Volume 2 · Number 1 · 1992
GENE EXPRESSION

The start site of the Acanthamoeba castellanii ribosomal RNA transcription unit

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The 39S ribosomal RNA (rRNA) precursor has been isolated from Acanthamoeba castellanii. In vitro capping of the isolated RNA verified that it is the primary transcript and identified the 5′ nucleotide as pppA. The position of the 5′ coding nucleotide on the rRNA repeat unit sequence was identified using Northern blot, R-loop, and S1 nuclease mapping techniques. Dinucleotide priming of an in vitro transcription system stalled because of low initiating nucleotide concentration revealed that ApA maximally stimulates initiation of transcription. All of these results show that the underlined A in the sequence 5′-TATATATAAAGGGAC (RNA-like strand) coincides with the 5′ nucleotide of the primary transcript. This identification is compatible with in vitro transcription experiments mapping the promoter for this transcription unit. The initiation sequences of rRNA genes from 14 species are compared, and a weak consensus for the initiator derived:

$$n\ \frac{gg}{rr}\ GttaTnTAggG\frac{a}{r}g\frac{a}{r}n$$

The start sites for transcription of over one dozen precursor ribosomal RNA (prRNA) genes have been identified. We report here the start site for another, along with the sequence surrounding it, and a comparison of the sequences from all of the species reported to date. This comparison supports the notion that the divergence in prRNA start site sequence between species is significant, but that a weak consensus sequence can be discerned.

Materials and methods

RNA isolation

RNA was isolated by the guanidinium thiocyanate (GuSCN) procedure of Chirgwin et al. (1987) and purified by rate zonal sedimentation in isokinetic sucrose gradients. One liter of exponentially growing Acanthamoeba cells was cooled and pelleted by centrifugation in a Beckman JA10 rotor for 10 minutes at 5000 rpm, 4°C. The cell pellets were rapidly homogenized in 20 ml GuSCN lysis buffer (4M GuSCN, 500 mM NaCl, 0.5 N-Lauryl sarcosine, 50 mM Tris-HCl pH 7.9, 200 mM 2-mercaptoethanol, 10 mM EDTA) with a Dispax tissuemizer, followed by five rounds of phenol/chloroform extraction and two rounds of chloroform extraction. Nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol and storage at −20°C overnight. The crude RNA pellet was obtained by centrifugation in a Beckman JA20 rotor at 5000 rpm, 4°C for 20 minutes. Ribosomal RNA (35–39S) was isolated by rate zonal sedimentation in isokinetic sucrose gra-

Received September 30, 1991; revision accepted November 21, 1991.

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 1052-2166/92/0201/071-08$2.00

dients (5%–28.4% sucrose in 40 mM Tris-HCl, pH 7.9, 500 mM NaCl, 10 mM EDTA). Gradients were centrifuged for 12 hours at 20,000 rpm in a Beckman SW-28 rotor at 20°C. The gradients were fractionated, and the amount of RNA was determined by the absorbance at 254 nm. Fractions containing 35–39S RNA were pooled and precipitated by ethanol as before.

Capping of 39S rRNA

Fifty micrograms of high molecular weight RNA was incubated with 10 units of gunaylyltransferase (BRL), 50 μCi α-[^{32}P]-GTP in 150 μl of 40 mM Tris HCl pH 7.9, 2 mM $MgCl_2$ at 37°C for 90 minutes. The capped RNA was isolated from the unincorporated α-[^{32}P]-GTP by 10% trichloracetic acid precipitation two times. The pellet was resuspended in 50 mM Tris HCl pH 7.9, 500 mM NaCl followed by ethanol precipitation.

Selection of capped 39S rRNA

Precursor rRNA capped in vitro was selected by hybridization to cloned rDNA (D'Alessio et al., 1981) immobilized on nitrocellulose. Cloned rDNA (pAr4) was linearized with EcoR I, followed by phenol extraction and ethanol precipitation. The pellet was dissolved in 10 mM Tris HCl pH 7.4, 1 mM EDTA. The binding of DNA to nitrocellulose paper, hybridization of capped RNA, and elution of the rRNA were performed as described by Maniatis et al. (1982).

Detection of CAP structure

After ethanol precipitation of capped hybrid-selected rRNA, the RNA pellet was resuspended in 20 μl of 50 mM sodium acetate pH 5.6, and 35 units of P1 nuclease (BRL) were added and incubated at 37°C for 30 minutes. Five μl of this mixture were applied to a PEI cellulose TLC plate (J. T. Baker), and the plate was developed with 0.6 M ammonium sulfate and autoradiographed; unlabeled standards were located with an ultraviolet Mineralight lamp.

S1 mapping

S1 mapping was carried out using the Weaver and Weissmann modification (1979) of the Berk and Sharp procedure (1977).

In vitro transcription

RNA was transcribed in vitro by the procedure of Paule et al. (1984). All nucleoside triphosphates were set at 10 μM except for α-[^{32}P]-CTP, which was 2.5 μM. Dinucleoside phosphates were added at 4.8 mM.

Results

Ribosomal RNA gene clones

A single complete copy of the ribosomal RNA repeat unit from A. castellanii was cloned into lambda charon 9 and characterized (D'Alessio et al., 1981). A subclone in pBR322 of a 6.5 Kbp BamH I/EcoR I fragment containing the 3′ end of the 26S RNA coding region, the entire intergenic spacer (IGS), the external transcribed spacer (ETS) and the majority of the 5′ end of the 18S RNA coding region was constructed (pArl) (D'Allesio et al., 1981). A 3.3 Kbp Hinc II fragment containing the 5′ half of the 18S coding region plus the entire ETS and about 600 bp of IGS was subcloned into the EcoR I site of pBR322 using EcoR I synthetic linkers (pAr3). The region between the Hind III site in pBR322 and a Hind III site just downstream of the 5′ end of the 18S coding region was excised from pAr3 to give pAr4, which was used for all of the mapping experiments described below.

Isolation of the 39S precursor ribosomal RNA

Acanthamoeba cells were pulse labeled with [^{32}P]-sodium phosphate for 10 minutes. Cellular RNA was prepared from the labeled cells by a modification of the guanidinium thiocyanate procedure developed by Chirgwin et al. (1979). High molecular weight RNA was isolated by rate zonal sedimentation in isokinetic sucrose gradients. During the short pulse time, high molecular weight RNAs which sediment at about 39S are preferentially labeled. When the 39S RNA is isolated and used to probe a Southern blot of a Pst I/Kpn I digest of pAr4, it is found to hybridize only to the fragments containing the rRNA insert (data not shown). Significantly, the RNA is complementary to the DNA fragment upstream of the single Pst I site in the rRNA insert—a fragment which contains only the 5′ end of the ETS and no stable coding regions. Thus, this 39S RNA potentially contains the 5′ end of the precursor rRNA.

The 5′ end of primary transcripts in prokaryotes and primary rRNA transcripts in eukaryotes have a 5′ triphosphorylated nucleotide,

usually a purine. RNAs which have been endonucleolytically processed at their 5′ ends will no longer end in a triphosphorylated nucleotide. Thus, a method of analysis designed to discriminate a 5′ triphosphorylated RNA from a 5′ monophosphorylated RNA will determine whether a transcript has been processed at its 5′ end or whether it is a primary transcript.

Eukaryotic messenger RNAs are modified at their 5′ end by the addition of a "cap," 7mG(5′)ppp(5′)NpN . . . (Shatkin, 1976). The enzyme complex which co- or posttranscriptionally adds the cap to the primary transcript has been isolated from vaccinia virus (Ensinger et al., 1975; Venkatesan et al., 1980) and can be used to cap RNAs which contain a di- or triphosphorylated 5′ end. The complex is inactive with monophosphorylated RNAs; thus, the ability to be capped in vitro is diagnostic of primary transcripts.

Unlabeled 39S RNA from Acanthamoeba was incubated with guanylyl transferase and α-[^{32}P]-GTP, the RNA was isolated, and an aliquot assayed for trichloroacetic acid precipitable radioactivity (64,500 cpm per 0.5 μg RNA). If the RNA were pure rRNA precursor, this represents an approximately 7% efficiency of labeling. This low efficiency suggests the 39S RNA is not pure rRNA precursor. A series of enzymatic treatments were performed and the products analyzed by PEI cellulose thin layer chromatography and autoradiography (Fig. 1A). Lane a is untreated material: residual α-[^{32}P]-GTP is present along with labeled RNA which remains at the origin. Bacterial alkaline phosphatase

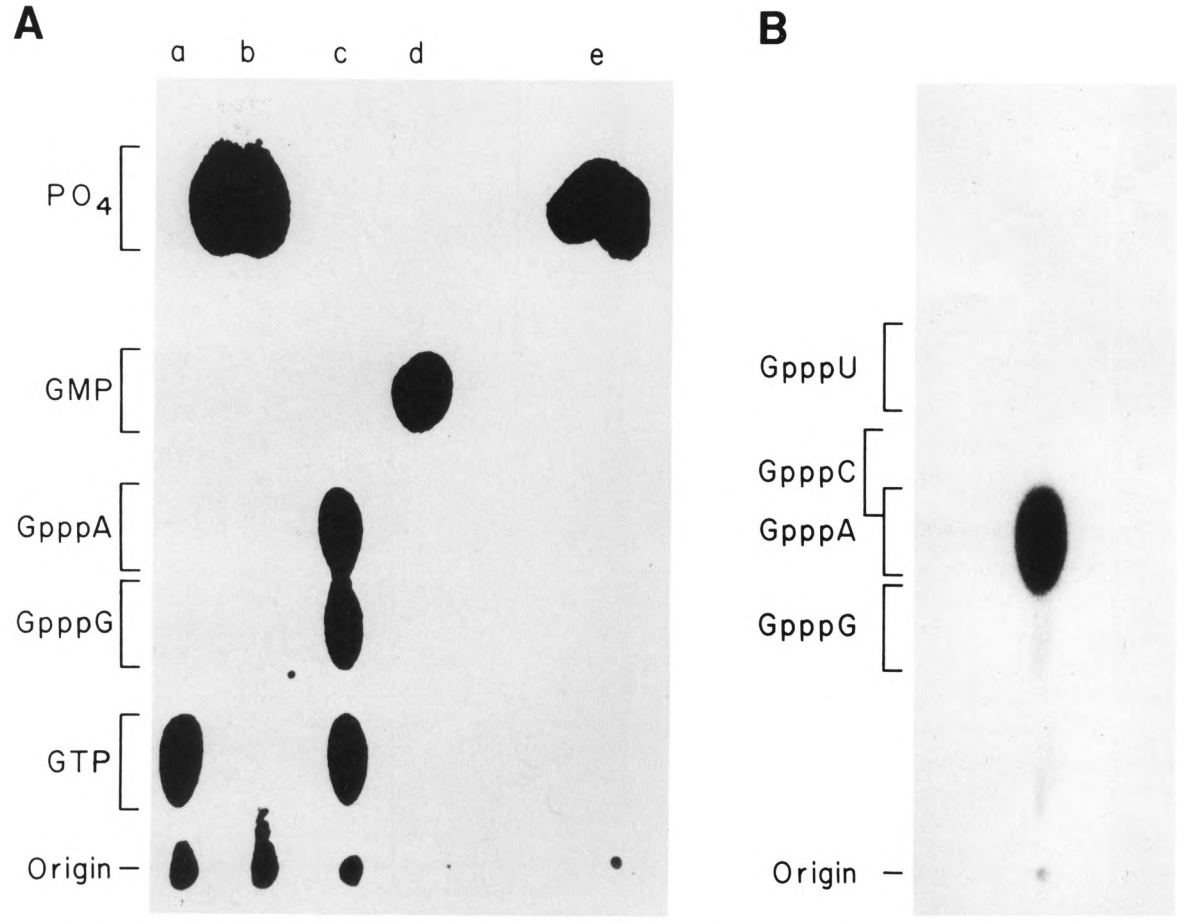

Figure 1. Analysis of in vitro capped 39S RNA. **A.** Isolated 39S RNA from Acanthamoeba was capped with α-[^{32}P]-GTP as described in Materials and Methods, treated as described below, and the products separated by thin layer chromatography. Lane a: untreated. Lane b: bacterial alkaline phosphatase treated. Lane c: P1 nuclease treated. Lane d: P1 nuclease + snake venom phosphodiesterase. Lane e: P1 nuclease + snake venom phosphodiesterase + bacterial alkaline phosphatase treated. **B.** Capped 39S RNA was hybrid-selected on cloned rRNA and treated with P1 nuclease before separation by PEI thin-layer chromatography.

treatment results in [^{32}P]-PO_4 release from α-[^{32}P]-GTP, but the labeled RNA at the origin is resistant (Lane b), indicating that the RNA is not merely phosphorylated. P1 nuclease, acting as both an exonuclease and endonuclease, hydrolyzes phosphodiester bonds of single-stranded DNA and RNA to 5′ mononucleotides. It will not cleave di- or triphosphates and will thus release intact cap structures from capped RNAs. Lane c shows, in addition to the presence of α-[^{32}P]-GTP (unaffected by P1 nuclease), two major products following P1 nuclease treatment. One of the products comigrates with an unlabeled GpppG standard, and the other comigrates with a GpppA standard. (The standards, which were chromatographed together, are unmethylated cap analogues; since S-adenosyl methionine was not included during the capping reaction, the product is expected to be unmethylated.) Treatment of the P1 digested material with snake venom phosphodiesterase yields 5′-[^{32}P]-GMP (lane d). In lane e, the 5′-[^{32}P]-GMP derived from P1 and snake venom phosphodiesterase treatment of the capped RNA has been degraded with bacterial alkaline phosphatase to [^{32}P]-PO_4. These products are consistent with the labeled high molecular weight material being capped RNA. The results shown in Figure 1A indicate that the 39S RNA contains primary transcripts which can be converted to capped RNAs by guanylyltransferase in vitro, and further, that the 5′ nucleotide of the primary transcripts is either pppG or pppA.

In order to determine which of the two nucleotides is the 5′ end of the prRNA transcript, 39S RNA capped in vitro was hybrid-selected, eluted from the filter, treated with P1 nuclease, chromatographed on PEI cellulose, and autoradiographed (Fig. 1B). The majority of the hybrid-selected labeled cap is GpppA; the majority of the GpppG was removed by the selection. This result, coupled with the data presented below, shows that the transcript is initiated with pppA.

Localization of the start site by Northern blotting analysis and R-loop mapping

The approximate start site for the transcription unit was localized by hybridizing a series of labeled DNA probes to Northern blots of Acanthamoeba and by R-loop mapping. These mapping techniques placed the start site within a 122 bp region characterized by three closely spaced Xma III sites which are approximately centered within a 915 bp Hha I fragment of the rRNA repeat unit (data not shown).

Localization of the start site by S1 nuclease mapping

The start site was precisely mapped using S1 nuclease (Weaver and Weissmann, 1979; Berk and Sharp, 1977). Four hundred base pairs of the start site region were sequenced, and a 161 bp Fnu4H I/Fnu4H I fragment was used as a probe. The S1 product resulting from this probe was electrophoresed next to a sequencing ladder to localize the exact base on the sense strand of the template which codes for the precursor's start site. The products were resolved to within 1/2 nucleotide. The start was found to be within the sequence 3′-ATA<u>T</u>TTCCC, corresponding to 5′-TAT<u>A</u>AAGGG (RNA-like strand) after correcting for the 1 1/2 base slower migration of the 3′ unphosphorylated S1 product compared

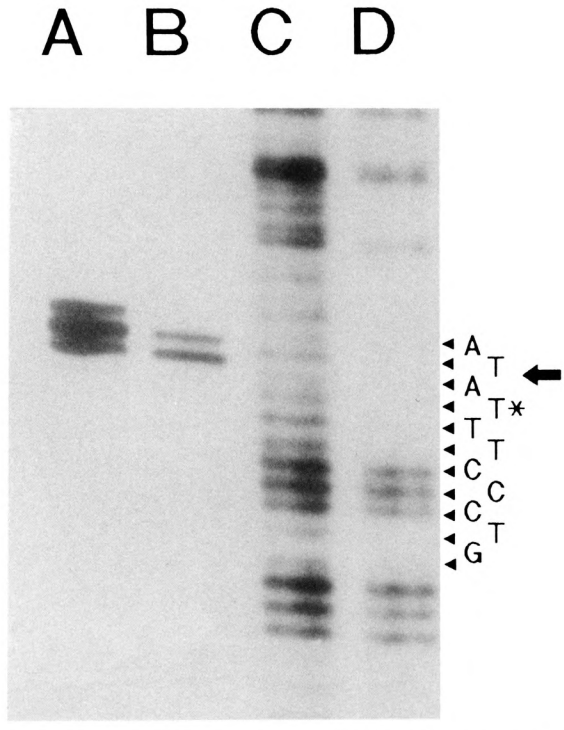

Figure 2. High resolution S1 nuclease map of 39S RNA. The 161-base Fnu4H I fragment was used for S1 nuclease mapping, and the products were electrophoresed next to a Maxam and Gilbert "C+T" (lane C) and "C > T" (lane D) sequencing ladders of the template DNA strand. Lanes A and B represent the products obtained with increasing S1 nuclease. The T representing the first base in the coding strand is marked with an asterisk, taking into account the 1 1/2 base correction relating the sequencing lanes (which are 3′ phosphorylated) to the S1 nuclease lanes (which are not 3′ phosphorylated).

to the 3′ phosphorylated sequencing ladder (Fig. 2; see Sollner-Webb and Reeder, 1979). The capping data determined that the primary transcript starts with an A, and so the S1 and capping data are in accord. Since a small amount of GpppG was seen in the hybrid-selected, capped RNA, however, a final methodology was used to determine whether transcripts could initiate with G as well as A.

Verification of the initiating base by primed synthesis in vitro

The Km for the initiating purine of an RNA transcript is considerably higher than that for bases added internally in the RNA chain. If the total nucleotide pool concentration is reduced below a critical level, the efficiency of chain initiation is radically decreased, while elongation of initiated chains is relatively unaffected. A transcription system in such a state can be stimulated by adding an RNA primer that is complementary to the first two bases of the normal transcript. Studies in several in vitro systems (Learned and Tjian, 1982; Wilkinson et al., 1983; Wilkinson and Sollner-Webb, 1982) have shown that only the correct dinucleotide stimulates maximally; thus a survey of the possible dinucleotide primers can be used to identify the first two bases of the transcript.

We have developed an in vitro transcription system (Paule et al., 1984) which, when the pAr4/Hha I DNA fragment is used, yields a runoff RNA of 495 nucleotides plus a slightly shorter prematurely terminated product. When the nucleoside triphosphate concentration is reduced below 100 μM, the efficiency of the in vitro transcription system drops precipitously (data not shown). At 10 μM, the 495 nucleotide runoff RNA is barely detectable (e.g., see Fig. 3, lane 2). In order to identify the initiating nu-

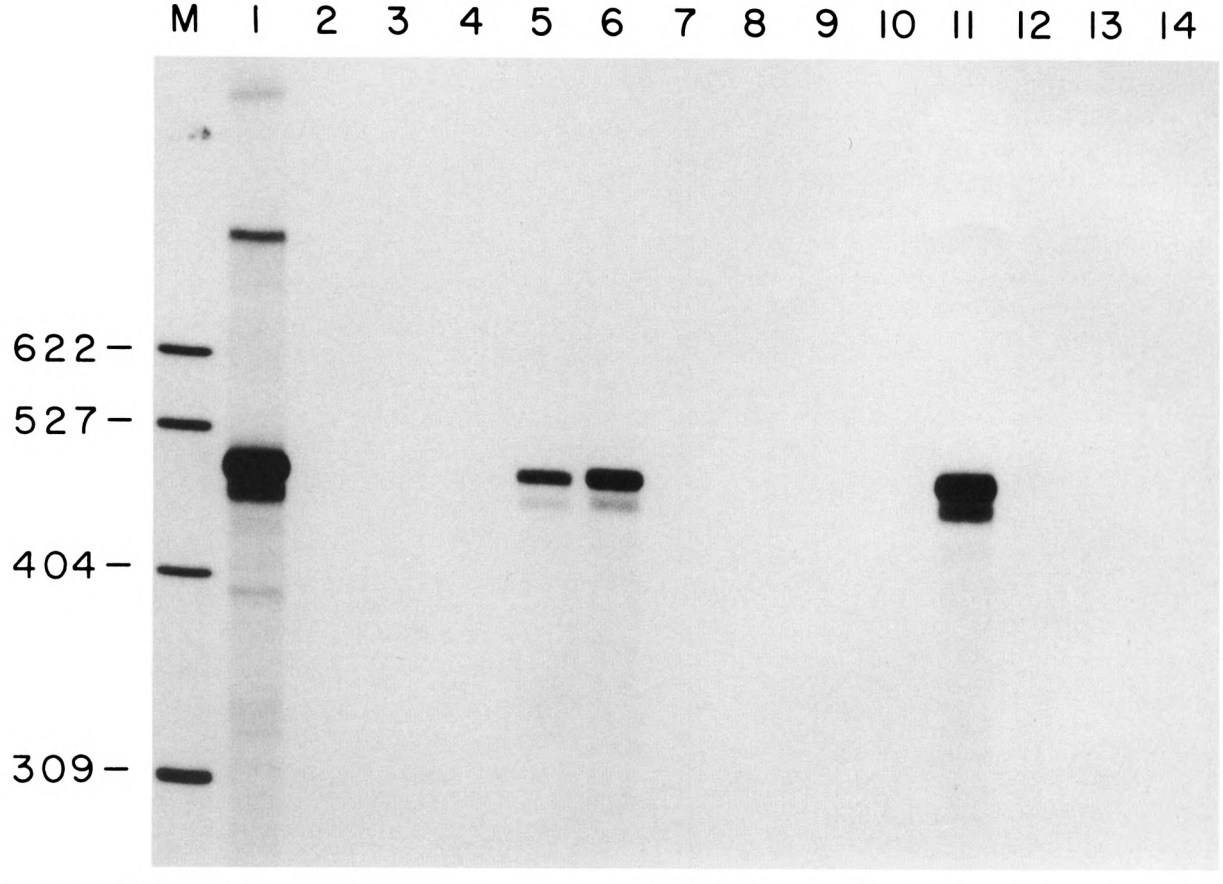

Figure 3. Mono- or dinucleotide primed synthesis of RNA in a cell free system. In vitro transcription was carried out as described by Paule et al. (1984), except that the concentrations of ATP, GTP, and UTP were set at 10 μM, and 480 μM dinucleoside phosphates were added to some lanes. Lane 1: nucleotides were increased to 600 μM. Lane 2: no additions. Lane 3: ApU. Lane 4: ApG. Lane 5: UpA. Lane 6: ApA. Lane 7: GpA. Lane 8: GpG. Lane 9: GpC. Lane 10: GpU. Lane 11: ATP was increased to 600 μM. Lane 12: GTP was increased to 600 μM. Lane 13: UTP was increased to 600 μM. Lane 14: CTP was increased to 600 μM.

cleotide, the concentrations of ATP, GTP, UTP, and CTP were individually increased to 600 μM. Only ATP stimulated production of the runoff transcript (Fig. 3, lane 11); increasing the other nucleotide concentrations had no effect (Fig. 3, lanes 12–14). Thus, in agreement with the capping data above, ATP appears to be the initiating nucleotide for Acanthamoeba rRNA synthesis (There is no requirement for ATP other than as an RNA precursor [A. Lofquist, H. Li, and M. R. Paule, unpublished data].)

The eight dinucleotides appearing in the start region were tested for their ability to stimulate the system (Fig. 3). ApA addition resulted in a maximal increase in transcription (lane 6). UpA also stimulated (lane 5), but less than ApA. Since GpppU did not appear in the capped products, and UTP does not stimulate transcription (lane 13), we believe the stimulation by UpA to be an artifact of the system. Dinucleoside phosphates representing the −1 and +1 positions commonly stimulate (Learned and Tjian, 1982). UpA is the correct dinucleoside phosphate for positions −1, −3, −5, and −7. While this multiplicity of possible sites might allow it to stimulate anomalously, ApU does not stimulate (lane 3), though it is correct for starts at −2, −4, and −6. Thus, multiplicity alone does not lead to stimulation. This result is consistent with two possible start sites, but in conjunction with the S1 nuclease data, only the first A of the sequence AAAGGG is wholly consistent as the initiating nucleotide.

Discussion

We have isolated a 39S ribosomal RNA from Acanthamoeba. It has been identified as the primary transcript by indirectly demonstrating that it has a polyphosphorylated 5′ end. The prRNA was used in Northern blot, R-loop, and S1-nuclease mapping experiments to identify the transcription unit start site sequence in cloned rDNA. Analysis of the cap structure and in vitro stimulation of transcription with dinucleoside phosphates were used to verify the exact starting base. All of these results show that the underlined A in the sequence 5′TATATAT<u>A</u>AAGGG is the initiating nucleotide.

We have subcloned the Xma III fragment extending from −55 to +19 and have shown that it is sufficient to promote faithful transcription of runoff transcripts in an in vitro system (Paule et al., 1984) and bind auxiliary proteins needed for transcription in a sequence-specific manner (Iida et al., 1985; Bateman et al., 1985). This is compatible with the finding that sequences from about −50 to +15 are necessary and sufficient for faithful transcription initiation by RNA polymerase I in in vitro systems from a number of organisms. The finding that deletions or point mutagenesis of the sequence around the start site identified above have dele-

```
   -10        +1       +10
.    .    .    .    .    .    .
TCCGAAAGTATATATAAAGGGACGGGTCCGGC  Acanthamoeba
TGCGGGCAGGAAGGTAGGGGAAGACCGGCCCT  Xenopus
GCCGCCGGGTTATATGCTGACACGCTGTCCTC  human
GACCTGGAGATAGGTACTGACACGCTGTCCTT  mouse
TACCTGGAGATATATGCTGACACGCTGTCCTT  rat
GTTGGGAGGTACTTCATGCGAAAGCAGTTGAA  Saccharomyces
TAAAAATGCATATTTAAGAAGGGGAAACATCT  Tetrahymena
CCGTCCGGACTTTTGAGACTTAGAGAAAATTT  Neurospora
TTCAAAAACTACTATAGGTAGGCAGTGGTTGC  Drosophila
   TAAGTGTTATATAGGGGGTAGGCA       radish
  CCTCAGGTATAGTAGGGGGTAGGGA       maize
  CCTCGGGTATAGTAGGGAGGAGGGG       wheat
AAATCAAGCTTATATAGGGGGAGGCC        Pisum sativum
TGTTTTGCTAAAACTCGTGTCTGAGACAAGCA  Crithidia

A    444145725-02216274
G    46781--24126785633
C    41-21-2--1-3-2-3-5
T    -111673830-131112-

     n[gg]GttaTnTAggG[a]g[a]n
       [rr]          [r] [r]
```

Figure 4. Sequences of the regions surrounding the transcription start sites (RNA-like strands) for Acanthamoeba and 13 other organisms. The first nucleotide of the transcript (+1) is underlined. Transcription initiation sequences are from Acanthamoeba (this paper), Xenopus (Sollner-Webb and Reeder, 1979; Bakken et al., 1982), human (Financsek et al., 1982), mouse (Grummt, 1981), rat (Rothblum et al., 1982; Harrington and Chikaraishi, 1983), Saccharomyces (Klemenz and Geiduschek, 1980), Tetrahymena (Saiga et al., 1982), Neurospora (Niles et al., 1981), Drosophila (Lond et al., 1981), radish (Delcasso-Tremousaygue et al., 1988), maize (McMullen et al., 1986), wheat (Barker et al., 1988), Pisum sativum (Piller et al., 1990), Crithidia (Grondal et al., 1990). The matrix at the bottom compiles the number of times a given nucleotide appears at the position, using 0 to indicate ten times and - to indicate zero times. The consensus shown at the bottom is derived with the following rules: *n* = any nucleotide; *r* = purine ⩾75%; upper case letters ⩾67% of the time; lower case letters ⩾50% of the time.

terious effects on transcription in vitro is strong evidence that the site is, indeed, the initiation sequence (Kownin et al., 1985; Kownin et al., 1988; Kownin et al., 1987). Studies with Xenopus laevis (Sollner-Webb et al., 1983; Moss, 1982) Drosophila (Kohorn and Rae, 1983), mouse (Grummt, 1982), and human (Learned et al., 1983) rRNA genes have identified a similar sequence surrounding the initiation site which cannot be deleted without significantly decreasing the efficiency of transcription. Thus, both the structural approaches described in the present paper and the functional analysis presented in numerous other manuscripts support the notion that the start site for A. castellanii prRNA is as identified herein.

The start sites for prRNA have been identified for 14 organisms. These are listed in Figure 4. If one omits pea and Crithidia from this list, a consensus for the initiator sequence can be discerned (Fig. 4), in which all transcripts start with a purine, almost always an A, usually preceded by a pyrimidine, usually a T. Further, the six positions upstream of the starting nucleotide are very AT-rich (83%); exceptions are Saccharomyces (67%) and Xenopus and mouse, (both 50%); for comparison, the first six positions (excluding +1) within the external transcribed spacer are only 40% AT. Positions −7, −8, and −9 are usually purines, often Gs. If the starting nucleotide for pea is shifted one base pair to the right, pea would also fit this pattern. For both pea (Piller et al., 1990) and Crithidia (Grondal et al., 1990), identification of the start was determined solely by S1 nuclease mapping, with no corroboration of the presence of a 5′ triphosphate by capping, or the use of dinucleoside phosphate stimulation in vitro to verify the starting dinucleoside. For the latter organism, which deviates most from the consensus, the critical S1 data are unpublished. Further, these are the only two rDNA transcription units reported to initiate with pyrimidines. In light of this, it seems possible that the start sites for these organisms were incorrectly identified.

Acknowledgments

Supported by Grants GM26059 and GM22580 from the U.S. Public Health Service.

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