Volume 12 Number 18 1984 Nucleic Acids Research

Processing of the external transcribed spacer of murine rRNA and site of action of actinomycin D

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Received 15 March 1984; Revised 20 July 1984; Accepted 5 September 1984

ABSTRACT

The primary rRNA transcript contains a large external transcribed spacer (ETS) approximately 4,000 nucleotides in length. We have used subcloned DNA probes derived from the 5' end of the ETS in conjunction with Northern blot analysis of murine nuclear RNA to examine processing of this region. In agreement with the results of previous investigators, we find that the large rRNA precursor lacks part of the ETS region. These ETS sequences are also missing from subsequent rRNA processing intermediates. Experiments using actinomycin D confirm that the excision of portion of the ETS is an early event in rRNA processing. In addition, in the presence of actinomycin D small RNA species accumulate which hybridize to a probe specific for the 5' end of the ETS. The length of these abbreviated transcripts defines a region of rDNA which is probably a target for this drug.

INTRODUCTION

The primary transcript of ribosomal DNA (rDNA) contains a large external transcribed spacer (ETS) and the 18S, 5.8S and 28S rRNAs which are separated by two internal transcribed spacers (see Fig. 1). The synthesis and processing of this RNA has been a popular topic of scientific investigation for several years. Early studies followed rRNA processing by kinetic analysis, methylation patterns or secondary structure maps of the various intermediates (1,2). More recently, recombinant rDNA clones have been used to examine rRNA processing. The results of Bowman et al. (3) indicate that there are three major rRNA processing pathways (see Fig. 1). To simplify matters we will only discuss intermediates leading to the formation of 18S rRNA. One pathway first removes the entire ETS, yielding a 41S RNA intermediate (1-3). The 41S RNA is processed to a 20S RNA species which is the immediate precursor to 18S rRNA. An alternative pathway cleaves the primary transcript 5' to 5.8S rRNA resulting in a 34S RNA precursor to 18S. Removal of the ETS sequences from 34S RNA generates 20S RNA. In a third processing scheme, the initial cleavage occurs 5' to 28S rRNA resulting in a 37S RNA species. The 37S RNA is then processed to a 26S precursor by the excision

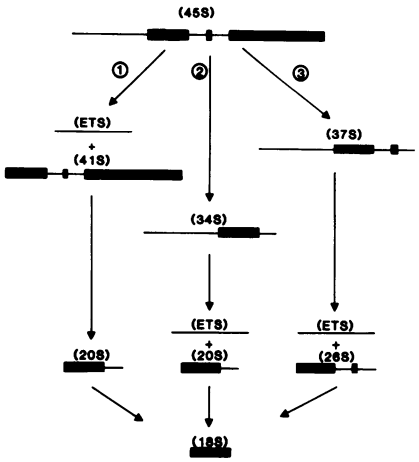

Fig. 1.
Ribosomal RNA processing pathways.

of the ETS. Cleavage of the 26S RNA liberates 18S rRNA.

Studies employing a variety of techniques demonstrate that additional events in rRNA processing involve cleavages within the ETS sequences. For example, early attempts to label 45S RNA using vaccinia virus capping enzyme met with little success indicating that sequences had been removed from the 5' terminus (4,5). Subsequently, S1 nuclease mapping of the large rRNA precursor revealed two species, one mapping to the initiation site and a second molecule lacking 650 nucleotides from the 5' end (6,7). These in vivo results were extended by demonstrating that, using an in vitro transcription assay, truncated rRNA transcripts which contain only ETS sequences are clipped 650 nucleotides downstream from the initiation site (6-8).

We have used subcloned DNA probes derived from the 5' end of the ETS to examine processing of this region. Our results confirm the experiments of Miller and Sollner-Webb (7) and others (6,8) and extend them to show that 37S and 34S rRNA intermediates also lack part of the ETS region.

MATERIALS AND METHODS

Enzymes and reagents.

Restriction endonucleases were purchased from Bethesda Research Laboratories or New England Biolabs and used as recommended by the manufacturer. T4 DNA ligase and E. coli DNA polymerase I were obtained from Bethesda Research Laboratories. [^{32}P]dCTP (800 Ci/mmole) was from New England Nuclear. Actinomycin D was purchased from Boehringer Mannheim.

Plasmids.

The murine rDNA plasmid, p5'Sal (5), containing the transcription initiation site, 160 base pairs (bp) of the nontranscribed spacer and approximately 3.0 kilobase pairs (kbp) of the ETS region was generously provided by Dr. R. Reeder (Fred Hutchinson Cancer Research Center, Seattle, Washington). To obtain a probe specific for the 5' end of the ETS, p'5Sal was digested with PvuII and electrophoresed on a 0.7% agarose gel cast in 89mM Tris-borate, 10mM EDTA. A 3.4 kbp fragment containing 160 bp of the nontranscribed spacer region, the initiation site for rRNA synthesis, the first 300 bp of ETS and adjacent pBR22 sequences was eluted, blunt-end ligated and used to transform E. coli HB101 (9). The resulting recombinants were designated pNTS. pETS was constructed by inserting the 1.4 kbp XhoI-SalI fragment of p5'Sal located 1.6 kbp downstream from the transcription start site into the SalI site of pBR322. The composition of these plasmids was confirmed by restriction endonuclease digestion and hybridization to Southern blots of p5'Sal DNA.

The ribosomal clone, λgtWES MR100, was obtained from Dr. P. Leder (Harvard University, Cambridge, MA). The insert into λgtWES MR100 is a 6.6 kbp EcoRI genomic fragment containing part of 18S rDNA, both internal transcribed spacers, 5.8S and most of 28S rDNA (10). An EcoRI-SalI digest of λgtWES MR100 was ligated to pBR322 cleaved at the EcoRI and SalI sites. Transformants were screened by the method of Grunstein and Hogness (11). Plasmids were isolated from positive colonies and identified by restriction endonuclease digests. pλr1.8 contains sequences complementary to the 3' end of 18S rRNA, all of the first internal transcribed spacer, 5.8S rRNA and approximately one-half of the second internal transcribed spacer.

A fragment specific for the internal transcribed spacer region separating 18S rRNA from 5.8S rRNA was obtained from a Sau3A digest of pλr1.8 (3).

Cell culture and RNA isolation.

Murine myeloma cells, P3K, were grown in spinner culture in Dulbecco's modified Eagle medium (GIBCO) supplemented with 5% fetal calf serum, 100 units/ml penicillin, 75 μg/ml streptomycin and 40 units/ml mycostatin.

Nuclear RNA was isolated from log-phase cells essentially as described by Glisin et al. (12). Briefly, cells were lysed in a hypotonic buffer (RSB) containing 10mM Tris (pH 7.6), 20mM NaCl, 1mM $MgCl_2$ by the addition of Nonidet P-40 to 0.5% (v/v). Nuclei were pelleted, resuspended in RSB plus 5% N-lauroyl sarcosinate and homogenized 8 times in a tight-fitting Dounce

homogenizer. Following the addition of 0.25 gm of CsCl/ml, the nuclear homogenate was layered over a 5.7M CsCl cushion and centrifuged 16 hours at 15°C in a Beckman SW41 rotor at 76,000 x g. The RNA pellets were resuspended in a buffer containing 10mM Tris (pH 7.4), 7M urea, 0.15M NaCl, 1mM EDTA and 1% sodium dodecyl sulfate (SDS), extracted with phenol-chloroform (1:1) and ethanol precipitated.

Northern blot analysis.

Nuclear RNA was fractionated by electrophoresis in agarose/formaldehyde gels (13) containing 20mM Na-Mops (3-(N-Morpholino)propane-sulfonic acid; pH 7.0 , 6% formaldehyde, 5mM sodium acetate, 1mM EDTA. Prior to electrophoresis, samples were heated at 55°C for 15 minutes in gel buffer containing 50% formamide. The gels were soaked for one hour in 2 changes of 20X SSC (1X SSC is 0.15M NaCl, 0.015M sodium citrate) and transferred to nitrocellulose filters (14). In some cases, gels were sandwich blotted (15). The filters were prehybridized for 12 hours at 42°C in 50% formamide, 5X SSC, 5X Denhardts (1X Denhardts is 0.02% each bovine serum albumin, polyvinyl-pyrrolidone, Ficoll (16)), 50 mM $NaPO_4$ (pH 6.5), 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridization reactions were performed in the same buffer and included 1-2 x 10^7 cpm of nick-translated DNA (17). The specific activity of the ^{32}P-labeled DNA was generally 1 x 10^8 cpm/µg. The filters were washed at room temperature in 4 changes of 2X SSC, 0.1% SDS for 5 minutes each followed by four 15 minute rinses in 0.1X SSC, 0.1% SDS at 42°C. Autoradiography was performed at -70°C with an intensifying screen.

Actinomycin D treatment and filter hybridizations.

P3K cells were pre-incubated for 15 minutes in the presence of 0.1 µg/ml actinomycin D (AMD), concentrated 10 fold and incubated an additional 30 minutes with 200 µCi/ml of (^3H)uridine (25 Ci/mmol, New England Nuclear). AMD was also present during the labeling period. This concentration of AMD preferentially inhibits rRNA synthesis and does not affect transcription by RNA polymerase II. Nuclear RNA was isolated as described above and hybridized to nitrocellulose filters containing p5'Sal DNA or λgtWES MR100 DNA. Hybridization reactions were incubated for 24 hours at 68°C in 0.6M NaCl, 0.1M Tris (pH 8.0), 8mM EDTA, 10X Denhardts, 0.1% SDS and 10 µg/ml yeast tRNA. The filters were processed as described by Schibler et al. (18). A variety of RNA concentrations were used for hybridization to insure that the reactions were performed in DNA excess.

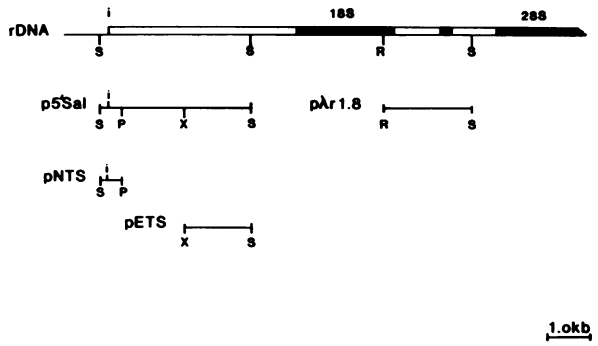

Fig. 2. Mouse ribosomal clones. Regions of murine rDNA, subcloned into pBR322, are shown in the bottom of the figure in relationship to part of a murine rRNA cistron (top line). The letters refer to the following restriction enzymes: P=PvuII, X=XhoI, S=SalI, R=EcoRI. Only the relevant restriction enzyme sites required to generate the subcloned fragments are shown. (i) is the initiation site for transcription.

RESULTS

The derivation of the rDNA clones used in this study are shown in Fig. 2. The plasmid pλr1.8 was constructed from λgtWES MR100 and contains 181 bp of 18S rDNA, the entire first internal transcribed spacer, 5.8S rDNA and 430 bp of the second internal transcribed spacer. p5'Sal consists of 160 bp of the nontranscribed spacer region as well as the initiation site for rRNA synthesis and approximately 3.0 kbp of the ETS sequence. Two subclones of p5'Sal, designated pNTS and pETS, were constructed (see Materials and Methods). pNTS contains part of the nontranscribed spacer region, the initiation site and the first 300 bp of the ETS. The pETS insert consists of a 1.4 kbp XhoI-SalI fragment derived from the 3' end of p5'Sal.

Northern analysis of murine nuclear RNA fractionated on an agarose/formaldehyde gel is shown in Fig. 3. Individual nitrocellulose strips were hybridized to pλr1.8 (lane 1), p5'Sal (lane 2), pETS (lane 3), pNTS (lane 4).

Miller and Sollner-Webb (7) as well as others (6,8) have shown that a 650 nucleotide fragment is excised from the primary rRNA transcript. Hence, the "45S" rRNA precursor actually consists of two species, one 650 nucleotides shorter than the other with the processed transcript being more abundant. The largest RNA which hybridized to pNTS appears to migrate slightly slower than the 45S bands observed with the other rDNA probes (Fig. 3, compare lane 4 with lanes 1-3). Thus, this RNA probably corresponds to the primary transcript while the 45S-like bands (45S*)

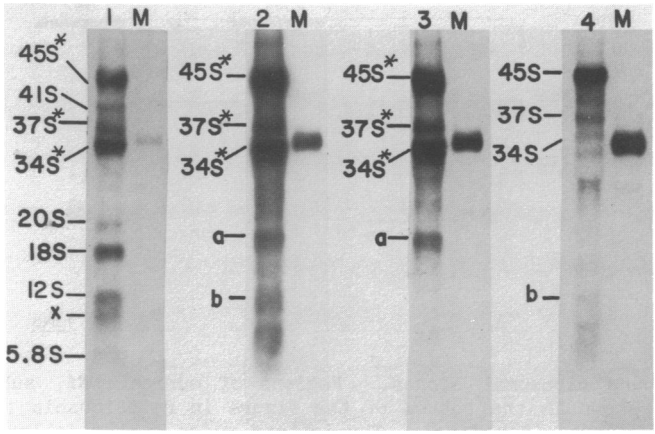

Fig. 3. Northern blot analysis of nuclear RNA from P3K cells. Nuclear RNA was separated on a 1.2% agarose/formaldehyde gel and blotted onto nitrocellulose. Individual strips were hybridized to the following nick-translated DNA probes; pλr1.8, lane 1; p5'Sal, lane 2; pETS, lane 3 and pNTS, lane 4. Linearized pBR322 DNA was included in adjacent lanes (m) to serve as a reference for standardization of the autoradiograms.

hybridizing to pλr1.8, p5'Sal and pETS may represent the processed transcript. Although the primary transcript should have been detected by all the rDNA probes, the low amount of this RNA in combination with the short exposure time of blots hybridized to p5'Sal, pETS and pλr1.8 may have precluded our ability to detect it.

As expected, only pλr1.8 and a 28S rDNA probe (data not shown) detected 41S RNA indicating that this species lacks the entire ETS. In addition, the pλr1.8 probe hybridized to 20S, 18S, 12S and 5.8S rRNAs. Previous investigators have shown that 20S RNA contains 18S rRNA sequences and the internal transcribed spacer region separating 18S from 5.8S while 12S RNA consists of 5.8S plus the adjacent downstream internal transcribed spacer (see Fig. 1 and ref. 3). A unique band (labeled x in Fig. 3, lane 1) was also detected by pλr1.8. This RNA hybridized to a DNA fragment derived from the internal transcribed spacer separating 18S and 5.8S rRNA (data not shown). Based on size (800 nucleotides) and hybridization characteristics, this band probably corresponds to an intact excised spacer fragment.

The pλr1.8 probe detected two large molecular weight RNAs in addition to 41S and the processed transcript, which we assumed were the 37S and 34S rRNA precursors. If 37S and 34S RNAs contain an intact ETS then we would expect these pre-rRNAs to hybridize to p5'Sal, pETS and pNTS. Although the

37S and 34S-like bands hybridized to p5'Sal and pETS, they did not hybridize to pNTS (Fig. 3, lanes 2-4). Since the pNTS probe encodes the 5' terminal 300 nucleotides of the primary rRNA transcript (7) the RNA species, designated 37S* and 34S*, must lack part of the ETS.

RNAs that contained only ETS sequences included a 24-26S species, which may correspond to an intact excised ETS, and two discrete RNAs approximately 2,400 and 1,000 nucleotides in length (Fig. 3, bands a and b, respectively). The 2.4 kb band contained sequences homologous to pETS (Fig. 3, lane 3), but not to pNTS. This RNA species could correspond to ETS sequences that were subsequently excised from 37S* and 34S* to generate 26S and 20S RNAs, respectively. The relatively minor 1,000 nucleotide RNA that was only detected by p5'Sal and pNTS may represent that part of the ETS which is removed prior to the formation of 37S* or 34S*.

The origin of the additional high molecular weight bands seen with ^{32}P-labeled pNTS is not clear (Fig. 3, lane 4). The bands marked 37S and 34S likely represent molecules containing the entire ETS (as depicted in Fig. 1, pathways 2 and 3) based on the hybridization probes we have used. We have observed these RNAs only with the pNTS probe. However, 37S and 34S RNAs may be in such low abundance in steady state nuclear RNA that they are not detectable with the other rDNA probes. Size estimates of the putative 37S and 34S (lane 4) versus 37S* and 34S* (lanes 1 to 3) determined by relative mobility plots indicate that the 37S* and 34S* species are approximately 1,000 nucleotides smaller than their nonprocessed precursors. This size difference suggests an additional ETS cleavage site approximately 400 nucleotides downstream from that observed at +650 by Miller and Sollner-Webb (7). Our detection of a minor band at approximately 1,000 nucleotides (band b in Fig. 3, lanes 2 and 4) using p5'Sal and pNTS strengthens this possibility. However, 5' end mapping of the 37S* and 34S* species is required to prove this suggestion and explain the size discrepancies we have noted.

Additional evidence that the large rRNA precursors lack portions of the ETS region was derived from studies using actinomycin D (AMD). Nuclear RNA was isolated from cells treated for 0, 15, 30 and 45 minutes with 0.1 ug/ml AMD. The results of a Northern analysis using this RNA probed with p5'Sal and pNTS are shown in Fig. 4A and B. After a 45 minute incubation with AMD, nuclear RNA still contained large molecular weight rRNA precursors as demonstrated by hybridization to p5'Sal (Fig. 4A, lane 4). The largest rRNA was more apparent in the AMD-treated samples after an overnight exposure. How-

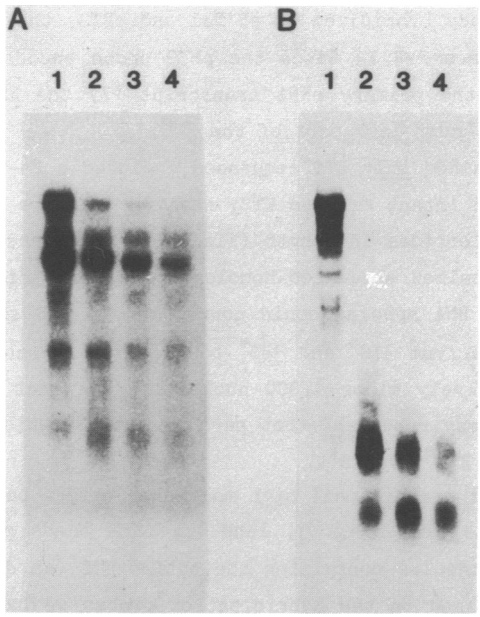

Fig. 4. Hybridization of nuclear RNA from actinomycin D-treated cells. Nuclear RNA from cells treated for 0 (lane 1), 15 (lane 2), 30 (lane 3) or 45 (lane 4) minutes with 0.1 µg/ml AMD, was fractionated on 1% agarose/formaldehyde gels and transferred to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled p5'Sal DNA (A) or pNTS DNA (B). (A) was a 2 hour exposure while (B) corresponds to an 18 hour exposure.

ever, none of the pre-rRNAs were observed when RNA from AMD-treated cells was probed with ^{32}P-labeled pNTS (Fig. 4B, lanes 2-4), even after a 4 day exposure or if RNA larger than 28S rRNA was probed with pNTS (data not shown). Since p5'Sal contains the same sequences as pNTS, rRNA species hybridizing to p5'Sal, but not to pNTS, must lack the 5' end of the ETS region. Therefore, we conclude that in addition to 37S* and 34S*, the 45S-like RNA present in AMD-treated cells has also been processed.

Surprisingly, nuclear RNA from AMD-treated cells contained a substantial amount of small, heterogeneous RNAs which hybridized to ^{32}P-labeled pNTS (Fig. 4B, lanes 2-4) and p5'Sal, but not to pETS (data not shown). These species may be due to: (1) decreased turnover of excised ETS sequences or (2) an AMD-induced block in rDNA transcription downstream from the initiation site which still permits continued synthesis of RNA from the ETS region. To distinguish between these two possibilities, cells were pre-treated for 15 minutes with AMD and then labeled in the presence of AMD for

TABLE I. Hybridization of labeled nuclear RNA from control and AMD-treated cells to filters containing p5'Sal DNA or λgtWES MR100 DNA[a].

	p5'Sal		λgtWES MR100	
	counts	percentage	counts	percentage
Control	2957	3.0	11,266	12.0
+AMD	209	0.6	116	0.3

[a]Hybridizations to p5'Sal and λgtWES MR100 DNAs were performed in separate reactions containing equal amounts of nuclear RNA. Total counts in the reactions were 98,000 (control) and 35,000 (+AMD). Counts bound to a filter containing pBR322 DNA have been subtracted.

30 minutes with (^{3}H)uridine. Labeled nuclear RNA from control and AMD-treated cells was hybridized to filters containing either p5'Sal or λgtWES MR100 DNA. A variety of RNA concentrations were tested to insure that the hybridization reactions were performed in DNA excess. Only one RNA concentration is shown. A 15 minute pretreatment with AMD caused a 40-fold decrease in the percentage of labeled RNA binding to the λgtWES MR100 DNA filters, but only a 5-fold decrease in the percentage hybridizing to p5'Sal (Table I). This concentration of AMD did not affect the transcription of RNA polymerase II genes as determined by filter hybridization to a kappa light chain cDNA clone (data not shown). Although we cannot entirely exclude the possibility of decreased turnover, these results suggest that limited transcription of the rRNA ETS region can occur in the presence of AMD.

DISCUSSION

A detailed model for rRNA processing is outlined in Fig. 5. For the sake of clarity and simplicity we have omitted some of the intermediates involved in the generation of 28S and 5.8S rRNAs. Based on the differential hybridization of the various rRNA precursors to ETS DNA probes and the results obtained with RNA from AMD-treated cells, we propose two pathways for the removal of ETS sequences. In agreement with previous processing schemes (1-3), in one pathway the entire ETS is removed from 45S rRNA generating a 41S precursor. Alternatively, only part of the ETS region may be removed yielding 45S* rRNA. This alternative processing model agrees with results obtained by several other laboratories. For example, early studies on the migration of short-term labeled nuclear RNA in polyacrylamide gels detected rRNA species larger than 45S that could correspond to a

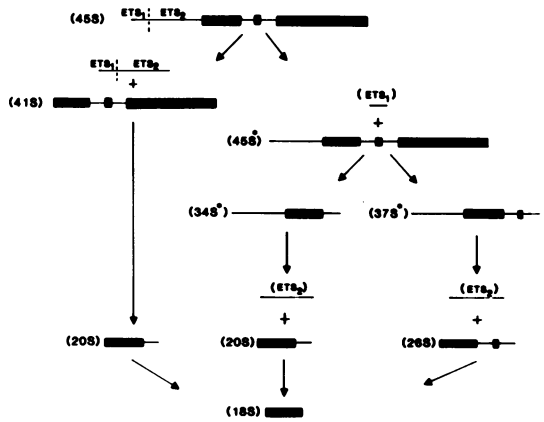

Fig. 5. Ribosomal RNA processing model. Only the intermediates involved in the generation of 18S rRNA are shown.

primary transcript containing an intact ETS (19). In addition, only 10-15% of the 45S-size rRNAs possess polyphosphate termini (4,5) suggesting that most of these species have been processed. Finally, S1 nuclease mapping of large rRNA precursors using p5'Sal DNA has detected two species, one mapping to the initiation site and a second molecule which is 650 nucleotides shorter (6,7). Our observations confirm these earlier studies and extend them to show that the processed 45S molecules (or 45S* by our nomenclature) can be cleaved to form either 37S* or 34S*.

It is possible that part of the ETS is removed after the formation of conventional 37S or 34S. In our hands, the 37S* and 34S* are major rRNA precursors in steady state nuclear RNA. However, this does not necessarily imply that these species are preferred intermediates in rRNA processing.

Bowman et al. (3) did not detect a 37S* or 34S* species. However, these investigators used p5'Sal DNA as a probe to follow ETS sequences and thus would not have observed processing of only part of the ETS. Preliminary analysis of nuclear RNA from other murine cell lines (NIH 3T3, P815, YAC-1 and S49.1) indicates that the 37S* and 34S* species are not unique to P3K cells. The 37S* and 34S* RNAs are presumably cleaved to 26S and 20S RNAs, respectively, plus the remainder of the ETS sequences. We have been unable to detect 26S RNA in our nuclear RNA preparations, however this species is reportedly very minor (3). Alternatively, in P3K cells the 37S* could be converted to 34S* and then to 20S. The final processing step removes the internal transcribed spacer from 20S to liberate 18S rRNA and an

intact spacer fragment. The spacer fragment was a predominant RNA species in P3K cells which was not identified in L cells (3,20). This may simply reflect a difference in the turnover rate for this fragment in L cells versus P3K cells.

In the presence of AMD, we observed an accumulation of small heterogeneous RNAs which were derived from the 5' end of the ETS (see Fig. 4B). The hybridization data using labeled RNA from AMD-treated cells indicated that transcription of 18S and 28S rDNA was inhibited, but that limited transcription of sequences from the 5' end of the rDNA still occured. Although low concentrations of AMD selectively block the transcription of rRNA genes by interacting with G/C base pairs, evidence suggests that the drug acts by inhibiting elongation of polynucleotide chains and not initiation (21). Thus, the small RNA species synthesized in the presence of AMD were probably actual transcripts of the ETS region.

The AMD-generated RNAs migrated as two heterogeneous groups of approximately 800 and 400 nucleotides. An inspection of the rDNA sequence within these regions reveals the presence of a highly G/C rich (67%) area beginning 700 nucleotides and ending 900 nucleotides downstream from the initiation site (4,5). Therefore, this region probably serves as the site of action for AMD. Termination of transcription at various sites within this region would generate a heterogeneous group of RNAs (approximately 800 nucleotides) which hybridize to pNTS.

The derivation of the smaller (400 nucleotides) group of AMD-RNAs is not as clear. Since these species hybridize to the pNTS probe, they must contain sequences from the 5' end of the ETS. There could be a second site of AMD action located 400 nucleotides downstream from the initiation site. However, this explanation is unlikely since DNA sequence analysis (7,8) reveals that this region contains a series of T-stretches which probably would not interact with AMD. It is more interesting to speculate that this area within the 800 nucleotide RNAs is recognized (specifically or non-specifically) by a ribonuclease and that the smaller RNAs are actually cleavage products of these abbreviated transcripts. More detailed studies will be required to precisely map the region of AMD action and determine the relationship between the two groups of AMD-generated RNAs.

ACKNOWLDEGEMENTS

This research was supported by NSF Grant PCM 80 20326 to R. J. Patterson. This is manuscript #11044 from the Michigan Agriculture Experiment Station.

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