Sequence organization of the *Acanthamoeba* rRNA intergenic spacer: identification of transcriptional enhancers

Qin Yang, Michael G. Zwick and Marvin R. Paule*

Department of Biochemistry and Molecular Biology, and The Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO 80523, USA

Received June 6, 1994; Revised and Accepted September 11, 1994

GenBank accession no. L07635

ABSTRACT

The primary sequence of the entire 2330 bp intergenic spacer of the *A. castellanii* ribosomal RNA gene was determined. Repeated sequence elements averaging 140 bp were identified and found to bind a protein required for optimum initiation at the core promoter. These repeated elements were shown to stimulate rRNA transcription by RNA polymerase I *in vitro*. The repeats inhibited transcription when placed in *trans*, and stimulated transcription when in *cis*. In either orientation, but only when upstream of the core promoter. Thus, these repeated elements have characteristics similar to polymerase I enhancers found in higher eukaryotes. The number of rRNA repeats in *Acanthamoeba* cells was determined to be 24 per haploid genome, the lowest number so far identified in any eukaryote. However, because *Acanthamoeba* is polyploid, each cell contains approximately 600 rRNA genes.

INTRODUCTION

Ribosomal RNA (rRNA) genes exist in multiple copies in the eukaryotic genome (1). The intergenic spacer (IGS) separates successive adjacent rRNA genes and plays an important role in regulating rRNA transcription. The partial or complete nucleotide sequence of the IGS is known for a number of rDNAs: *Xenopus* (2,3), human (4–6), rat (7,8), mouse (9,10), yeast (11), wheat (12), *Drosophila* (13), *Neurospora* (14) and *Arabidopsis* (15). Despite large variation in their primary sequence, when analyzed these IGSs usually contain functionally similar regulatory elements. These include the core promoter, the upstream control element, transcriptional terminators, spacer promoters, and enhancers [reviewed in (16–18)].

Transcriptional enhancers were first reported in genes transcribed by RNA polymerase II (19–22). These DNA sequence elements can stimulate transcription from a variable, and even a long, distance away from the promoter, either upstream or downstream, and in either orientation (20,23,24). Later, RNA polymerase I-specific enhancer elements were identified in the rRNA IGS of *Xenopus* (25–28) where they exist as 60 or 81 bp multiply repeated sequences. Functionally similar

DNA elements were subsequently found in rodents (29–31), and yeast (32–34) and elements which stimulate rat rRNA transcription by another mechanism have been identified (35–37). In *Drosophila* (38), multiple spacer promoters serve as enhancers, and artificial reduplication of the *Xenopus* gene promoter results in enhancers (16). These DNA elements are often present in multiple repeats in the IGS, except in yeast and rat. Polymerase I enhancers cause both *cis*-enhancement of rRNA transcription and *trans*-competition in both *in vivo* or *in vitro* assays (31,39,40). In *Xenopus*, the 60/81 repeat elements have more limited position independence than polymerase II enhancers because they do not function when located further than about 2 kbp away from the core promoter. In fact, when located at distances greater than ~2 kbp, they inhibit as though in *trans* (28).

In *Acanthamoeba castellanii*, the transcription unit of ribosomal RNA has been identified to be 12 kbp in length, of which 9.7 kbp includes the external transcribed spacer (ETS), internal transcribed spacers (ITS), 18S rDNA, 5.8S rDNA, and 26S rDNA (41). The remaining 2.3 kbp is the intergenic spacer. In *Xenopus*, size heterogeneity between rRNA repeats reflects variable numbers of 60/81 repeats within the IGS. Southern blot analysis indicated that there is only one size of rRNA transcription unit in *Acanthamoeba* (41). Therefore, there is not heterogeneity in the number of repeated elements in the IGS, nor in the coding region of *Acanthamoeba* rDNA.

The core promoter sequence of the rRNA gene in *Acanthamoeba* has been well characterized (42,43). Here, we report the complete sequence of the intergenic spacer and the copy number of the rRNA gene in *A. castellanii*. Multiple repeated DNA sequence elements were found in the IGS. These repeated elements were identified as polymerase I enhancers in an *in vitro* transcription system on the basis of the characteristics of these elements discussed above.

MATERIALS AND METHODS

Restriction endonucleases were obtained from Bethesda Research Laboratory or New England Biolabs and were used according to the manufacturer's instructions. DNA polymerase I and DNase I were from International Biotechnologies, Inc.. Sequenase (modified T7 DNA polymerase) was from United States

*To whom correspondence should be addressed

Biochemical Co.. GeneScreenPlus membrane was from DuPont Company. Deoxy- and dideoxynucleotides were from Pharmacia LKB Biotechnologies. Standard M13 sequencing primers were from New England Biolabs; custom made oligonucleotides were from Operon Technologies, Inc.. α -[32 P]-dATP (300 Ci/mmol), γ -[32 P]-ATP (6000 Ci/mmol) and α -[32 P]-UTP (3000 Ci/mmol) were from Amersham Corporation. All other chemicals or reagents were from either Sigma Chemicals or Fisher Scientific.

Determination of rRNA gene copy number

A modification of the method reported by Fyrberg *et al.* (44) was used to determine the copy number of the rRNA gene. Various amounts, representing different numbers of copies of the rRNA gene, of a 2.3 kbp EcoRI/HindIII fragment cut from cloned rDNA were run on a 0.7% agarose gel along with known amounts of genomic DNA, completely digested with BamHI, EcoRI or HindIII. The gel was blotted to Gene Screen Plus, and blocked in 50% formamide (deionized), 1% SDS, 1 M sodium chloride, and 10% dextran sulfate with constant agitation for two to four hours at 42°C. Denatured salmon sperm DNA (final concentration: 220 μ g/ml) and saturating amounts of nick translated EcoRI/HindIII rDNA were added in blocking buffer, denatured and incubated with constant agitation for 12–24 hours at 42°C. After hybridization, the membrane was washed twice with 100 ml of 2 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) at room temperature for 5 minutes, twice with 200 ml of 2 $\times$ SSC and 1% SDS at 65°C for 30 minutes, and twice with 100 ml of 0.1 $\times$ SSC at room temperature for 30 minutes. All washes were done with constant agitation. Following autoradiography, the radioactive regions of the Southern blot were excised and radioactivity was estimated by scintillation counting.

Subcloning of the IGS regions of the rRNA gene

The plasmid pAr1, which contains the entire intergenic spacer, and parts of the 18S and 26S rRNA genes (41) was digested with BamHI and PstI. The 3.4 kbp fragment, which contains part of the 26S rRNA gene, the entire IGS and part of the external transcribed spacer, was cloned into the BamHI and PstI sites of pUC18 and pUC19 to produce pU18BPY and pU19BPY, respectively. The BamHI/HincII fragment from pAr1 was cloned into BamHI + HincII cut pUC18, M13mp18 and pUC19 producing pU18BHY, M13mp18BHY and pU19BHY, respectively. A third fragment (EcoRI/DdeI) was isolated from pAr4 (41) and cloned into EcoRI and SmaI cut pUC8. The EcoRI/HindIII fragment of this derived plasmid (pU8EDY) was subcloned into M13mp18 and M13mp19 respectively, and the new constructs were named M18EDY and M19EDY, respectively. Plasmids (pUC vector derivatives) were purified by 2 rounds of a CsCl gradient banding method. The M13 single stranded DNAs were prepared as described (45,46).

Sequencing

Purified double-stranded plasmid DNAs or single-stranded M13 DNAs were used as templates for sequencing. All sequencing was done by modifications of the Sanger method (47) using Sequenase. Standard M13 sequencing primer was used to get the first 300 to 400 nucleotides of sequence data at the 5' end or 3' end of the cloned IGS region of the rRNA. Then new primers were synthesized which would hybridize to the region on the DNA that was sequenced, and were used for further sequencing.

This process was repeated multiple times. Both strands of the entire intergenic spacer of rDNA were sequenced by this method except for one region (from –1.9 kbp to –1.1 kbp relative to the transcription start site) where the DNA sequence is highly repeated so that the primers hybridized to more than one site. The fragment which contains the repeated sequence region (E fragment) was isolated from an AvaI digest and cloned into pUC18. E fragment was also cut into seven smaller fragments with ApaLI, the seven fragments were made blunt by filling using Klenow fragment, cloned into pUC19 at the HincII site, and sequenced. By this method, clear sequence data in the highly repeated region was obtained. The DNA sequencing strategy is presented in Fig. 2.

Sequence analysis

Sequencing gels were read with a Beckman Sonic Digitizing System using MicroGenie nucleic acid sequence entry and merge programs. The assembled sequence was analyzed by PC/GENE nucleic acid sequence analysis program (Intelligenetics, Inc.).

In vitro transcription

In vitro transcription was carried out by a modification of a published method (43,48). Two approaches were used: (1) Repeated sequences from the IGS were added on separate DNA molecules to test whether they would inhibit transcription from the promoter by competing for a common DNA-binding factor (*trans*-competition assay). (2) The repeated sequence was moved closer to the core promoter, both upstream and downstream and in both orientations. These constructs were compared as templates with the core promoter alone.

Trans-competition. Fragments A, B, D and E (Figure 2), which contain different regions of the IGS, but not the core promoter, were incubated with protein samples at 25°C for 10 minutes. 200, 400, 800, 1200 fmol of each were used. Then 80 fmol of the template DNA, a NdeI digest of pEBH10 containing promoter sequences from –120 to +80 were added, and the reaction mixtures were incubated for another 10 minutes. Nucleotides were added to start transcription, which proceeded for 25 min. RNA products were analyzed as described (43,48).

Cis-enhancement. ApaLI digestion of fragment E gave rise to seven smaller fragments. Except the one which is closest to the 3' end of the E fragment (–1160 to –1087), the other six fragments (–1960 to –1161) have over 80% sequence similarity (see the sequencing results). They were named E₁ through E₆, with E₁ at the 5' (upstream) end of fragment E. E₃, the largest in size (174 bp), was isolated and cloned into pUC18 at its SmaI site producing pU18E₃Y. A BamHI/PstI fragment which contains the core promoter (–120 to +80) was isolated from pEBH10 (48) and cloned into pU18E₃Y at its BamHI/PstI site in the correct orientation relative to E₃ generating pU18E₃PY; the 3' end of E₃ starts 137 bp upstream of the initiation site. E₃ fragment was also cloned into pEBH10 at its SmaI site in the reverse orientation from the core promoter producing pU8rE₃PY. The entire fragment E was subcloned upstream of the promoter in pEBH10 following the procedure described above. The derived construct was named pU18rEPY as fragment E was in reverse orientation relative to the core promoter. E₃ and E₄ (151 bp) were separately cloned into pEBH10, but at its HindIII site, so that they are located downstream of the core

promoter giving pU8PE₃Y and pU8PE₄Y (correct orientation) and pU8PrE₃Y and pU8PrE₄Y (reverse orientation). The structures of the different templates are shown in Fig. 6.

RESULTS

Copy number

The number of copies of the rRNA gene was determined by the method of Fyrberg *et al.* (44) in which the amount of a specific labelled probe that anneals to a known amount of genomic DNA is compared to a set of standard artificial genome mixtures made up to contain the equivalent of different numbers of gene copies. Fig. 1A shows an autoradiogram of the Southern blot. Lanes marked B, E, and H were genomic DNA (10 μ g) digested with BamHI, EcoRI, and HindIII, respectively. Lanes 1 through 40 contained the amount of rDNA which would be present in this 10 μ g of *Acanthamoeba* DNA if there were between 1 and 40 copies of the rRNA gene per haploid genome, respectively. Fig. 1B shows the relationship between copy number and radioactivity in the annealed bands on the Southern blot determined by excising the bands and scintillation counting. The estimate of copy number is 24 per haploid genome. *Acanthamoeba* is a polyploid organism, estimated to be 25 n (Byers, 1986); thus, each cell contains 600 copies (24 $\times$ 25) of the rRNA gene. Though the number of rRNA gene copies per haploid genome is the lowest we know of, the copy number estimate of 600 copies per cell is similar to that found in other eukaryotic organisms (1).

Sequence of the *Acanthamoeba* rDNA IGS

The sequence of *Acanthamoeba* rDNA IGS (RNA-like strand) flanked by 126 bp of the 3' end of 26S rRNA coding sequence and 219 of bp the 5' ETS was determined (GeneBank Sequence accession number L07635). The IGS is 2330 bp long, which makes it one of the shortest rDNA IGS sequences known. The transcription start site, which is located at +1 bp was determined

previously (49). The core promoter extends from -47 to +8 and was defined by deletion mutants and single-point mutants by Kownin *et al.* (50,51). Determination of the location of the last 126 bp at the 3' end of the 26S rRNA coding sequence was based on the sequence of isolated *Acanthamoeba* 26S rRNA (M. Gray, personal communication).

The IGS has a G+C content of 49.0%, which is considerably lower than the *Acanthamoeba* genomic average (64%). Interestingly, the G+C content is not constant throughout the IGS of *Acanthamoeba* rDNA. The G+C content of the region between the 3' end of the 26S rRNA coding sequence and the enhancer repeats (see below) (-2330 to -1992) is only 35%, while fragment E, which contains the repeated DNA elements, has a G+C content of 46%. Fragment B, which is closest to the transcription start site, has a high G+C content (55%). The ETS sequence between the transcription start site and the 18S coding region is also very rich in G+C (58%). However, the 10 bp surrounding the transcription start site (-9 to +1) are unusually A+T rich, and is predicted to be the most thermally unstable 10 bp sequence block in the entire IGS (52,53). This may have significance for the DNA strand-separation step of initiation (50,54).

Repeated DNA sequences

A dot-matrix self-comparison of the IGS sequence revealed the presence of internal repeated sequences. The region between -1991 and -1161 consists of six imperfect repeats with an ApaLI site (GTGCAC) at approximately the border of each repeat (Fig. 2). The six repeats were named E₁ through E₆ according to their position in the IGS, with E₁ starting at the 5' (upstream) end. The size of each repeat ranges from 106 bp to 174 bp, with an average size of about 140 bp. Fig. 3 shows the sequence alignment of the six repeats. The region (about 100 bp) close to the 5' end of each repeat is highly conserved. The 3' end consists of a stretch of d(GT)_n, n = 1-5, subrepeats. The main

Figure 1. Determination of the number of rRNA gene copies per haploid *Acanthamoeba* genome. A. Ten μ g of *A. castellanii* DNA previously digested with BamHI (lane B), EcoRI (lane E) or HindIII (lane H), or artificial genome mixtures containing known amounts of a 2.3 kbp EcoRI/HindIII rRNA gene fragment equivalent to 1 to 40 copies per haploid genome (lanes 1, 10, 20, 30, 40) were electrophoresed, blotted and annealed to a radiolabelled rDNA probe. An autoradiogram of the resulting blot is shown. B. The amount of radioactivity in each band is plotted versus the copy number.

difference between the repeats is the number of 3' d(GT_n) subrepeats, though there is also some variation in the d(CA_n) repeats at the 5' end of each repeat. The region between -2195 and -2074 seems to be another repeat element in reverse orientation, but with a larger deviation from the consensus.

Spacer repeats in *Acanthamoeba* have characteristics of rRNA transcriptional enhancers

Repeated sequences were found in the rDNA IGS of a number of different organisms. These repeated DNA elements have been identified as transcriptional enhancers of the polymerase I system. Identification of repeated sequences in the IGS of *Acanthamoeba* rDNA suggested that the same transcriptional enhancement mechanism might also apply to the *Acanthamoeba* system. To test this possibility, the effects of the spacer repeats on rRNA transcription when the repeats are on the same or different DNA molecules with respect to the core promoter were examined in our *in vitro* transcription system.

Trans-competition

'Order-of-addition' experiments (43) were performed to determine whether elements of the IGS bind protein factors required for core promoter function (Fig. 4). Increasing amounts of the test DNA (DNA1) were preincubated with the proteins required for *in vitro* transcription, then a template DNA (DNA2) was added and incubated for 10 min before transcription was initiated by nucleotide addition. The experiments shown here are complex because there are several different types of equilibria taking place. The committed complex formed on the promoter is a stable complex (43). On the other hand, the complex formed between the repeated sequences and the protein which interacts with it is not a stable complex (Yang *et al.*, submitted). The protein which binds the repeats is a functional homolog of vertebrate upstream binding factor, UBF (Yang *et al.*, submitted), and unstable binding of UBF to the enhancers is common. Thus, though competitor DNA may be added before template DNA, some of the protein bound to competitor may switch to the template during subsequent incubation, including during the transcription reaction itself. Furthermore, we and others have found that rRNA transcription is particularly susceptible to inhibition by nonspecific DNA-binding proteins in the factor preparations. Inclusion of additional DNA which binds these

inhibitors can stimulate transcription (55). Finally, the amount of end-to-end transcription obtained from different DNAs is dependent upon the structure on their ends. More end-to-end transcription decreases the amount of RNA polymerase available for transcription. Thus, all of these competing reactions makes it difficult to obtain quantitative correspondence between the amounts of competitors added and inhibition. We consider these assays to be only qualitatively useful.

In the control experiment (Fig. 4A, lanes 1–6), two promoter-containing DNAs, S (giving a Shorter 242 nt RNA product) and L (giving a Longer 309 nt RNA product), were added at different times in the *in vitro* transcription assays. Template S, added first, virtually eliminated transcription of the 309 nt RNA from template L, added 10 minutes later (cf. Fig. 4A, lane 6 with lanes 2–5). The products seen near the top of lanes 4 and 5 are end-to-end transcripts of S. The same *trans*-competition effect was found when the entire IGS less the promoter (fragment A) was used to compete against S. When increasing amounts of fragment A (-2360 bp to -96 bp) were pre-incubated with protein samples, the amount of the 242 nt transcript from template S added 10 minutes later decreased (Fig. 4A, lanes 8–11) and almost completely disappeared as the amount of fragment A reached the 12 nM level (Fig. 4A, lane 11). The results suggest that the IGS of *Acanthamoeba* rDNA contains DNA elements which can bind to protein factor(s) required for rRNA synthesis. To verify that the competition is sequence-specific, two pBR322 fragments (pBR322/EcoRI–NdeI) with lengths equivalent to fragment A were used for competition. As seen in Fig. 4B (lanes 6–9), when this pBR322 DNA was preincubated, there was no change in the amount of transcript from template S.

To determine which region(s) of the IGS competes, three fragments (E, which contains the repeats, D, and B; Fig. 2) were used in a competition experiment against S. Fragment E competes the 242 nt runoff of S better than the other two fragments; the

Figure 2. Structure and sequencing strategy of the IGS. The arrows show the length and direction of sequencing runs. The scale is in base pairs starting at the BamHI site. Fragments A, B, D and E are discussed in the text.

E1	CACAAACACACACACACACACATCTGCTCGGGGGCTTGGCCCAACG	50
E3	C-----ACACACA---AAACATGTTTGTCTTGGGGGCTTGGCCCAACG	39
E4	C-----ACAC--A---AAACATGTTTGTCTTGGGGGCTTGGCCCAACG	37
E2	CACAAACACACACACACA---AAACATCTGCTTGGGGGCTTGGCCCAACG	48
E5	C-----ACACA---AAACATGTTTGTCTTGGGGGCTTGGCCCAACG	37
E6	C-----ACACACA-AAACATGTTTGTCTTGGGGGCTTGGCCCAACG	39
	* ** *** * *****	
E1	ACTTGGCGTTTTCGACGGGTCGTGCGGAAAA-TAGGGGTCGGTTGTGT-	98
E3	ACTTGGCATTTTTCGCGGGTCGTGCGGAAAA-TAGGGGTCGGTTGTGT	88
E4	ACTTGGCATTTTTCGACGGGTCGTGCGGAAAAATAGGGGTCGGTTGTGT	86
E2	ACTTGGCATTTTTCGATGGGTCGTGCGGAAAA-TAGGGGTCGGTTGTGT	97
E5	ACTTGGCATTTTTCGACGGGTCGTGCGGAAAA-TGGGGTCGGTTGTGT	86
E6	ACTTGGCATTTTTCGACGGGTCGTGCGGAAAAATAGGGGTCGGTTGTGT	89
	*****.*****.***** *.******	
E1	-----GTTTTGTGTTTGTGTGTTTGTGTGTGTT	127
E3	TTTGTGTTTGTGTGTTTGTGTGTTTGTGTGTTTGTGTGTTGTT	138
E4	-----GTTTTGTGTTTGTGTTTGTGTGTTTGTGTTTGTGTT	115
E2	TGTTTGT-----GTTTTGTGTTTGTGTTTGTGTTTGTGTT	104
E5	T-----T-----T-----T-----T-----T-----	87
E6	T-----T-----T-----T-----T-----T-----	92
E1	T--GTGTTTGTGTGT--TTTGTGTTT--TGTGTTTGTG	159
E3	TTTGTGTTTGTGTGTGTTTGTGTTTGTGTTTGTGTTTGTG	174
E4	TTTGTGTTTGTGTGTGTTTGTGTTTGTGTTTGTGTTTGTG	151
E2	-----GTTTTGTGTTTGTGTTTGTGTTTGTGTTTGTG	127
E5	-----TTGTGTTTGTGTTTGTGTTTGTGTTTGTGTTTGTG	106
E6	TTTGTGTTTGT-----TTGTGTTTGTGTTTGTGTTTGTG	114

Figure 3. Alignment of repeated sequence elements. The six repeated sequence elements, E₁ to E₆, are shown aligned. Asterisks indicate invariant base pairs, periods indicate highly conserved base pairs and dashes indicate missing base pairs. Numbers to the right indicate the position in the repeat of the last base pair listed on that line. The RNA-like strand is shown.

B fragment competes more weakly and the D fragment does not compete at all (Fig. 4C). Other bands seen in Figure 4B and C are end-to-end transcripts and paused products from DNAs A,

pBR322, E, B, and D. On a molar basis, three times more E fragment than A fragment (Figs. 4B and C) is needed to compete transcription. The reason for this is not clear, though as noted

Figure 4. The repeated sequence elements inhibit transcription *in vitro* when placed *in trans* to the core promoter. In each experiment, transcriptional proteins were preincubated with DNA 1 for 10 min, then template DNA (DNA 2) was added and preincubated for an additional 10 min before transcription was started by addition of nucleotide substrates. **A.** DNA 1 = template DNA giving a short runoff (S, 242 nt) or ETS fragment A (see Fig. 2). DNA 2 = template DNA giving a long runoff (L, 309 nt) or template S. **B.** DNA 1 = template S, fragment A or pBR322 (pBR) fragments. DNA 2 = template S. **C.** DNA 1 = template S, fragments E, B, or D. DNA 2 = template S.

above, these competitions involve complex equilibria. Furthermore, electrophoretic-mobility shift assays (EMSA) have shown that there are several additional binding sites for the factor in the full length IGS outside of the E region (e.g. in the B fragment). Also, only four of the six imperfect repeats in E can bind the protein.

These results suggested that the spacer repeats contained in the E region of the IGS are the target sites for the binding of transcription factor(s) required at the promoter for specific initiation.

Cis-enhancement

Repeated sequences found in *Xenopus* (27), mouse (39), and rat (56) rDNA IGSs which bind a transcription factor utilized by the core promoter have been found to enhance rRNA transcription *in vivo* and *in vitro*. To test whether the spacer repeats of *Acanthamoeba* are enhancers of polymerase I transcription, a single repeat (E_3) was cloned close to the core promoter (–120 to +80), either upstream or downstream, and in both orientations. Fragment E, which contains six repeats was also cloned upstream of the core promoter in the reverse orientation. The transcriptional activities of these constructs were tested and compared with that

of the template bearing the core promoter only (–120 to +80). As shown in Fig. 5, when E or E_3 was located upstream of the core promoter, they stimulated transcription approximately 2 fold, independent of orientation (lanes 2–4 and 8–10). [Note that slightly different RNA products (309 and 313) were obtained in these experiments. These differences are beyond +80 and do not affect transcriptional activity.] This stimulation is only observed at a low template concentration (5 ng/assay) (data not shown). The same DNA-dependence was also observed for enhancers from rat (L.Rothblum, personal communication) and mouse [B.Sollner-Webb, personal communication,(57)]. When E_3 was moved downstream of the core promoter (causing the runoff product to be 484 nt), it had no effect when in its correct orientation (lanes 5 and 11), but it inhibited runoff transcription when downstream in reverse orientation (lanes 6 and 12). In some experiments, similar position dependence has been found for *Xenopus* enhancers (28). In all cases, stimulation did not appear time dependent in that 10 minute (lanes 1–6) or 45 minute (lanes 7–12) incubations gave equal stimulation. Thus, the enhancer does not appear to affect the rate of active complex assembly. The possibility of rate effect less than 10 minutes cannot be ruled out by these experiments, however.

Figure 5. The repeated sequence elements are transcriptional enhancers. Fragment E (large shaded box) or E_3 (small shaded box) were cloned upstream or downstream of the core promoter (small hatched box) in the orientations shown by the arrowhead on each shaded box and tested in an *in vitro* transcription runoff assay. Runoff products were 309 nt (lanes 1, 4, 7, 10), 313 nt (lanes 2, 3, 8, 9) or 484 nt (lanes 5, 6, 11, 12) depending upon the construct (see text). The percent transcriptional activity relative to lane 1 (lanes 2–6) or lane 7 (lanes 8–12) is shown at the bottom of each lane. Lanes 1–6 were incubated for 10 min, lanes 7–12 for 45 min. The band marked recovery is an internal control for product loss.

DISCUSSION

The IGS sequence of *Acanthamoeba* rDNA has been completely determined. Sequence comparison with other known IGS sequences did not reveal any significant overall homology.

The *Acanthamoeba* IGS contains a repeated enhancer element. Sequence analysis of the IGS revealed the existence of six repeated sequences located between about 2 kbp and 1.2 kbp upstream of the transcription start site. The size of the six repeats ranges from 106 bp to 174 bp, with an average of 140 bp. The 5' region (about 100 bp) is highly conserved in all six repeats. The 3' region of each repeat consists of subrepeats of d(GT)_n, n = 1 to 5, with the number of subrepeats varying from one enhancer repeat to another. Similar repeated sequences in the IGS of rRNA genes have been reported in a number of organisms. Those spacer repeats were identified as enhancers of polymerase I transcription [see reviews (16, 58–60)]. Since polymerase I enhancers bind a transcription factor required at the core promoter for optimal activity, they inhibit transcription when placed in *trans* to their promoter. Similarly, *Acanthamoeba* IGS repeats located in *trans* inhibited rRNA synthesis initiating from the core promoter in our *in vitro* transcription system, while pBR322 fragments had no effect. When one or more of the spacer repeats were located on the same DNA molecule close to the core promoter, in the upstream position, they stimulated transcription independent of orientation. In the downstream position, they had no effect in the correct orientation, and inhibited transcription in the reverse orientation. However, the *cis*-stimulatory effect was not very strong: the spacer repeats enhanced transcription by only about two-fold. Considering that the assays were done under *in vitro* conditions, the results were not surprising because most polymerase I enhancers are notoriously inactive *in vitro* (58). Enhancers function very poorly in purified *in vitro* systems because in the absence of specific or nonspecific inhibitors which depress basal level transcription, expression is already at a high level without the enhancer (57). In *Xenopus*, the 60/81 bp repeats stimulated rRNA transcription much more strongly *in vivo* than *in vitro* (28). The development of an *in vivo* transcriptional assay in *Acanthamoeba* will help to further characterize the enhancer-like properties of the spacer repeats. Nevertheless, the results of both *trans*-competition and *cis*-enhancement *in vitro* showed that the repeated sequences in the IGS of *Acanthamoeba* rRNA gene are enhancer-like elements similar to those found in other polymerase I systems.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service grants GM22580 and GM39901.

REFERENCES

- Long, E.O. and Dawid, I.B. (1980) *Annu. Rev. Biochem.* **49**, 727–764.
- Moss, T., Boseley, P.G. and Birnstiel, M.L. (1980) *Nucleic Acids Res.* **8**, 467–485.
- Labhart, P. and Reeder, R.H. (1987) *Nucleic Acids Res.* **15**, 3623.
- La Volpe, A., Simeone, A., D'Esposito, M., Scotto, L., Fidanza, V., De Falco, A. and Boncinelli, E. (1985) *J. Mol. Biol.* **183**, 213–223.
- Sylvester, J.E., Petersen, R. and Schmickel, R.D. (1989) *Gene* **84**, 193–196.
- Dickson, K.R., Braaten, D.C. and Schlessinger, D. (1989) *Gene* **84**, 197–200.
- Yavachev, L.P., Georgiev, O.I., Braga, E.A., Avdonina, T.A., Bogomolova, A.E., Zhurkin, V.B., Nosikov, V.V. and Hadjiolov, A.A. (1986) *Nucleic Acids Res.* **14**, 2799–2810.
- Yang-Yen, H. and Rothblum, L.I. (1986) *Nucleic Acids Res.* **14**, 5557–5558.
- Bach, R., Grummt, I. and Allet, B. (1981) *Nucleic Acids Res.* **9**, 1559–1569.
- Braaten, D.C., Thomas, J.R., Little, R.D., Dickson, K.R., Goldberg, I., Schlessinger, D., Ciccodicola, A. and D'Urso, M. (1988) *Nucleic Acids Res.* **16**, 865–881.
- Skryabin, K.G., Eldarov, M.A., Larionov, V.L., Bayev, A.A., Klootwijk, J., de Regt, V.C.H.F., Veldman, G.M., Planta, R.J., Georgiev, O.I. and Hadjiolov, A.A. (1984) *Nucleic Acids Res.* **23**, 2955–2968.
- Lassner, M., Anderson, O. and Dvorak, J. (1987) *Genome* **29**, 770–781.
- Simeone, A., La Volpe, A. and Boncinelli, E. (1985) *Nucleic Acids Res.* **13**, 1089–1101.
- Dutta, S.K. and Verma, M. (1990) *Biochem. Biophys. Res. Commun.* **170**, 187–193.
- Gruendler, P., Unfried, I., Pascher, K. and Schweizer, D. (1991) *J. Mol. Biol.* **221**, 1209–1222.
- Reeder, R.H. (1992) In McKnight, S.L. and Yamamoto, K.R. (Eds.) *Transcriptional Regulation*, Cold Spring Harbor Laboratory Press, New York, pp. 315–347.
- Paule, M.R. (1993) *Gene Exp.* **3**, 1–9.
- Lofquist, A.K. and Paule, M.R. (1993) In Zimmermann, R.A. and Dahlberg, A.E. (Eds.) *Ribosomal RNA: structure, evolution, gene expression and function in protein synthesis*, CRC Press, New York, NY.
- Gruss, P., Dhar, R. and Khoury, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 943–947.
- Levinson, B., Khoury, G., Woude, G.V. and Gruss, P. (1982) *Nature* **295**, 568.
- Wasylyk, B., Wasylyk, C., Augereau, P. and Chambon, P. (1983) *Cell* **32**, 503–514.
- Jongstra, J., Rerdelhuber, T.L., Oudet, P., Benoist, C., Chae, C., Jeltsch, J., Mathis, D.J. and Chambon, P. (1984) *Nature* **307**, 708–714.
- Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981) *Nucleic Acids Res.* **9**, 6047.
- Imperiale, M.J., Feldman, L.T. and Nevins, J.R. (1983) *Cell* **35**, 127–136.
- Busby, S.J. and Reeder, R.H. (1983) *Cell* **34**, 989–996.
- Reeder, R.H., Roan, J.G. and Dunaway, M. (1983) *Cell* **35**, 449–456.
- Labhart, P. and Reeder, R.H. (1984) *Cell* **37**, 285–289.
- Pape, L.K., Windle, J.J., Mougey, E.B. and Sollner-Webb, B. (1989) *Mol. Cell. Biol.* **9**, 5093–5104.
- Cassidy, B.G., Yang-Yen, H. and Rothblum, L.I. (1986) *Mol. and Cell. Biol.* **6**, 2766–2773.
- Dixit, A., Garg, L.C., Chao, W. and Jacob, S.T. (1987) *J. Biol. Chem.* **262**, 11616–11622.
- Pikaard, C.S., Pape, L.K., Henderson, S.L., Ryan, K., Paalman, M.H., Lopata, M.A., Reeder, R.H. and Sollner-Webb, B. (1990) *Mol. Cell. Biol.* **10**, 4816–4825.
- Elion, E.A. and Warner, J.R. (1984) *Cell* **39**, 663–673.
- Elion, E.A. and Warner, J.R. (1986) *Mol. Cell. Biol.* **6**, 2089–2097.
- Johnson, S.P. and Warner, J.R. (1989) *Mol. Cell. Biol.* **9**, 4986–4993.
- Jacob, S.T., Zhang, J., Garg, L.C. and Book, C.-B. (1991) *Mol. Cell. Biochem.* **104**, 155–162.
- Ghosh, A.K., Hoff, C.M. and Jacob, S.T. (1993) *Gene* **125**, 217–222.
- Hoff, C.M. and Jacob, S.T. (1993) *Biochem. Biophys. Res. Commun.* **190**, 747–753.
- Grimaldi, G. and Di Nocera, P.P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5502–5506.
- Kuhn, A., Deppert, U. and Grummt, I. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7527–7531.
- Grimaldi, G., Fiorentini, P. and Di Nocera, P.P. (1990) *Mol. Cell. Biol.* **10**, 4667–4677.
- D'Alessio, J.M., Harris, G.H., Perna, P.J. and Paule, M.R. (1981) *Biochemistry* **20**, 3822–3827.
- Kownin, P., Iida, C.T., Brown-Shimer, S. and Paule, M.R. (1985) *Nucleic Acids Res.* **13**, 6237–6247.
- Iida, C.T., Kownin, P. and Paule, M.R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1668–1672.
- Fyrberg, E.A., Kindle, K.L., Davidson, N. and Sodja, A. (1980) *Cell* **19**, 365–378.
- Schreier, P.H. and Cortese, R. (1979) *J. Mol. Biol.* **129**, 169–172.
- Sanger, F., Coulson, R., Barrell, B.G., Smith, A.J.H. and Boe, B.A. (1980) *J. Mol. Biol.* **143**, 161–178.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Bateman, E., Iida, C.T., Kownin, P. and Paule, M.R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8004–8008.

49. Perna, P.J., Harris, G.H., Iida, C.T., Kownin, P., Bugren, S. and Paule, M.R. (1992) *Gene Exp.* **2**, 71–78.
50. Kownin, P., Bateman, E. and Paule, M.R. (1987) *Cell* **50**, 693–699.
51. Kownin, P., Bateman, E. and Paule, M.R. (1988) *Mol. Cell. Biol.* **8**, 747–753.
52. Breslauer, K.J., Frank, R., Blocker, H. and Markey, L.A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3746–3750.
53. Rychlik, W. and Rhoads, R.E. (1989) *Nucleic Acids Res.* **17**, 8543–8551.
54. Bateman, E. and Paule, M.R. (1988) *Mol. Cell. Biol.* **8**, 1940–1946.
55. Radebaugh, C.A., Matthews, J.L., Geiss, G.K., Liu, F., Wong, J., Bateman, E., Camier, S., Sentenac, A. and Paule, M.R. (1994) *Mol. Cell. Biol.* **14**, 597–605.
56. Yang-Yen, H.-F. and Rothblum, L.I. (1988) *Mol. Cell. Biol.* **8**, 3406–3414.
57. Kuhn, A. and Grummt, I. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7340–7344.
58. Reeder, R.H. (1989) *Curr. Opin. Cell. Biol.* **1**, 466–474.
59. Reeder, R.H. (1990) *Trends Genet.* **6**, 390–395.
60. Paule, M.R. (1994) In Conaway, R. and Conaway, J. (Eds.) *Transcription*, Raven Press, New York, pp. 83–106.