. These subclones contain unique restriction enzyme sites allowing the production of a number of runoff templates which were not available in pAr4.

Seven different runoff RNAs ranging in length from 302 to 1143 nucleotides were obtained (data not shown, for examples, see Figure 3, lanes 2 and 4). To distinguish between faithful initiation on the sense strand and processing of nonspecific end to end transcripts, a number of DNA fragments were isolated from these clones and transcribed in vitro (Figure 3).

Only the correct in vivo template strand is specifically transcribed, and no processing of end to end transcripts can be demonstrated. We reasoned that if transcription were correctly initiated and elongated along the appropriate strand of isolated fragments of the template, an RNA transcript size would be predictable from each fragment (see Figure 3A). If the putative start site were an RNA-processing site and the apparent runoff RNA actually resulted from endonucleolytic cleavage of an end to end transcript, at least two discrete RNA products should be observed (Figure 3A). (It is unlikely that more than one specific processing site would be contained within the end to end products of these DNA fragments since most of the sequence derives from the prokaryotic vector pBR322.) To test this hypothesis, restriction fragments which contain the insert were isolated from these plasmids and were used in the runoff assay. The fragments which were used are diagrammed in Figure 3A; the result of each transcription experiment is shown in Figure 3B. The experimental results clearly show, in all cases, that transcription initiates at the start site and proceeds only along the strand corresponding to the in vivo sense strand. As an example, from the known orientation of the 74 bp insert in pSBX60, a 1117 bp *Bam*HI–*Ava*I fragment is expected to yield a 500-base runoff if the proper site and strand are transcribed. Processing of an end to end transcript would yield RNAs of 500 and 617 bases. The 500-base band in lane 5 of Figure 3B and the lack of a 617-base RNA clearly indicate that transcription in this system initiates specifically and continues along the correct strand. Further, the data show that the RNAs found do not arise from processing of an end to end RNA. The same restriction

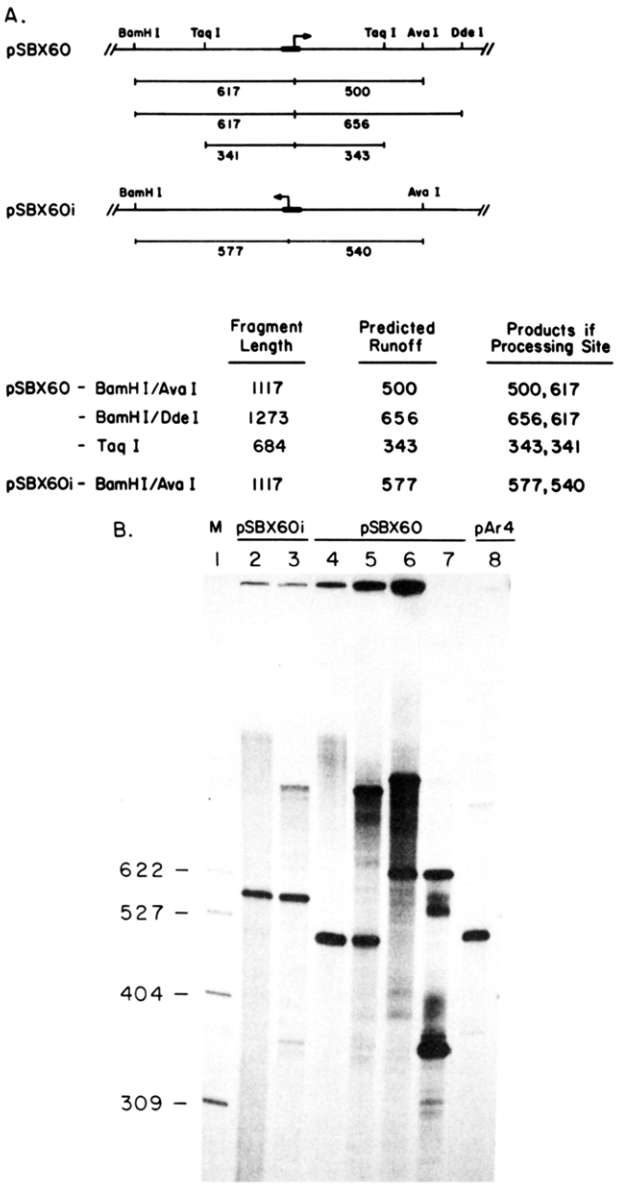

FIGURE 3: Structure, predicted runoff RNAs, and transcription products arising from transcription of isolated fragments of the constructs pSBX60 and pSBX60i (see text). (A) Fragment runoffs. The structure of the regions of pSBX60 (top) or pSBX60i (middle) containing the transcription start site (bent arrow) within the inserted *Xma*III fragment (heavy line) and its surrounding vector sequences (thin lines) are shown. Below each map are shown the DNA fragments which were isolated and used as templates. On each is marked the length of the predicted runoff product and the product to be expected from processing of an end to end transcript. The information for each fragment is tabulated in the lowest panel of (A). (B) Transcription of the templates listed below was carried out and analyzed as described under Materials and Methods. (Lane 1) pBR322/*Msp*I markers; (lane 2) pSBX60i linearized with *Bam*HI; (lane 3) pSBX60i *Bam*HI–*Ava*I fragment; (lane 4) pSBX60 linearized with *Ava*I; (lane 5) pSBX60 *Bam*HI–*Ava*I fragment; (lane 6) pSBX60 *Bam*HI–*Dde*I fragment; (lane 7) pSBX60 *Taq*I–*Taq*I fragment; (lane 8) pAr4/*Hha*I fragment for comparison.

fragment of pBR322 (lacking any insert) failed to yield a specific runoff transcript (data not shown).

In summary, one major RNA runoff is obtained (in addition to an end to end product when DNA fragments are transcribed), and it is always the one predicted by the insert orientation previously determined by restriction enzyme mapping and primary sequencing. The same runoff is not produced from the equivalent pBR322 fragment. Therefore, specific transcription initiation occurs in this system, and the

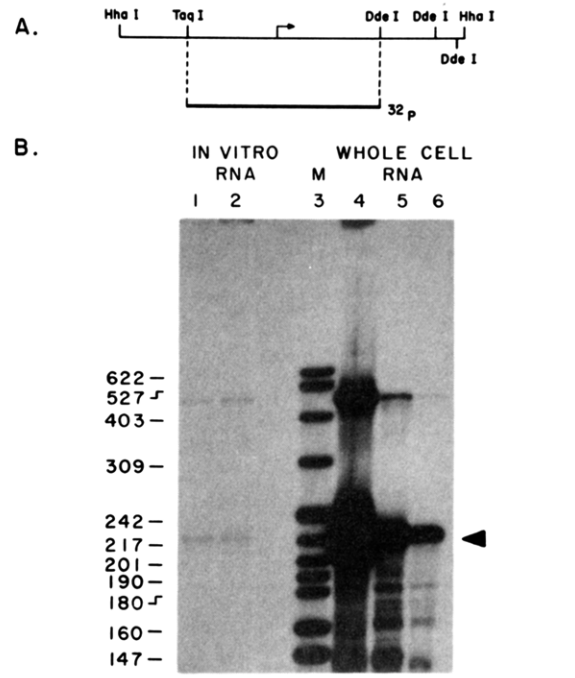

FIGURE 4: S1 nuclease mapping of in vitro and in vivo RNAs. (A) Map of the region of pAr4 containing the transcription start site (bent arrow) and showing the *Taq*I and *Dde*I sites used to produce the 5′-end-labeled probe used in the S1 nuclease mapping experiment. (B) (Lanes 4–6) RNA extracted from whole *Acanthamoeba* cells or (lanes 1–2) from 125-μL in vitro transcription reaction mixtures (unlabeled; pAr4/*Hha*I DNA template) was hybridized to pAr4/*Taq*I–*Dde*I DNA (−238 to +216) 5′-^{32}P-labeled at the *Dde*I site, treated with increasing amounts of S1 nuclease, and analyzed by polyacrylamide gel electrophoresis and autoradiography. (Lane 3) Markers whose lengths are shown in the left margin. The arrowhead in the right margin indicates the position of the protected fragment.

74 bp surrounding the start site are necessary and sufficient to direct faithful initiation in vitro. Further, the above results argue against the runoff RNA arising from processing of an end to end transcript since the other half of the end to end RNA is never observed. A similar argument can be made for the pAr4/*Hha*I template since no 420-base product is obtained (Figure 2A).

Cellular Pre-rRNA and the in Vitro Transcribed RNA Initiate at the Same Site. To further test that the sites of in vitro and in vivo initiation are identical, the 5′-ends of the in vivo and in vitro RNA products were localized on the DNA template by the single-strand nuclease method of Berk & Sharp (1977). Whole-cell RNA (in vast excess) and RNA transcribed in vitro from pAr4/*Hha*I DNA (−420 to +495) were hybridized to a *Taq*I–*Dde*I DNA fragment (−240 to +216) which had previously been ^{32}P-end-labeled at the *Dde*I site (see Figure 4). Following digestion of the unpaired regions of the resulting hybrid with S1 nuclease, the length of the protected DNA fragment (which corresponds to the distance between the *Dde*I site and the transcription start site) was determined electrophoretically. When either cellular RNA (Figure 4, lanes 4–6) or in vitro RNA (Figure 4, lanes 1 and 2) is used, a band of 216 bases is obtained (arrow) along with a small amount of undigested double-stranded probe 456 bp in length. Thus, in vitro initiated RNA has the same 5′-terminus as preribosomal RNA transcribed in vivo.

All the results presented above show that the in vitro transcription system correctly and specifically initiates at the in vivo ribosomal RNA start site.

In Vitro RNA Is Transcribed by RNA Polymerase I. The S100 extract contains significant amounts of all three RNA

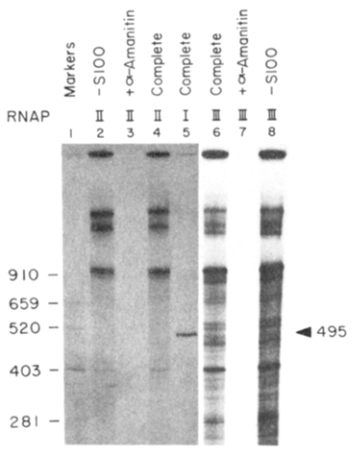

FIGURE 5: Specificity of the in vitro system for RNA polymerase I. Different purified *Acanthamoeba* RNA polymerase classes were substituted for RNA polymerase I in the standard assay. Reaction mixtures were the same as those described under Materials and Methods with pAr4/*Hha*I DNA as template, except in lanes 2–4 RNA polymerase II substituted for RNA polymerase I and in lanes 6–8 RNA polymerase III substituted for polymerase I. (Lane 1) pBR322/*Alu*I markers whose lengths are shown in the left margin; (lane 2) minus S100 with RNA polymerase II; (lane 3) complete with RNA polymerase II + 600 μg/mL α-amanitin; (lane 4) complete with RNA polymerase II; (lane 5) complete with RNA polymerase I; (lane 6) complete with RNA polymerase III; (lane 7) complete with RNA polymerase III + 600 μg/mL α-amanitin; (lane 8) minus S100 with RNA polymerase III. In each case, 8.4 milliunits of the indicated RNA polymerase were added to the assay. Due to the high background in lanes 6–8, these lanes in the figure were printed for one-third the time of the other lanes so that detail of the transcripts could be seen. All eight lanes of the figure were run on the same gel in the order seen in the figure.

polymerase classes. These are distinguishable by α-amanitin titration—in *Acanthamoeba*, 1 μg/mL completely inhibits RNA polymerase II and 600 μg/mL eliminates RNA polymerase III activity. The class I enzyme is insensitive to the mushroom toxin (Detke & Paule, 1976). Compatible with the notion that the specific transcription observed in the assay is carried out solely by polymerase I, complete insensitivity to α-amanitin is seen in the assay. When purified polymerase I is added to the system, neither low nor high amanitin levels inhibit the appearance of the runoff RNA (Figure 2A, lanes 9 and 10). Omission from the complete system of the exogenously added, purified RNA polymerase I resulted in a significant reduction in the intensities of the RNA products (Figure 2A, lane 5). Under these conditions of low polymerase activity, the specific runoff RNA remains completely insensitive to amanitin inhibition (data not shown). Thus, the specific transcription observed in the unsupplemented S100 is catalyzed by the class I RNA polymerase. The extensive stimulation of transcription by the added RNA polymerase I further suggests that the rate-limiting component in the S100 extract is the RNA polymerase I, not the transcription factor(s). However, this result does not rule out the possibility that other polymerase classes could participate in rRNA transcription in vitro.

To further test the specificity of the system for RNA polymerase I, the exogenously added polymerase I was replaced with equal units of highly purified RNA polymerase II (D'Alessio et al., 1979b) (Figure 5, lane 4) or polymerase III (Spindler et al., 1978b) (Figure 5, lane 6). RNA polymerase III initiates more efficiently on double-stranded DNA templates than either of the other enzymes; thus, the total amount of RNA transcribed in lanes 6–8 of Figure 5 is much higher. (In order to reveal details of the transcripts in the polymerase III experiments, lanes 6–8 of the figure, though run on the same gel as the other lanes, are printed for one-third the time of the other lanes in the figure.) Substitution of neither RNA polymerase II nor RNA polymerase III results in transcription of the correct runoff RNA (cf. lane 5). In contrast to the results observed when polymerase I is used to supplement the assays, α-amanitin completely inhibits all transcription by the class II and III enzymes (Figure 5, lanes 3 and 7), and the addition or deletion of S100 has little effect, if any, upon the pattern of transcripts observed (lanes 2 and 8). Other workers have shown that rRNA transcription in vitro is insensitive to α-amanitin, but this is the first direct demonstration that neither the class II nor the class III polymerase can substitute for polymerase I.

This result also argues strongly against the putative runoff transcript arising from processing of an end to end RNA since both polymerases II and III produce high levels of the 915-base end to end product but no specific product.

We occasionally observe high molecular weight products which are longer than the input DNA template. These seem to be RNAs since they are destroyed by RNase and appear to be produced mainly by polymerases II and III.

Optimum Conditions for in Vitro Transcription. Transcription using different levels of added template DNA shows that the system goes through a plateau of activity between 0.05 and 0.75 μg of DNA per 25-μL assay. The activity of the transcription system varies with ionic strength and with the concentration and type of divalent cation present. The optimum KCl concentration for rRNA transcription is 150 mM. For magnesium, transcription is optimum at 7.5–12.5 mM. There is a relationship between the optimum concentrations of the monovalent and the divalent cations in that an increase in the concentration of one of them results in a lower optimum concentration for the other. This suggests, but does not prove, that each can serve to complex with the charged groups on the nucleotide substrates and on the DNA template. Each must have some specific function as well, however, since both must be present for specific initiation. When manganese is substituted for magnesium, at any concentration, faithful initiation is completely lost. This also suggests some type of specific role for the divalent metal ion in initiation since we have shown that RNA polymerase I from *Acanthamoeba* can use manganese and magnesium equally well when nonspecifically transcribing calf thymus DNA (Detke & Paule, 1978), presumably a measure of elongation from nonspecific start sites. The optimum conditions determined here differ somewhat from the conditions reported by other groups working on polymerase I in vitro systems. In particular, the optimum KCl and $MgCl_2$ concentrations are higher than those for other systems.

It is also worth noting that the optimum concentration for end to end transcription is different than that for faithful initiation. The optimum for KCl is 200 mM and for $MgCl_2$ is 15–20 mM. This KCl concentration is also optimum for transcription of damaged calf thymus DNA by RNA polymerase I; however, the optimum $MgCl_2$ concentration for transcription of the latter template is 5 mM.

Transcription Factor Is Heat Labile. Heating the S100 to 50 °C for 10 min results in elimination of specific initiation. The factor is stable for 10 min at 45 °C, however (data not shown). The factor directing faithful initiation appears to have the heat inactivation properties of a macromolecule, probably a protein.

Acknowledgments

We thank Barry Davison, Jeff Corden, and Claude Kedinger for many helpful discussions and Pierre Chambon for providing

facilities and encouragement during the early part of this study.

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

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