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Transcription of the Mouse Ribosomal Spacer Region

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This paper describes experiments designed to test the hypothesis that DNA sequences upstream from the mouse rRNA promoter are transcribed in vivo or in vitro. Plasmid pB28 contains a *Sal*I restriction fragment that extends from −169 to −1,894 base pairs, with respect to the origin of transcription of pre-rRNA. Labeled RNA synthesized in intact cells does not hybridize to this region. Neither S1 nuclease mapping nor RNA dot blot hybridization revealed the presence of sequences complementary to this region. Transcriptional studies carried out in vitro indicated that this region is not transcribed under conditions that are optimal for utilization of the authentic rRNA promoter. Moreover, this region does not appear to form stable transcription complexes with RNA polymerase I transcription components. These data indicate that the mouse rDNA repeating unit differs from those of *Xenopus* spp. and *Drosophila melanogaster* in that reduplicated RNA polymerase I promoters are not found in the mouse rDNA spacer region.

The rDNA genes of higher eucaryotes are organized into tandem arrays composed of transcriptional units encoding pre-rRNA interspersed with spacer regions of various lengths. Until recently, it was believed that the spacer regions were not transcribed. However, it has been demonstrated that the nontranscribed spacers (NTS) of *Xenopus* spp. and *Drosophila melanogaster* contain RNA polymerase I promoters and are actively transcribed in vivo (3, 7, 9, 13, 17, 19–21). The NTS of these organisms contain multiple polymerase I promoters within restriction endonuclease "islands"; termination apparently occurs upstream from the pre-rRNA promoter to yield multiple transcripts ranging in size from ca. 200 to 2,000 nucleotides (nt). The *D. melanogaster* NTS enhances transcription in vitro (13), and deletion of the NTS promoters of *Xenopus* spp. results in a corresponding decrease in transcription from the microinjected, pre-rRNA promoter (19). Such data suggest that the NTS promoters function as polymerase I "docking" sites to increase the utilization of the pre-rRNA promoter.

A recent paper describes a small nucleolar RNA that the authors believe originates from polymerase I promoters within the mouse NTS (22). Unfortunately, the authors were mistaken with respect to the location of the 45S pre-rRNA promoter of mouse NTS, and it is possible that the RNA they described is a 650-nt processing product cleaved from the 5′ end of 45S RNA (18). Moreover, the sequence of the mouse NTS has been determined and contains no apparent pre-rRNA promoter homologies (14). From available data, it is not possible to unequivocally state that the mouse NTS is transcribed. If the mouse NTS is transcribed, it must contain polymerase I promoters which differ from the pre-rRNA promoter in sequence and perhaps in regulatory properties. This laboratory group is involved in studying the regulation of initiation of rRNA synthesis, and we therefore undertook to determine whether the mouse NTS region is transcribed in vivo or in vitro or both. We report here that by several criteria this does not occur in a mouse cell culture line.

MATERIALS AND METHODS

Preparation of cellular and nuclear RNA. The cell line used in these experiments was derived from murine lymphosarcoma P1798 (8, 24; A. H. Cavanaugh, P. K. Gokal, R. P. Lawther, and E. A. Thompson, Proc. Natl. Acad. Sci. U.S.A., in press). The cells were maintained in RPMI 1640 containing 5% fetal bovine serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10 mM glucose, 6 mM glutamine, and 2×10^{-5} M 2-mercaptoethanol. Cultures were grown in suspension in a humidified atmosphere of 95% air–5% CO_2, and mid-log-phase cultures containing 0.5×10^6 to 1×10^6 cells per ml were used for all experiments. Labeled RNA, or unlabeled cellular RNA was prepared as follows. Labeled RNA was prepared by incubating cells with [^{3}H]uridine or $^{32}P_i$ as described below. Cells (10^7) were washed three times in Dulbecco phosphate-buffered saline (Flow Laboratories, Inc.) and transferred to a 1.5-ml plastic microfuge tube. The cell pellet was suspended in 0.5 ml of extraction buffer (containing 0.25 M NaCl, 50 mM Tris-hydrochloride [pH 7.4], 5 mM EDTA, 1% sodium dodecyl sulfate, and 1 mg of proteinase K [EM Biochemicals] per ml), incubated for 30 min at 37°C, sonicated for three 5-s bursts to shear DNA, and extracted with a solution containing 0.5 ml of phenol and 0.25 ml of chloroform. The aqueous phase was extracted again with phenol-chloroform (2:1), washed twice with chloroform, and precipitated with ethanol. The pellet was suspended in 0.1 M sodium acetate buffer (pH 5.1, containing 4 mM $MgCl_2$), digested for 30 min at 37°C with 2 μg of RNase-free DNase (25) per ml, extracted with phenol-chloroform, and precipitated with ethanol. Nuclear RNA was prepared from mouse L-cell nuclei by a similar procedure.

Recombinant plasmids. Pertinent aspects of the organization of the mouse rDNA repeating unit are shown in Fig. 1A. The area of interest contains three *Sal*I restriction sites which divide the gene into the 3.2-kilobase (kb) *Sal*I B fragment and the *Sal*I-D region (1, 4, 26). The *Sal*I D fragment is variable in length and contains most of the so-called NTS. The *Sal*I D fragment has been cloned in pBR322 as a 1.7-kb *Sal*I insert (2) and has been sequenced (14). The cloned *Sal*I D fragment is designated pB28 and was obtained from Norman Arnheim (State University of New York at Stony Brook). The identity of this plasmid was confirmed by restriction mapping with *Pvu*II and *Sal*I (2). The authentic pre-rRNA promoter (designated P45 in Fig. 1A) lies within the *Sal*I B fragment, which contains ca. 3.0 kb of external transcribed spacer (ETS) DNA (1). The *Sal*I B fragment, inserted into pBR322, is designated pI23 and was also

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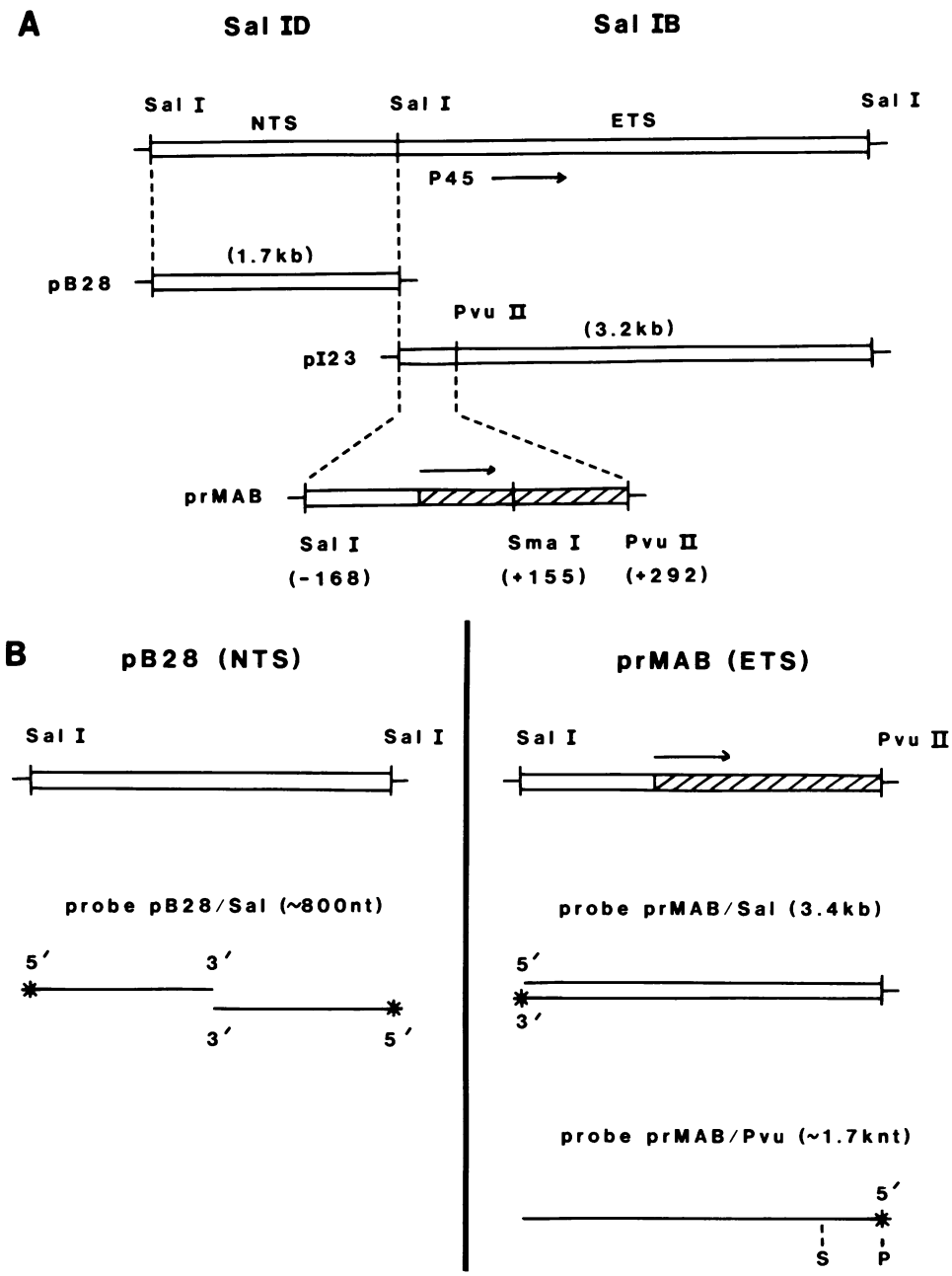

FIG. 1. (A) Restriction map of the 5'-flanking region of mouse rDNA. The mouse rDNA promoter (designated P45) is located within the *Sal*I B fragment. Transcription of pre-rRNA proceeds in the direction indicated by the arrow. The transcribed portion of the gene, contained in prMAB, is indicated by crosshatching. The arrow indicates the direction of transcription. (B) Ribosomal DNA probes prepared from pB28 and prMAB as described in the text. The size of the probes was estimated by gel electrophoresis of the 5'-end-labeled fragments. The positions of the *Sal*I and *Pvu*II restriction sites on single-stranded prMAB/*Pvu*II are indicated by S and P, respectively.

obtained from Norman Arnheim. Both pB28 and pI23 were originally isolated by Marshall Edgel (University of North Carolina at Chapel Hill).

Transcription of mouse rDNA initiates at an A residue 168 base pairs (bp) downstream from the promoter-proximal *Sal*I restriction site of pI23 (18). This region has been sequenced (4, 26) and contains a unique *Sma*I restriction site at +155 bp and a unique *Pvu*II restriction site at +292 bp (with respect to the initiation site). A subclone, designated prMAB, contains the 45S promoter and origin of transcription within a *Sal*I-*Pvu*II fragment that replaces the *Sal*I-*Pvu*II fragment of pBR322. Plasmid prMAT2 (not shown in Fig. 1A) was made by inserting a *Bgl*II linker into the unique *Pvu*II site.

Probes for S1 nuclease mapping. Single-stranded probes were prepared with exonuclease III (New England BioLabs) as described by Miller and Sollner-Webb (18). The 1.7-kb *Sal*I insert of pB28 was digested with exonuclease III under conditions recommended by the manufacturer and 5' end labeled (16) to yield two half-length linear fragments of ca. 800 nt (Fig. 1B). prMAB and prMAT2 were digested with *Pvu*II and *Bgl*II, respectively, and exonuclease III and 5' end labeled to yield single-stranded probes of ca. 1,700 nt.

Single-stranded probes (0.1 pmol, ca. 10^7 dpm/pmol of DNA) were hybridized overnight at 65°C in the presence of mouse RNA in a solution containing 0.3 M NaCl, 0.1 M Tris-hydrochloride (pH 8.0), 1 mM EDTA, and 0.3 mg of *Escherichia coli* tRNA per ml. The hybridization reaction was diluted 10-fold into S1 buffer (to a final concentration of 5% glycerol, 0.25 M NaCl, 0.03 M sodium acetate [pH 4.5], 1 mM $ZnSO_4$, and 100 μg of phenol-extracted calf thymus DNA per ml at 4°C) and digested for 50 min at room temperature with 20 to 100 U of S1 nuclease (P-L Biochemicals, Inc.). The reaction was extracted with phenol-chloroform (2:1), precipitated with ethanol, dissolved in 4 M urea containing 90 mM Tris · borate–EDTA buffer and 0.02% each of bromophenol blue and xylene cyanol, and resolved on 5% denaturing polyacrylamide gels (15). Standards were 5′-end-labeled fragments of *Hae*III-digested φX174 replicative-form DNA (φX174RF).

The large fragment of DNA polymerase I was used to 3′ end label *Sal*I-digested prMAB (Fig. 1B) under conditions recommended by the manufacturer (New England Nuclear Corp.). Cellular RNA and 0.1 pmol of the probe (ca. 10^7 dpm/pmol) were dissolved in 80% formamide containing 0.4 M NaCl, 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8), and 1 mM EDTA (5). Hybridization was carried out for 2 h as described in the legend to Fig. 3, and the products were digested with S1 nuclease and resolved on denaturing polyacrylamide gels as described above.

Filter hybridization. Nick-translated probes were prepared with DNA polymerase I (New England Nuclear) as described by Rigby et al. (23). Dot blot hybridization and hybridization of labeled RNA to filter-immobilized plasmids were carried out as described previously (8).

Transcription in vitro. Unless otherwise noted, transcription was carried out for 30 min at 30°C in a 50-μl reaction containing 5 to 10 μCi of [α-^{32}P]UTP, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 25 μM UTP, 30 mM HEPES (pH 7.9), 100 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and ca. 0.4 pmol of plasmid DNA (Cavanaugh et al., in press). Transcription was initiated by the addition of 10 μl of S100 extract prepared from P1798 cells as previously described (Cavanaugh et al., in press). Reactions were terminated by the addition of 5 μl of 2% sodium dodecyl sulfate with 5 μg of *E. coli* tRNA per ml plus 5 μl of proteinase K (1 mg/ml). After digestion at 65°C for 15 min, the reaction was diluted 10-fold with water, and RNA was precipitated in 10 mM spermine (12). The precipitate was washed with 75% ethanol, dried, and resolved on 5% denaturing polyacrylamide gels as described above.

Reagents and enzymes. Nick translation and 3′-end-labeling reactions were carried out with [α-^{32}P]dATP and [α-^{32}P]dCTP (New England Nuclear; 600 Ci/mmol); [α-^{32}P]UTP (>600 Ci/mmol) and [^{3}H]uridine (45 Ci/mmol) were purchased from New England Nuclear; 5′-end-labeling reactions contained crude [γ-^{32}P]ATP from ICN Pharmaceuticals, which also provided $^{32}P_i$. Restriction endonucleases were purchased from New England BioLabs, and reaction conditions were those recommended by the manufacturer. Other enzymes were purchased from the sources indicated.

RESULTS

Studies with cellular RNA. Pulse-labeling studies were carried out to estimate the amount of NTS transcripts synthesized in P1798 cells. Labeled RNA was synthesized by incubating cells for 15 min with [^{3}H]uridine (250 μCi/ml). Nucleic acids were extracted and hybridized to pB28 or pBR322 immobilized on nitrocellulose filters. Seven preparations of pulse-labeled RNA were tested, and none contained ^{3}H-labeled RNA, which bound to pB28 to a significantly greater extent than it did to pBR322 (Table 1, experiments a through g). Two RNA preparations (a and b) were simultaneously hybridized to filter-immobilized pI23 (Table 1), and ca. 1.4% of the labeled RNA bound to the cloned ETS DNA. Labeled RNA was also synthesized by labeling cells for 6 h in the presence of $^{32}P_i$ in phosphate-free medium. Of this RNA, ca. 1.2% bound to pI23, whereas binding to pB28 was never more than 30% greater than binding to pBR322 (data not shown).

RNA dot blot hybridization was used to determine whether NTS transcripts could be detected by this more sensitive approach. Unlabeled RNA was extracted from P1798 cells or mouse L-cell nuclei, annealed to nitrocellulose filters, and hybridized to nick-translated pB28 (4.6×10^8 ^{32}P cpm per μg) or pI23 (1.8×10^8 ^{32}P cpm per μg). The results are shown in Fig. 2. Transcripts from the ETS region were detectable in 0.1 μg of cellular RNA after 40 h of exposure and in 0.1 μg of nuclear RNA after 24 h of exposure. However, after 120 h of exposure, no detectable hybridization occurred with pB28.

As a final attempt, S1 nuclease mapping was employed to detect NTS transcripts. Single-stranded DNA was prepared from the 1.7-kb *Sal*I insert of pB28 (Fig. 1B). The 5′ ends of such fragments were labeled with ^{32}P, hybridized to cellular RNA, and subjected to S1 mapping. No portion of the 5′ end of the probe was protected by cellular RNA (Fig. 3A, lanes a through d). Cellular RNA was hybridized to the 5′-end-labeled prMAB/*Pvu*II probe (Fig. 1B) and subjected to S1 mapping. Cellular RNA protected a fragment of ca. 300 nt that corresponds to the 5′ end of 45S RNA (Fig. 3A, lane e). In addition, the ETS subclone prMAB was linearized with *Sal*I, and the 3′ ends were labeled with the large fragment of DNA polymerase (Fig. 1B). This probe was hybridized with cellular RNA and subjected to S1 mapping. The 3′ end of this probe was not protected by cellular RNA, indicating that there are no prevalent cellular transcripts which overlap by more than 50 nt the *Sal*I site upstream from the 45S promoter (Fig. 3B).

Transcription of pB28 in vitro. The inability to detect RNA

TABLE 1. Hybridization of ^{3}H-labeled RNA to pB28 and pI23[a]

DNA	Expt	^{3}H cpm	
		Added (10^5)	Bound
pB28	a	4.8	0, 29, 8
	b	5.3	0, 0
	c	3.7	0, 44
	d	2.0	0, 6
	e	3.6	0, 12
	f	16.0	0, 0
	g	7.6	9, 0
pI23	a	4.8	14,300, 4,800, 4,600
	b	5.3	7,000, 6,200

[a] P1798 cells were incubated for 15 min at 37°C in the presence of [^{3}H]uridine. RNA was extracted and hybridized to cloned DNA probes immobilized on nitrocellulose filters, as described in the text. Replicate filters were used, and all data are corrected for the amount of ^{3}H-labeled RNA which bound to immobilized pBR322 (22 to 40 cpm per filter). ^{3}H counts per minute bound to pI23 are rounded off to the nearest 100 cpm. Efficiency of hybridization to pI23 was estimated with a [^{32}P]cRNA internal probe (8) and was 6 to 9%. Data are not corrected for efficiency of hybridization.

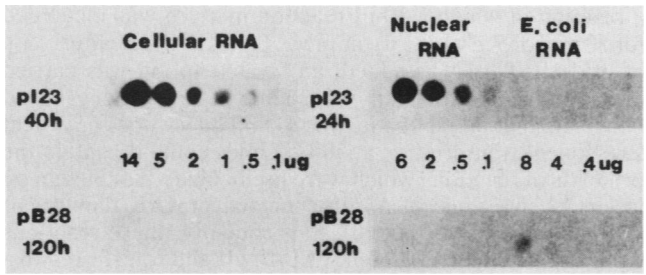

FIG. 2. Dot blot hybridization assay for ETS and NTS RNAs. Cellular RNA from P1798, *E. coli* RNA, or nuclear RNA from mouse L cells was prepared, immobilized on nitrocellulose filters, and hybridized with ^{32}P-labeled probes as described in the text. Filters were exposed to X-ray film by using intensifying screens for the time periods indicated in the figure.

complementary to the mouse NTS region could be due to extremely rapid degradation of NTS RNA in intact cells. S100 extracts from P1798 cells contain very low levels of nuclease activity and degrade newly synthesized transcripts very slowly. Under reaction conditions, transcripts of longer than 1,000 nt are degraded with a half time of longer than 1.5 h, and within a 30-min incubation there is no detectable degradation of specific transcripts from prMAB, a 5S RNA gene, or a tRNA gene (data not shown). We therefore compared the products formed when pB28 and prMAB were transcribed in cell extracts. Transcription of *Pvu*II-truncated prMAB (prMAB/*Pvu*II) yields a predominant RNA of ca. 300 nt (Fig. 4, lane a). This corresponds to the 292-nt runoff transcript reported by Miller and Sollner-Webb (18). The identity of this transcript has been confirmed by S1 nuclease mapping which demonstrates that the 5′ end of this RNA is identical to the 5′ end of authentic pre-rRNA (data not shown). No RNA of greater than 118 nt could be detected when *Sal*I-truncated pB28 was transcribed under identical conditions (Fig. 4, lane b).

Miller and Sollner-Webb (18) reported that supercoiled mouse rDNA is transcribed ca. 5 to 10 times more effectively than the corresponding linear plasmids. Quantitative S1 mapping experiments carried out in this laboratory are in agreement with this observation. S1 mapping of transcripts from supercoiled plasmids is therefore 5 to 10 times more sensitive than runoff transcription. An S1 mapping assay was used to determine whether transcripts from pB28 were synthesized at low levels. Supercoiled prMAT2, a derivative of prMAB containing a *Bgl*II linker inserted into the unique *Pvu*II site, was used as a control. Since endogenous pre-rRNA does not contain sequences complementary to the *Bgl*II recognition sequence, the 5′ end of *Bgl*II-truncated prMAT2 is not protected by endogenous pre-rRNA. RNA was extracted from transcription reactions, hybridized to single-stranded, 5′-end-labeled probes, and digested with S1 nuclease. The products were resolved on denaturing polyacrylamide gels as shown in Fig. 5. Lane a contains the fragment of the 5′-end-labeled prMAT2 probe protected by the products of a 25-μl transcription reaction containing supercoiled prMAT2. As predicted, a fragment of ca. 300 nt was protected. No protection was observed when the prMAT2 probe was hybridized to RNA extracted from a

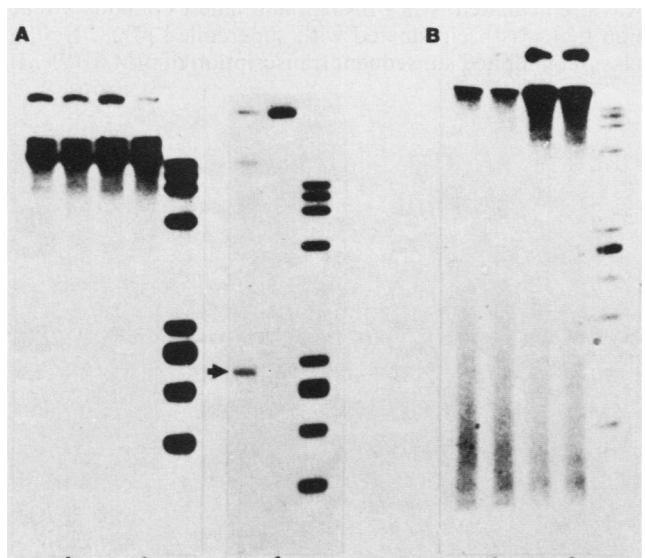

FIG. 3. S1 nuclease mapping of cellular RNA. (A) Cellular RNA was hybridized to single-stranded, 5′-end-labeled probes. Lanes a through d contain 6.5, 13, 20, and 0 μg, respectively, of RNA hybridized to the pB28/*Sal*I probe. The marker lane (designated m) contains *Hae*III-cut ϕX174RF fragments; the bottom band is 194 nt. Lanes e and f contain 1 and 0 μg, respectively, of cellular RNA hybridized to prMAB/*Pvu*II. The arrow indicates the position of the 292-nt protected fragment. All reactions were digested with 20 U of S1 nuclease, and gels containing pB28/*Sal*I (lanes a through m) were intentionally overexposed. (B) Cellular RNA was hybridized to 3′-end-labeled prMAB/*Sal*I. The probe was hybridized to 20 μg of RNA (lanes a and c) or 0 μg of RNA (lanes b and d), and reactions were digested with 20 U of S1 nuclease (lanes a and b) or 100 U of S1 nuclease (lanes c and d). Lane m contains *Hae*III-cut ϕX174RF markers; the bottom band is 118 nt.

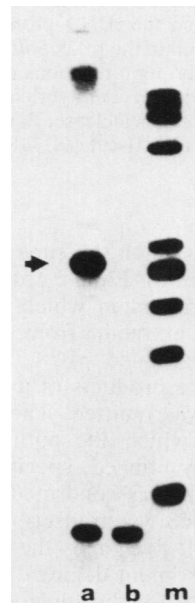

FIG. 4. Transcription of prMAB/*Pvu*II (lane a) and pB28/*Sal*I (lane b) in vitro. The bottom band in lane m is 118 nt, and the position of 292-nt runoff transcript from prMAB/*Pvu*II is indicated by the arrow. The gel was intentionally overexposed to reveal any pB28/*Sal*I transcripts which might be formed. The labeled nucleic acid of ca. 110 nt (lanes a and b) is a terminal elongation product formed by terminal deoxynucleotidyltransferase acting upon an unidentified endogenous nucleic acid (unpublished data).

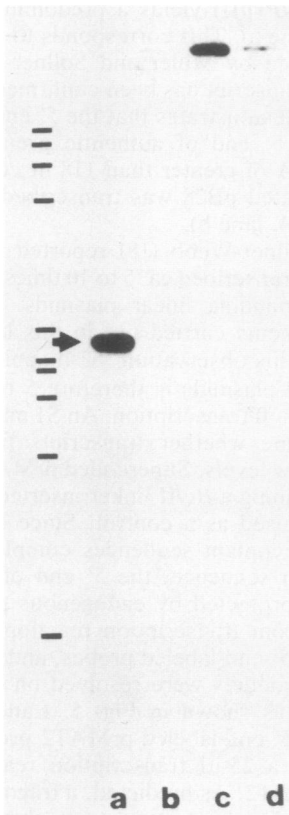

FIG. 5. S1 nuclease mapping of transcripts from prMAT2 (lane a) and pB28 (lane c) in vitro. Transcription was carried out as described in the text. Lanes a and b contain the prMAT2/*Bgl*II probe hybridized to the products of 25-μl reactions containing 0.25 and 0 μg of supercoiled prMAT2, respectively. The position of the 292-nt fragment transcribed from the rDNA promoter is indicated by an arrow. Lanes c and d contain the pB28/*Sal*I probe hybridized to the products of 100-μl transcription reactions containing 1 and 0 μg of supercoiled pB28, respectively. All hybridization reaction products were digested with 20 U of S1 nuclease. Bars to the left of the figure indicate the positions of *Hae*III-cut ϕX174RF markers, the smallest of which is 118 nt.

parallel reaction from which the supercoiled prMAT2 template was omitted (lane b). Lane c contains the products of an S1 mapping experiment in which the pB28 probe was hybridized with RNA extracted from a 100-μl transcription assay containing supercoiled pB28. Lane d is a control reaction containing the products of a 100-μl reaction from which the template was omitted. These data indicate that under conditions in which the authentic 45S pre-rRNA promoter is effectively utilized, specific initiation does not occur upon pB28. This was confirmed by an experiment in which supercoiled pB28 was transcribed in the presence of [α-^{32}P]UTP (1 mCi/ml; five times the concentration of isotope used in the experiment depicted in Fig. 4). RNA was extracted, hybridized to the unlabeled, single-stranded pB28/*Sal*I probe (Fig. 1B), and subjected to S1 nuclease digestion analysis. No labeled RNA fragment was protected by pB28 DNA (data not shown).

Competition experiments were undertaken to determine whether pB28 inhibited transcription of prMAB. The basis of this experiment is illustrated by Fig. 6. Lane a contains the products of a control transcription reaction (50-μl reaction transcribing prMAB/*Pvu*II). Lane b contains the products of a reaction in which a 50-μl reaction mixture was incubated for 30 min on ice with 1 μg of prMAB/*Pvu*II. Thereafter, 1 μg of prMAB/*Pvu*II was added, and transcription was carried out under standard conditions (30 min at 30°C). Lanes a and b indicate that preincubation for 30 min on ice does not affect subsequent transcription at 30°C. Lanes c and d contain the products of reactions which were preincubated for 30 min on ice in the presence of *Sma*I-truncated prMAB (lane c) or supercoiled prMAB (lane d). Subsequently, these reactions were fortified with 1 μg of prMAB/*Pvu*II and transferred to a 30°C water bath for 30 min. Preincubation with *Sma*I-truncated prMAB resulted in preferential synthesis of the expected 155-nt runoff transcript of prMAB/*Sma*I (open arrow, lane c). Although prMAB/*Pvu*II was present during the transcription reaction, little or no 292-nt product was formed. Likewise, preincubation with supercoiled prMAB inhibited transcription of prMAB/*Pvu*II and resulted in the synthesis of higher-molecular-weight RNA species.

The apparent decrease in transcription of prMAB/*Puv*II in the presence of pBR322 (lane g) is not reproducible and was observed with DNA from a unique pBR322 preparation. These data indicate that preincubation of transcription reaction mixtures with plasmids containing functional pre-rRNA promoters results in preferential transcription of those plasmids. A similar series of experiments was carried out to determine whether preincubation with pB28 inhibited transcription of prMAB/*Pvu*II. Lane e contains a control reaction that was not preincubated, and lane f contains products from a reaction that was preincubated on ice with prMAB/*Pvu*II before transfer to 30°C. Lane g contains a reaction that was preincubated with pBR322, and lane f contains a reaction that was preincubated with supercoiled pB28. Neither plasmid inhibited subsequent transcription of prMAB/*Pvu*II.

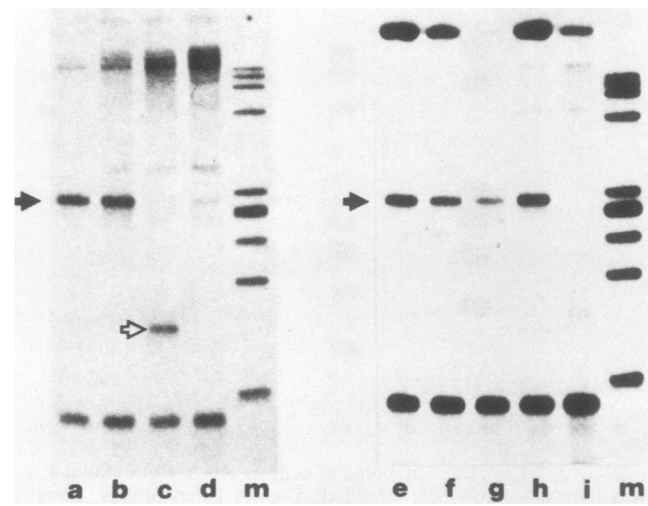

FIG. 6. Effect of preincubation upon transcription of prMAB/*Pvu*II. Lanes a and e are control reactions, and the position of the 292-nt runoff transcript is indicated by the closed arrow. Lanes b and f were preincubated with prMAB/*Pvu*II. Lane c contains the products of a reaction that was preincubated with prMAB/*Sma*I before the addition of prMAB/*Pvu*II. The position of the 155-nt runoff transcript is indicated by an open arrow. Lanes d, g, and h were preincubated with supercoiled prMAB, pBR322, and pB28, respectively, before the addition of prMAB/*Pvu*II. Lane i contains products of a reaction mixture incubated with pB28/*Sal*I for 30 min on ice and for 30 min at 30°C. The smallest *Hae*III-cut ϕX174RF fragment in lane m is 118 nt.

Lane i contains the products of a reaction in which *Sal*I-truncated pB28 was preincubated for 30 min on ice and then transferred to 30°C without the addition of prMAB/*Pvu*II.

DISCUSSION

Labeled RNA from mouse tissue culture cells does not contain detectable amounts of RNA complementary to filter-immobilized, cloned mouse NTS DNA. The minimum level of detection in these assays approaches 0.01% of the input RNA, assuming an average length of 3,000 nt. However, hypothetical NTS transcripts cannot be larger than 1,700 nt, and the sensitivity of the assay decreases in proportion to the length of the transcript. A worst-case analysis suggests that NTS transcripts are present at less than 10% of the level of ETS transcripts. Dot blot hybridization and S1 nuclease mapping procedures utilize more sensitive probes and, within the limits of stable duplex formation requirements, are not influenced by the size of the RNA. Neither approach revealed the presence of NTS transcripts. With respect to the S1 mapping experiments, it was assumed that transcripts were initiated within the 1.7-kb *Sal*I-D NTS DNA (pB28) and terminated within the 3.2-kb *Sal*I-B ETS DNA (pI23). Transcripts which terminated upstream from the 45S promoter-proximal *Sal*I site would not be detected by this approach.

Interpretation of dot blot hybridization results requires no assumptions about the sites at which the hypothetical NTS RNA originates or terminates. Even after prolonged autoradiographic exposure, mouse cellular or nuclear RNA contains no detectable sequences that hybridize with a pB28 probe with twice the specific activity of the pI23 probe (4.6×10^8 versus 1.8×10^8 cpm/μg, respectively). On this basis, it is concluded that if NTS transcripts exist in mouse RNA, they must be present at less than 1% of the amount of ETS RNA. This may indicate that initiation within the mouse NTS region is not a common event. Alternatively, these data may indicate that hypothetical NTS transcripts are degraded 100 times faster than those of ETS RNA.

Specific (i.e., preferential) degradation of RNA is assumed not to occur when cloned genes are transcribed in vitro. Nevertheless, the most sensitive techniques were unable to detect the presence of specific transcripts when the cloned NTS region was transcribed under optimal conditions for initiation at the authentic 45S promoter in vitro. Competition experiments indicate that preincubation with a plasmid containing a polymerase I promoter inhibits subsequent transcription of a second, promoter-containing plasmid. This is similar to the situation that obtains with the cloned 5S RNA genes in which preincubation results in formation of a stable transcription complex and depletion of transcription factor IIIA (6). Formation of stable transcription complexes with mouse rDNA has been reported (11, 27; V. Cizewski and B. Sollner-Webb, Nucleic Acids Res., in press). By analogy, it is proposed that limiting and as yet undefined initiation factors bind to polymerase I promoters to form complexes which do not dissociate under the reaction conditions. Data suggest that such complexes do not form with NTS DNA and are consistent with all other data, indicating that this region of the mouse rDNA repeat does not contain RNA polymerase I promoters.

The data described above indicate that DNA sequences upstream from the promoter-proximal *Sal*I restriction site (−168 bp) of the mouse rDNA repeat do not contain functional rRNA promoters and are not transcribed in vivo or in vitro. This is contrary to the situation which prevails in the rDNA repeat of *Xenopus* spp. and *D. melanogaster*. The data do not preclude the existence of enhancer sequences within this region. Neither do the data preclude the existence of reduplicated RNA polymerase I promoters in the region between +1 and −168 bp. Deletion studies indicate that sequences upstream from −39 bp are not required for transcription in vitro; however, competition experiments suggest that genes containing the sequences from −39 to −168 bp compete more effectively for transcription components than do genes deleted of this region (11). Hence, this region may play a significant role in some as yet unrecognized manner. The possibility that this region is transcribed has not been rigorously investigated. The single report dealing with transcripts arising from this region is largely uninterpretable since the authors were mistaken with respect to the origin of transcription of 45S RNA and used probes which overlapped the 5′ end of authentic pre-rRNA (22). Data from our laboratory as well as a number of others are not consistent with transcription in vitro of sequences upstream from the 45S promoter, since transcripts that are longer or shorter than those originating at +1 bp are not reproducibily detected (10, 11, 18; Cavanaugh et al., in press). Nevertheless, the role of those sequences upstream from −39 bp requires further investigation.

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