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Additional RNA Polymerase I Initiation Site within the Nontranscribed Spacer Region of the Rat rRNA Gene

BRANDT G. CASSIDY, HSIN-FANG YANG-YEN, AND LAWRENCE I. ROTHBLUM*
Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

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We identified and characterized an additional promoter within the nontranscribed spacer (NTS) of the rat ribosomal gene repeat that is capable of supporting initiation of transcription by RNA polymerase I in vitro. Within this promoter there is a sequence of 13 nucleotides which is 100% homologous to nucleotides −18 to −6 (+1 being the first nucleotide of 45S rRNA) of the major promoter of 45S pre-rRNA and is located between nucleotides −731 and −719. To identify the exact location of the upstream initiation site, the RNA synthesized in vitro from this new promoter was gel isolated and subjected to fingerprint analysis, Southern hybridization, and reverse transcriptase elongation. Based on these analyses, the in vitro-synthesized RNA initiates with an A at nucleotide −713. When compared individually, the upstream promoter was transcribed ninefold less efficiently than the major promoter. When templates which contain both promoters on the same piece of DNA were transcribed, the major promoter was at least 50-fold more efficient.

Eucaryotic ribosomal genes are arranged as tandemly repeating units. The transcription units that code for the RNA precursor are flanked by the nontranscribed spacer (NTS) regions. The transcribed portion of the repeat consists of an external transcribed spacer, the region that codes for 18S rRNA, an internal transcribed spacer, the 5.8S coding region, a second internal transcribed spacer, the region that codes for 28S rRNA, and a short 3′ external transcribed spacer (23).

Besides functioning to direct and regulate transcription, the NTS has been both proposed and shown to have roles in recombination (39), DNA replication (37), and chromatin structure (8).

By far the greatest number of studies on the NTS have focused on its role in transcription. The DNA sequences immediately upstream of the rRNA transcription initiation site have been intensively studied by several laboratories. A core promoter region, located between nucleotides −39 and +6, has been identified, and specific nucleotides within this region have been shown to be required for initiation (11, 18, 22, 25, 38, 43, 47). A second region, the upstream control element or upstream promoter element (between nucleotides −150 and −110), has been demonstrated to stabilize effectively the preinitiation complex (15, 19, 30, 44, 45; B. G. Cassidy, R. Haglund, and L. I. Rothblum, Biochim. Biophys. Acta, in press).

In yeasts and *Xenopus laevis*, regions several hundred to several thousand nucleotides upstream of the 5′ end of the ribosomal precursor have been shown to have profound effects on promoter utilization and the efficiency of transcription. In yeasts, a polymerase I promoter (42) or enhancer element (9) has an effect on the transcription of the 35S rRNA precursor (10). Two promoter-related elements present in multiple copies in the NTS of *X. laevis* have also been identified (reviewed in references 7 and 34). These elements, the Bam islands and the 60- or 81-base-pair (60/81-bp) repeats, affect the utilization of the polymerase I promoter and are themselves partial duplications or homologies of portions of the rRNA promoter (7, 20, 32).

We have analyzed the potential effects of the rat ribosomal NTS on RNA polymerase I initiation and transcription in vitro. In addition to defining the core and upstream elements of the promoter, analogous to those mentioned above, we identified an upstream activator element (4). This element was mapped between nucleotides −1018 and −286 (+1 being the initiation site of 45S rRNA). Experiments suggested that this region forms a stable complex with *trans*-activating factors.

In this manuscript, we report the identification of a potentially important regulatory element within the region identified as containing the upstream activator element. We present the localization of an RNA polymerase I initiation site 713 bp upstream of the 45S initiation site. The putative promoter of the upstream initiation site contains a 13-nucleotide sequence that is 100% homologous to nucleotides −18 to −6 of the major promoter previously characterized. In vitro this promoter is utilized an order of magnitude less efficiently than the major promoter. The in vitro transcripts from the upstream promoter continue into the transcribed portion of the 45S rRNA.

MATERIALS AND METHODS

Restriction enzymes were purchased from either New England BioLabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as recommended by the supplier. Radioactive nucleotides were purchased from Amersham Corp. (Arlington Heights, Ill.). [γ-^{32}P]ATP for kinase reactions was >3,000 Ci/mmol, and [α-^{32}P]UTP or [α-^{32}P]GTP for transcription was >400 Ci/mmol. The Klenow fragment of *Escherichia coli* polymerase and *Sph*I linkers were purchased from Boehringer Mannheim. Reverse transcriptase was purchased from International Biotechnology, Inc. The autoradiographic signals were quantitated with a Bio-Rad video densitometer.

Subclones. puKpn/Bam and puXba/Bam were constructed from *Kpn*I or *Xba*I digests of p3.4 (49) (Fig. 1). After digestion, the ends were made blunt with Klenow fragment, and *Sph*I linkers were added. The respective DNAs were then digested with *Bam*HI and *Sph*I, and the *Bam*HI-*Sph*I fragments from each were gel isolated and ligated into the *Bam*HI-*Sph*I-digested pUC18 vector. p1742 was constructed

* Corresponding author.

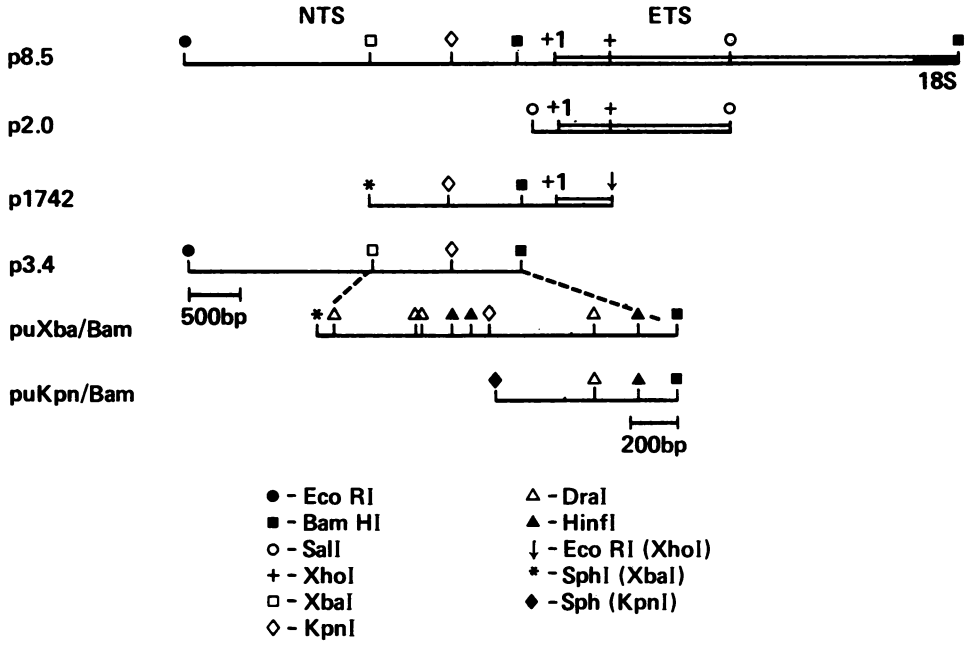

FIG. 1. Partial restriction maps of p8.5, p2.0, p1742, p3.4, puKpn/Bam, and puXba/Bam. These sites were determined from the sequence of the rat rDNA from −3686 to +8004 (48, 50) and by restriction enzyme mapping. Sites for these restriction enzymes within the pBR322 and pUC18 vectors have been omitted.

by ligating the *Xba*I-*Bam*HI fragment from p3.4 into *Sph*I-*Bam*HI-digested p5.1E/X. The *Xba*I site was converted into an *Sph*I site by blunt ending the *Xba*I site and adding *Sph*I linkers, and the fragment was isolated after digestion with *Bam*HI and *Sph*I. p5.1E/X contains the ribosomal sequences between nucleotides −286 and +638, and p1742 contains sequences between nucleotides −1742 and +638.

In vitro transcription. rDNA templates (0.25 to 1 μg) were transcribed in vitro in 50-μl reaction mixtures with a fractionated whole cell extract (DE-175) as previously described (4).

Southern analysis. p3.4 and p1742 were digested with *Dra*I and *Hin*fI, electrophoresed on a 2% agarose gel with φX174 *Hae*III fragments as markers, and transferred to nitrocellulose (41). The blot was hybridized with gel-isolated, in vitro-transcribed RNA for 24 h at 65°C. It was then washed two times for 30 min each with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate and 1.5× SSC–0.2% sodium dodecyl sulfate, followed by autoradiography at −80°C for 4 days with Kodak XAR-5 film and a Cronex Lightning-Plus intensifying screen.

RNA fingerprinting. puKpn/Bam was truncated with *Dra*I and transcribed with either [α-^{32}P]GTP or [α-^{32}P]UTP as the labeled precursor. The in vitro-synthesized RNA was fractionated by electrophoresis on a 4% polyacrylamide gel containing 8 M urea. The 122-nucleotide band was excised and eluted from the gel. T_1 RNase fingerprinting was done by the method of Brownlee et al. (2) as described by Busch et al. (3).

Reverse transcriptase elongation. Subclone puKpn/Bam was digested with *Sal*I and *Dra*I, and the 54-bp fragment was gel isolated, phosphatase treated, and kinase labeled. The labeled fragment was strand separated (26), and the single-stranded DNA was electroeluted from the gel. RNA was synthesized in vitro from *Bam*HI-truncated puKpn/Bam plasmid. In vitro-synthesized RNA was gel purified and isolated. Gel-purified single-stranded DNA and RNA were dried together and suspended in 10 μl of hybridization buffer (0.4 M NaCl, 0.025 M Tris hydrochloride [pH 7.5], 30 μg of tRNA). The hybrids were sealed in capillary tubes, heated at 68°C for 25 min, and then incubated at 42°C for 20 h. The

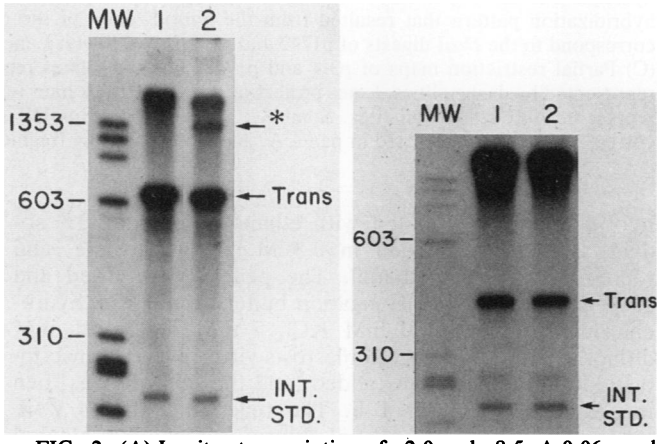

FIG. 2. (A) In vitro transcription of p2.0 and p8.5. A 0.06-pmol sample of p2.0 (0.26 μg) and of p8.5 (0.5 μg) truncated at the *Xho*I site was transcribed (lanes 1 and 2, respectively). The expected transcripts of 638 nucleotides are indicated (Trans). The asterisk designates the higher-molecular-weight transcript seen only when NTS sequences upstream of −286 were included in the assay. The molecular weight markers (MW) here and on subsequent gels are ^{32}P-labeled *Hae*III fragments of φX174 DNA. The internal standard (INT. STD.) here and on subsequent gels is an end-labeled DNA fragment of 240 bp added after transcription, as described previously (4). Numbers on left are expressed in nucleotides. (B) In vitro transcription of puXba/Bam and puKpn/Bam. A 0.43-pmol sample (1.0 μg) of puXba/Bam and puKpn/Bam truncated at the *Bam*HI site was transcribed (lanes 1 and 2, respectively). The 427-nucleotide transcript is marked (Trans). Molecular weight markers (MW) and internal standard (Int. Std.) are as described in panel A.

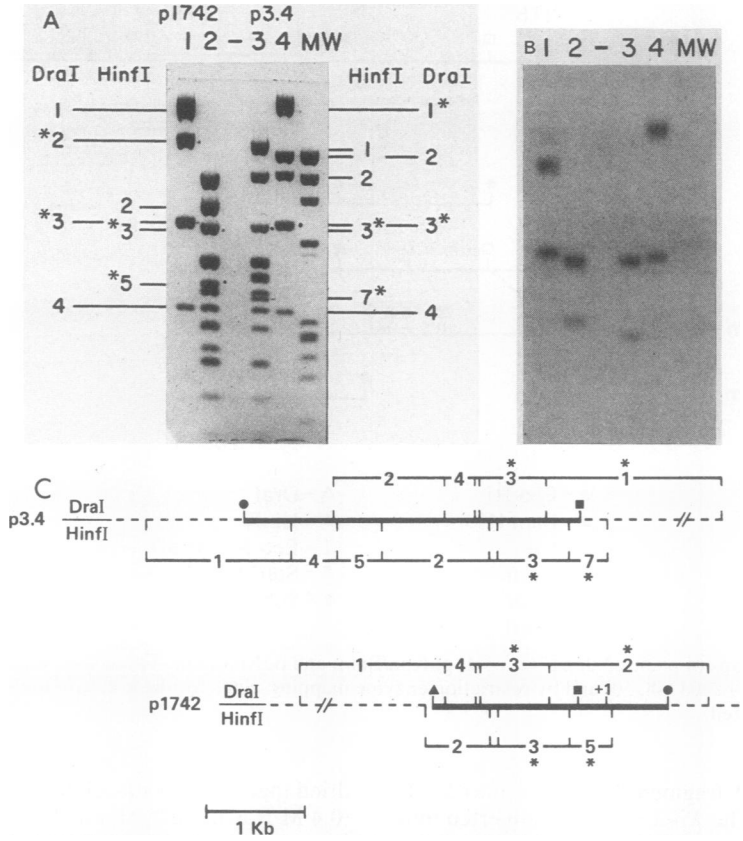

FIG. 3. Southern mapping of p1742 and p3.4 with in vitro-synthesized RNA as the probe. (A) p1742 and p3.4 were digested with *Dra*I (lanes 1 and 4, respectively) and with *Hin*fI (lanes 2 and 3, respectively), and the fragments were resolved on a 2% agarose gel. Numbers beneath the *Hin*fI and *Dra*I labels correspond to the numbered fragments depicted in panel C. The asterisks designate fragments which hybridized to the in vitro-synthesized RNA as shown in panel B. MW, Molecular weight markers. (B) Autoradiograph of the Southern hybridization pattern that resulted from the hybridization of the gel shown in panel A with in vitro-synthesized RNA. Lanes 1 and 4 correspond to the *Dra*I digests of p1742 and p3.4, respectively. Lanes 2 and 3 correspond to the *Hin*fI digests of p1742 an p3.4, respectively. (C) Partial restriction maps of p3.4 and p1742. The thick lines represent the rDNA NTS portions of the clones, and vector regions are represented by dashed lines. Lines projecting above the thick lines represent *Dra*I restriction sites, and lines projecting beneath the thick lines represent *Hin*fI restriction sites. Symbols: ●, *Eco*RI; ■, *Bam*HI; ▲, *Sph*I (*Xba*I). Numbered fragments above and below the thick lines correspond to those depicted in panel A. Asterisks mark the fragments which hybridized to the RNA. Kb, Kilobase.

hybrids were precipitated with ethanol containing 2% sodium acetate, suspended in 0.3 M sodium acetate, and reprecipitated with ethanol. The pellets were dried and suspended in 50 μl of elongation buffer (5 mM Tris hydrochloride [pH 8.0], 120 mM KCl, 7 mM $MgCl_2$, 10 mM dithiothreitol). Avian myeloblastosis virus reverse transcriptase (30 units; 3 μl) was added, and the mixture was then incubated at 42°C for 1 h. The reaction mixtures were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), precipitated with ethanol, dried, and suspended in 3 μl of sequencing dye mix. A total of 1,000 cpm were loaded on an 8% sequencing gel alongside Maxam and Gilbert (27) sequencing ladders of the corresponding *Dra*I-*Kpn*I, double-stranded, single-end-labeled DNA fragment.

DNA analysis. Micro Genie (Beckman Instruments, Inc., Fullerton, Calif.) was used on a Sperry microcomputer to identify homologies and search for restriction sites.

RESULTS

Subclones p2.0 and p8.5 were transcribed in vitro after truncation with *Xho*I (Fig. 2A, lanes 1 and 2, respectively). The individual RNA products from each reaction were resolved by gel electrophoresis. As expected, the transcript from the major promoter in both templates ran at 638 nucleotides (Trans). When the template used was p8.5, an extra, high-molecular-weight transcript (ca. 1,350 nucleotides) was seen (Fig. 2A, lane 2). In this experiment, relatively higher levels of template were used, conditions which have been shown to eliminate the effect of the upstream activator sequence (4). The synthesis of this RNA was insensitive to high levels of α-amanitin (300 μg/ml) in the transcription reaction, indicating that it was an RNA polymerase I transcription product. The size of this transcript suggested that it initiated approximately 700 bp upstream of the major promoter.

This region was further subcloned to perform a more detailed analysis of this upstream promoter. Two overlapping subclones containing the NTS sequences between −1742 and −286 (puXba/Bam) and −1018 and −286 (puKpn/Bam) were constructed (Fig. 1). Each of these subclones when truncated with *Bam*HI was capable of directing the synthesis of an RNA product of 428 nucleotides that was also resistant to 300 μg of α-amanitin per ml (Fig. 2B, lanes 1 and 2).

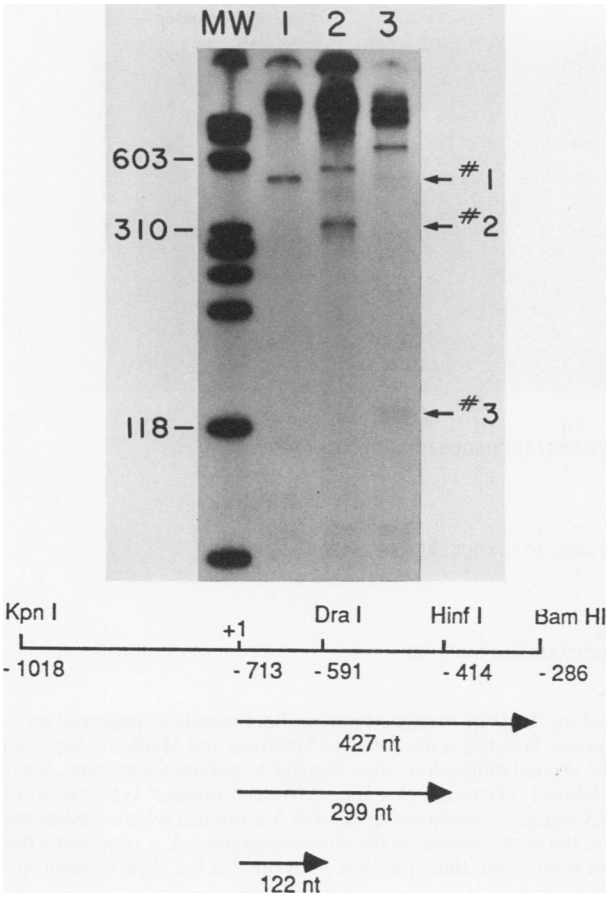

FIG. 4. Mapping of the in vitro initiation site in puKpn/Bam with truncated templates. A 0.43-pmol sample of puKpn/Bam truncated with *Bam*HI. *Hin*fI, or *Dra*I was transcribed in vitro (lanes 1 to 3, respectively). The predicted RNA transcripts from each truncated template are indicated. The *Bam*HI-truncated template (lane 1) yielded a 427-nucleotide transcript (#1). The *Hin*fI-truncated template (lane 2) yielded a 299-nucleotide transcript (#2). The *Dra*I-truncated template (lane 3) yielded a 122-nucleotide transcript (#3). The additional bands in lanes 2 and 3 appear to correspond to end-to-end transcripts. MW, Molecular weight markers; numbers on left are in nucleotides. Lower panel: Restriction map showing the truncated transcripts (nt, nucleotides).

From the size of the transcripts it appeared that a sequence upstream of the major promoter in the NTS could direct initiation of transcription. However, these results could also be due to initiation of transcription from within the vector DNA and termination elsewhere in the clones. Two experiments, described below, were designed to demonstrate that the size of the RNA being synthesized was the result of initiation within the NTS and runoff transcription because the template was truncated at the *Bam*HI site. In the first experiment, in vitro-synthesized RNA was isolated and used as a probe for Southern analysis (Fig. 3). Clones p3.4 and p1742 were digested with either *Dra*I or *Hin*fI. Figure 3A is a picture of the ethidium bromide-stained 2% agarose gel used to resolve the digested DNA fragments. These fragments were transferred to nitrocellulose and hybridized with radioactive, in vitro-synthesized RNA that had been gel purified (Fig. 3B). The DNA fragments that hybridized to this RNA correspond to overlapping regions of the two clones and are depicted in Fig. 3C. The *Dra*I fragments 1 and 3 from both clones hybridized, as did *Hin*fI fragments 3 and 5 from p1742 and fragments 3 and 7 from p3.4. No hybridization signal was detected from fragments that did not contain NTS DNA.

In the second experiment, restriction enzyme sites upstream of the *Bam*HI site (−286) were used to truncate the template. Theoretically, digestion further upstream from the *Bam*HI site should yield shorter RNA transcripts if the transcripts were terminating because of the truncation. Three different restriction enzymes were used to truncate the template for in vitro transcription. The template, puKpn/Bam, digested with *Bam*HI (−286) supported the synthesis of a 428-nucleotide transcript (Fig. 4). With *Hin*fI (−414)- and *Dra*I (−591)-truncated templates, the transcripts synthesized were 299 and 122 nucleotides, respectively. The results of in vitro transcription of these truncated templates were consistent with the possibility that the polymerase was initiating around −710 in the NTS and elongating downstream, toward the major promoter.

To confirm the identity of the *Dra*I transcript, we performed RNA fingerprint analysis on the 122-nucleotide transcript synthesized with puKpn/Bam truncated at the *Dra*I site as the template. RNA was synthesized in vitro with either GTP or UTP as the labeled precursor. The corresponding T_1 RNase fingerprints are shown in Fig. 5A and B, respectively. It was possible to assign each spot to a particular oligonucleotide expected from the sequence of this region of the DNA (Fig. 5C). Comigrating spots in the UTP-labeled RNA fingerprint confirmed the assignment of specific spots in the GTP-labeled RNA fingerprint. Spot A in the UTP-labeled map was a particularly unique spot which corresponded to the last 27 nucleotides of the transcript and consisted of two long stretches of T's which would be labeled U's in the RNA. From these results, we concluded that the NTS was directing transcription by RNA polymerase I and that the transcript was initiating within 5 nucleotides of −713.

A primer elongation assay was used to determine the initiation site of this transcript. A *Dra*I-*Sal*I fragment of puKpn/Bam corresponding to a region downstream of the putative initiation site was prepared as described in Materials and Methods. After strand separation, both strands were hybridized separately with the RNA synthesized in vitro from the *Bam*HI-truncated puKpn/Bam subclone. After hybridization and elongation with reverse transcriptase, the products of both reactions were analyzed by electrophoresis on an 8% sequencing gel (Fig. 6) alongside Maxam and Gilbert (27) sequencing reaction products of the corresponding region of DNA. The single-stranded DNA which was complementary to the RNA was elongated by 64 nucleotides (Fig. 6, lane 2). After correction for differences in mobility (24, 40), the 5′ terminus of the elongation product corresponded to the T on the sequence ladder, nucleotide −713. The incompletely elongated bands seen in Fig. 6, lane 2, may be due to pauses at G+C-rich regions or areas of secondary structure within the RNA. Alternatively, there may be weaker initiation start sites downstream of −713. However, based on the truncated template assays and the fingerprint analysis, it is unlikely that there are stronger initiation sites downstream or upstream of −713.

The polarity of the single-stranded DNA used in the elongation assay was determined by sequencing and by the fact that the asymmetric restriction cuts at either end of the DNA left the noncoding strand 4 nucleotides longer than the coding strand. This allowed us to unambiguously assign the polarity of the transcription. No elongation product was detected from the noncoding strand (Fig. 6, lane 1), demon-

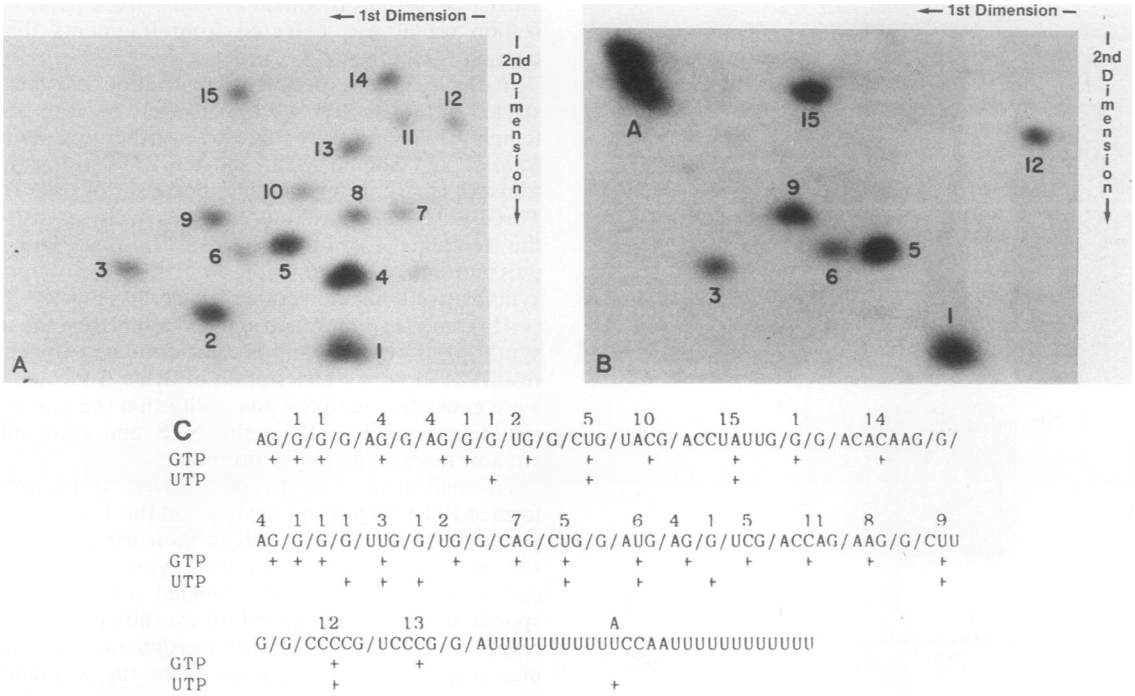

FIG. 5. T_1 fingerprint analysis of the 122-nucleotide transcript synthesized on the *Dra*I-truncated puKpn/Bam template. puKpn/Bam was truncated with *Dra*I and transcribed in vitro, and the 122-nucleotide transcript was isolated as described in Materials and Methods. Separation in the first dimension was by cellulose acetate electrophoresis and that in the second dimension, after transfer to polyethyleneimine, was by homochromatography. (A) RNA fingerprint of the product synthesized with labeled GTP as the precursor. (B) RNA product synthesized with labeled UTP as the precursor. (C) T_1 fragments are designated along the RNA sequence predicted by the DNA sequence where slashes mark the T_1 RNase cleavage sites. The numbers above each fragment correspond to the spots labeled on the chromatographs. A + represents those fragments which would be labeled by either GTP (top row) or UTP (bottom row). Note that spot A is seen only in the UTP fingerprint.

strating that the RNA product was being initiated at −713, in the same orientation as the 45S rRNA major promoter downstream.

Additional experiments were done to estimate the efficiency at which the upstream promoter is utilized in vitro in comparison with the major promoter. When the transcription of equal picomoles of puKpn/Bam (which contains only the upstream promoter) and p2.0 (which contains only the major promoter) were compared, p2.0 was transcribed ninefold more efficiently (Fig. 7). In contrast, when we compared the amount of both RNA products synthesized simultaneously from p8.5 (similar to that in Fig. 2A, lane 2), which contains both promoters, the major promoter was utilized at least 50-fold more efficiently than the upstream promoter. The implications of these differences are discussed below.

The sequence of the region capable of directing transcription was examined to understand its relationship to the major promoter. A 13-nucleotide sequence was found that is 100% homologous to the nucleotides of the major promoter between −18 and −6 (Fig. 8). This sequence lies exactly 6 nucleotides upstream of the upstream promoter initiation start site, just as it does with respect to the initiation site of 45S rRNA. No other homologies to the major promoter site were found in this region of the NTS.

DISCUSSION

We identified and characterized an additional RNA polymerase I initiation site upstream of the major promoter within the NTS of the rat ribosomal gene. The in vitro transcript directed by this promoter was characterized by Southern analysis, truncated template assays, fingerprint analysis, and reverse transcriptase elongation assays. The initiation site was determined to be 713 nucleotides upstream from the major promoter initiation site (+1) and begins with an A residue. Sequence analysis of this region revealed a 13-nucleotide sequence which is 100% homologous with the major core promoter (−18 to −6) and is located 6 nucleotides upstream of the upstream initiation site.

Evidence that this initiation site is utilized in vivo has been recently reported by Harrington and Chikaraishi (16) and by Grummt et al. (12). Utilizing in vitro nuclear runon experiments, Harrington and Chikaraishi demonstrated the synthesis of significant quantities of nascent RNA transcripts which hybridized to the NTS. Apparently, these transcripts are rapidly degraded in vivo, as they were not equally represented when the steady-state RNA was analyzed, a result consistent with the observations of Wood et al. (46). The in vivo transcripts detected by Harrington and Chikaraishi terminated before the 45S initiation site, near the *Sal*I box (−167). They suggested that the transcript is originating slightly upstream of, or within, the 130-bp repeats which constitute the variable region of the rat rDNA repeat. The upstream promoter which we identified lies 63 bp upstream of the *Sal*I-*Sal*I fragment which contains the variable region of the rat rDNA repeat. In the mouse rDNA repeat, the variable region is found within a 1.75-kilobase *Sal*I-*Sal*I fragment (12, 25). The analogous upstream promoter would lie approximately 65 bp upstream of this fragment, or 1,918 bp upstream of the mouse 45S initiation site, in agreement with the preliminary results of Grummt et al. (12).

Enhancers, upstream promoters, and upstream initiation sites have been previously described for the ribosomal genes of several nonmammalian eucaryotes. Elison and Warner (9)

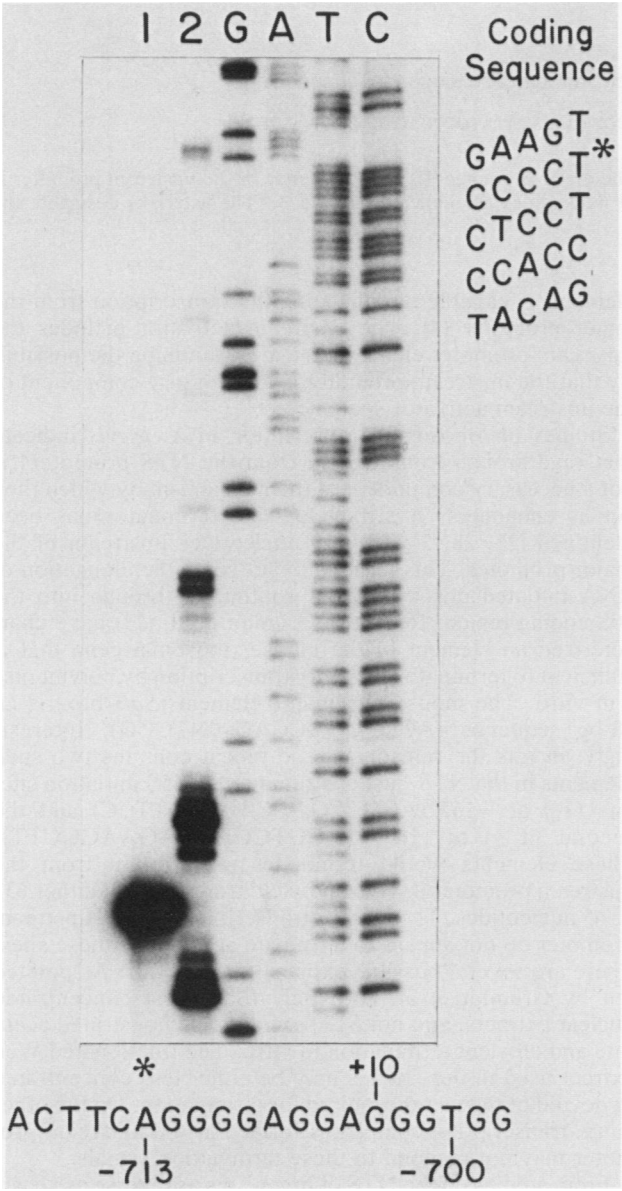

FIG. 6. Mapping of the 5′ end of the in vitro-synthesized RNA. Single-stranded end-labeled DNA corresponding to the noncoding and coding strands (lanes 1 and 2, respectively) was hybridized to the in vitro-synthesized RNA and elongated with reverse transcriptase. The reaction products were electrophoresed alongside sequence ladders of the corresponding 5′-end-labeled double-stranded DNA. The reactions were: G (G), A+G (A), T+C (T), and C (C). The asterisks designate the 5′ end of the in vitro elongation products on the DNA coding-strand sequence (along the side) and the noncoding strand (along the bottom).

have reported the characterization of an upstream activator sequence 2 kilobases upstream of the 5′ end of the yeast 30S pre-rRNA. In some strains of yeasts, this element could also direct the initiation of RNA polymerase I transcription. Kohorn and Rae (18) reported that the NTS of *Drosophila* rDNA contained several repeats of imperfect copies of the promoter region. Miller et al. (29) suggested that the role of these repeats is to facilitate initiation and provide a selective advantage to the downstream promoter.

The NTS of *Xenopus* rDNA also contains sequences homologous to the major promoter (for a review, see reference 34). Two elements of the rDNA promoter of *X. laevis* have been duplicated. The Bam islands represent imperfect copies of the promoter (nucleotides −145 to +4) (1); and the 60/81-bp repeats are homologous to the sequences between −72 and −114 of the promoter. It is clear from electron microscopic (31) and hybridization studies (33, 36) that the Bam islands are capable of directing initiation, albeit at a very low efficiency. Maximally efficient utilization of the downstream promoter occurs when upstream elements are colinear with it. The Bam islands and the 60/81-bp repeats appear to act synergistically. Labhart et al. (20) have demonstrated that the 60/81-bp repeats have enhancer properties, and it was suggested that these repeats of the promoter region activate the ribosomal genes (35). DeWinter and Moss (7) demonstrated that maximally efficient transcription requires the presence of at least one Bam island (NTS promoter). Therefore, both the Bam islands and the 60/81-bp repeats may have regulatory roles in the transcription of *Xenopus* rDNA.

We reported that an element capable of enhancing the in vitro transcription rate of the rat rRNA gene has a 5′ border within the *Kpn*I-*Bam*HI (−1018 to −286) fragment that contains the NTS promoter. The proximity of these two activities suggests that the NTS promoter is the enhancer, or a component of it. This would be remarkably similar to the nature of the ribosomal gene enhancers of yeast and *X. laevis* and would represent a striking example of parallel evolution. The relationship of the NTS promoter and the enhancer is under investigation.

The putative promoter of the upstream initiation site of the

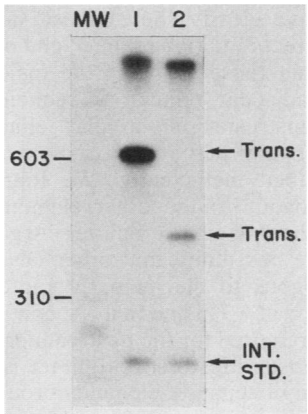

FIG. 7. Comparison of efficiencies of the upstream promoter and the major promoter. Equal picomoles of p2.0 truncated at the *Xho*I site and puKpn/Bam truncated at the *Bam*HI site were transcribed in vitro (lanes 1 and 2, respectively). The expected transcripts from each truncated template are marked (Trans.). The molecular weight markers (MW) and the internal standard (INT STD.) are those described in the legend to Fig. 2. Numbers on the left are expressed in nucleotides.

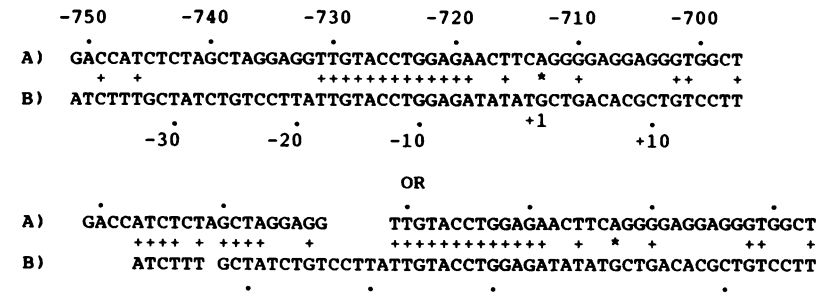

FIG. 8. Sequence homology between the upstream promoter (A) and the major promoter (B). The sequence of the upstream promoter is aligned above the homologous region of the major promoter. Conserved nucleotides are indicated with a +. The asterisks designate the initiation sites for each promoter (+1).

rat rDNA contains homology to only a part of the core promoter element and yet is still capable of initiating transcription. This homology includes the two conserved guanine nucleotides (−16 and −7) which have been shown to be essential for initiation at the major initiation site (17, 38). This region of the core promoter has been demonstrated to bind to a required initiation factor, TIF-IB (5). Clos et al. (6) also demonstrated that a point mutation at −16 eliminated the binding of this factor as well as initiation, whereas a point mutation at −7 effectively eliminated initiation without eliminating the binding of TIF-IB. On the basis of these observations, they proposed that the core promoter consists of two distinguishable parts. In addition to the core promoter element, the major promoter consists of several other DNA elements (11, 12, 15, 47) which together promote efficient initiation of transcription by polymerase I.

The upstream promoter must contain all that is necessary and sufficient to direct initiation. Finding that a region of this putative promoter 6 bp upstream of the initiation site is homologous to the core promoter element suggests (i) that this region is a part of the upstream promoter and (ii) that a protein, such as TIF-IB, is capable of recognizing this specific DNA sequence and directing the remaining steps involved in initiation of transcription by RNA polymerase I. This remains to be demonstrated. It is conceivable that the 13 conserved base pairs are capable of directing initiation by themselves. However, mutagenesis experiments of the major promoter have clearly shown these same 13 bp to be insufficient to direct initiation at the 5′ end of the 45S rRNA. It is possible that these 13 bp in the major promoter are influenced by adjacent regulatory sequences. Therefore, moving them upstream 700 bp may eliminate the other regulatory effects and permit RNA polymerase I to initiate transcription, albeit inefficiently. We found no other sequences homologous to the other elements of the major promoter near the upstream promoter. Arguing by analogy, this suggests the possibility that other DNA elements that are not homologous to the major promoter participate in effecting initiation at −713 and that a second set of transcription factors are required for the upstream promoter. Finally, it is possible that the upstream promoter is constructed by the juxtaposition of separate elements brought into proximity by DNA bending.

The upstream promoter is a weak promoter in comparison with the major promoter. This is especially evident when the two are colinear as they are in vivo. Thus, the function of the upstream promoter may not be to initiate transcription but to act as a separate signal to increase the efficiency of the utilization of the major promoter. We have previously demonstrated that a region of the NTS, the upstream activator element, is capable of enhancing the transcription from the major promoter (4). The upstream activator includes the upstream promoter element. We are examining the possibility that the upstream promoter is a necessary component of the upstream activator sequence.

Studies on ribosomal transcription in *X. laevis* indicate that readthrough transcription from the NTS promoters is not a necessary component of the mechanism by which they act as enhancers. A strong fail-safe terminator has been identified (21, 28, 31, 33) 213 nucleotides upstream of the major promoter. This terminator prevents the elongation of RNA initiated upstream from continuing through into the 40S coding region. Recently, Grummt et al. (13) have characterized an element of the mouse ribosomal gene that is sufficient to terminate efficiently transcription by polymerase I in vitro. The mouse terminator element (*Sal*I box) is an 18-bp sequence (AGGTCGACCAG$^{AA}_{TT}$NTCCG). Interestingly, in rats the ribosomal gene repeat contains two such elements in the NTS, just upstream of the 45S initiation site: one (T_0) at −167 (AGGTCGACCAGTTGTTCC) and the second (T_{-1}) at −635 (AGGTCGACCAGAAGGCTT). These elements would terminate transcription from the upstream promoter in vivo and yield transcripts of either 633 or 65 nucleotides. The in vitro transcripts from the upstream promoter do not appear to terminate at either of these sites. There are several possible explanations for this. As pointed out by Grummt et al. (14), only their most concentrated nuclear extracts, and not S100 extracts, demonstrated accurate and efficient termination in vitro. The fractionated Weil extract used in our studies may be either less concentrated or devoid of the factor required for termination. On the other hand, transcription complexes formed at the upstream promoter may not respond to these termination signals.

From studies on the NTS of organisms as diverse as yeasts and rats, the involvement of the NTS in the initiation of transcription seems to be a general phenomenon. It remains to be determined whether it is actually the initiation of transcription or simply the binding of transcription factors to the NTS which increases the efficiency of initiation at the major promoter. Perhaps both phenomena possess regulatory functions which allow for the rapid and accurate control of ribosomal gene expression.

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LITERATURE CITED

1. **Boseley, P., T. Moss, M. Machler, R. Portmann, and M. Birnstiel.** 1979. Sequence organization of the spacer DNA in a ribosomal gene unit of *X. laevis*. Cell **17**:19–31.
2. **Brownlee, G., F. Sanger, and B. Barrell.** 1968. The sequence of 5S ribosomal ribonucleic acid. J. Mol. Biol. **34**:379–412.
3. **Busch, H., Y.-C. Choi, Y. Dankal, C. D. Liarakos, M. R. S. Rao, T. S. Ro-Choi, and B. C. Wu.** 1976. Method for studies on messenger RNA. Methods Cancer Res. **13**:101–197.
4. **Cassidy, B., H.-F. Yang-Yen, and L. Rothblum.** 1986. Transcriptional role for the nontranscribed spacer of rat ribosomal DNA. Mol. Cell. Biol. **6**:2766–2773.
5. **Clos, J., D. Buttgereit, and I. Grummt.** 1986. A purified transcription factor (TIF-IB) binds to essential sequences of the mouse rDNA promoter. Proc. Natl. Acad. Sci. USA **83**:604–608.
6. **Clos, J., A. Norman, A. Ohrlein, and I. Grummt.** 1986. The core promoter of mouse rDNA consists of two functionally distinct domains. Nucleic Acids Res. **14**:7581–7595.
7. **DeWinter, R., and T. Moss.** 1986. Spacer promoters are essential for efficient enhancement of *X. laevis* ribosomal transcription. Cell **44**:313–318.
8. **Dhar, V. N., D. A. Miller, and O. J. Miller.** 1985. Transcription of mouse rDNA and associated formation of the nucleolus organizer region after gene transfer and amplification in Chinese hamster cells. Mol. Cell. Biol. **5**:2943–2950.
9. **Elion, E., and J. Warner.** 1984. The major promoter element of rRNA transcription in yeast lies 2 kb upstream. Cell **39**:663–673.
10. **Elion, E., and J. Warner.** 1986. An RNA polymerase I enhancer in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:2089–2097.
11. **Grummt, I.** 1982. Nucleotide sequence requirements for specific initiation of transcription by RNA polymerase I. Proc. Natl. Acad. Sci. USA **79**:6908–6911.
12. **Grummt, I., A. Kuhn, I. Bartsch, and H. Rosenbauer.** 1986. A transcription terminator located upstream of the mouse rDNA initiation site affects rRNA synthesis. Cell **47**:901–911.
13. **Grummt, I., U. Maier, A. Ohrlein, N. Hassouna, and J.-P. Bachellerie.** 1985. Transcription of mouse rDNA terminates downstream of the 3′ end of 28S RNA and involves interaction of factors with repeated sequences in the 3′ spacer. Cell **43**:801–810.
14. **Grummt, I., H. Rosenbauer, I. Niedermeyer, U. Maier, and A. Ohrlein.** 1986. A repeated 18 bp sequence motif in the mouse rDNA spacer mediates binding of a nuclear factor and transcription termination. Cell **45**:837–846.
15. **Haltiner, M., S. Smale, and R. Tjian.** 1986. Two distinct promoter elements in the human rRNA gene identified by linker scanning mutagenesis. Mol. Cell. Biol. **6**:227–235.
16. **Harrington, C. A., and D. M. Chikaraishi.** 1987. Transcription of spacer sequences flanking the 45S rDNA gene of rat. Mol. Cell. Biol. **7**:314–325.
17. **Kishimoto, T., M. Nagamine, T. Sasaki, N. Takakusa, T. Miwa, R. Kominami, and M. Muramatsu.** 1985. Presence of a limited number of essential nucleotides in the promoter region of mouse RNA gene. Nucleic Acids Res. **10**:3515–3532.
18. **Kohorn, B., and P. Rae.** 1982. Nontranscribed spacer sequences promoter *in vitro* transcription of *Drosophila* ribosomal DNA. Nucleic Acids Res. **10**:6879–6886.
19. **Kohorn, B., and P. Rae.** 1983. Localization of DNA sequences promoting RNA polymerase I activity in *Drosophila*. Proc. Natl. Acad. Sci. USA **80**:3265–3268.
20. **Labhart, P., and R. Reeder.** 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. Cell **37**:285–289.
21. **Labhart, P., and R. Reeder.** 1986. Characterization of three sites of RNA 3′ end formation in the *Xenopus* ribosomal spacer. Cell **45**:431–443.
22. **Learned, R., S. Smale, M. Haltiner, and R. Tjian.** 1983. Regulation of human ribosomal RNA transcription. Proc. Natl. Acad. Sci. USA **80**:3558–3562.
23. **Long, E., and I. Dawid.** 1980. Repeated genes in eukaryotes. Annu. Rev. Biochem. **49**:727–764.
24. **Long, E., M. Rebbert, and I. Dawid.** 1981. Nucleotide sequence of the initiation site for ribosomal RNA transcription in *Drosophila melanogaster*: comparison of genes with and without insertions. Proc. Natl. Acad. Sci. USA **78**:1513–1517.
25. **Mandal, R.** 1984. The organization and transcription of eucaryotic ribosomal RNA genes. Prog. Nucleic Acids Res. Mol. Biol. **31**:115–160.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
28. **McStay, B., and R. H. Reeder.** 1986. A termination site for *Xenopus* RNA polymerase I also acts as an element of an adjacent promoter. Cell **47**:913–920.
29. **Miller, J., D. Hayward, and D. Glover.** 1983. Transcription of the 'non-transcribed' spacer of *Drosophila melanogaster* rDNA. Nucleic Acids Res. **11**:11–19.
30. **Miller, K., J. Tower, and B. Sollner-Webb.** 1985. A complex control region of the mouse rRNA gene directs accurate initiation by RNA polymerase I. Mol. Cell. Biol. **5**:554–562.
31. **Morgan, G., R. Reeder, and A. Bakken.** 1983. Transcription in cloned spacers of *Xenopus laevis* ribosomal DNA. Proc. Natl. Acad. Sci. USA **80**:6490–6494.
32. **Moss, T.** 1983. A transcriptional role for the repetitive ribosomal spacer in *Xenopus laevis*. Nature (London) **302**:223–228.
33. **Moss, T., and M. Birnstiel.** 1979. The putative promoter of a *Xenopus laevis* ribosomal gene is reduplicated. Nucleic Acids Res. **6**:3733–3743.
34. **Reeder, R.** 1984. Enhancers and ribosomal spacers. Cell **38**:349–351.
35. **Reeder, R., J. Roan, and M. Dunaway.** 1983. Spacer regulation of *Xenopus* ribosomal gene transcription: competition in oocytes. Cell **35**:449–456.
36. **Runngger, D., H. Achermann, and M. Crippa.** 1979. Transcription of spacer sequences in genes coding for ribosomal RNA in *Xenopus* cells. Proc. Natl. Acad. Sci. USA **76**:3957–3961.
37. **Saffer, L, and O. Miller, Jr.** 1986. Electron microscopic study of *Saccharomyces cerevisiae* rDNA chromatin replication. Mol. Cell. Biol. **6**:1148–1157.
38. **Skinner, J., A. Ohrlein, and I. Grummt.** 1984. *In vitro* mutagenesis and transcriptional analysis of a mouse ribosomal promoter element. Proc. Natl. Acad. Sci. USA **81**:2137–2184.
39. **Smith, G.** 1976. Evolution of repeated DNA sequences by unequal crossover. Science **191**:528–535.
40. **Sollner-Webb, B., and R. Reeder.** 1979. The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in *X. laevis*. Cell **18**:485–499.
41. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
42. **Swanson, M., M. Yip, and M. Holland.** 1985. Characterization of an RNA polymerase I-dependent promoter within the spacer region of yeast ribosomal cistrons. J. Biol. Chem. **260**: 9905–9915.
43. **Toshimitsu, K., M. Nagamine, T. Sasaki, N. Takakusa, T. Miwa, R. Kominami, and M. Muramatsu.** 1985. Presence of a limited number of essential nucleotides in the promoter region of mouse ribosomal RNA gene. Nucleic Acids Res. **13**:3515–3532.
44. **Tower, J., V. C. Culotta, and B. Sollner-Webb.** 1986. Factors and nucleotide sequences that direct ribosomal DNA transcription and their relationship to the stable transcription complex. Mol. Cell. Biol. **6**:3451–3462.
45. **Wandelt, C., and I. Grummt.** 1983. Formation of stable preinitiation complexes is a prerequisite for ribosomal DNA transcription *in vitro*. Nucleic Acids Res. **11**:3795–3809.
46. **Wood, K., L. Bowman, and E. Thompson.** 1984. Transcription of the mouse ribosomal spacer region. Mol. Cell. Biol. **4**:822–828.

47. **Yamamoto, O., N. Takakusa, Y. Mishima, R. Kominami, and M. Muramatsu.** 1984. Determination of the promoter region of mouse ribosomal RNA gene by an *in vitro* transcription system. Proc. Natl. Acad. Sci. USA **81**:299–303.
48. **Yang-Yen, H.-F., and L. Rothblum.** 1986. Partial nucleotide sequence of a 3.4 kb fragment from the rat ribosomal DNA nontranscribed spacer. Nucleic Acids Res. **14**:5557.
49. **Yang-Yen, H.-F., C. Subrahmanyam, B. Cassidy, and L. Rothblum.** 1985. Characterization of rat ribosomal DNA. II. Identification of the highly repetitive DNA in the 3′ nontranscribed spacer. J. Mol. Biol. **184**:389–398.
50. **Yavachev, L. P., O. I. Georgiev, E. A. Braga, T. A. Avdonina, A. E. Bogomolova, V. B. Zhurkin, V. V. Nosikov, and A. A. Hadjiolov.** 1986. Nucleotide sequence analysis of the spacer regions flanking the rat rRNA transcription unit and identification of repetitive elements. Nucleic Acids Res. **14**:2799–2810.