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Analysis of Pre-rRNAs in Heat-Shocked HeLa Cells Allows Identification of the Upstream Termination Site of Human Polymerase I Transcription

KATHERINE A. PARKER†* AND URSULA BOND

Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510

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Human rRNA precursors from normal or stressed HeLa cells were studied by S1 nuclease mapping of unlabeled RNA and by antisense RNase mapping of RNA from cells that had been labeled in vivo with [^{32}P]PO_4. Heating cells to 43°C decreased the amount of newly synthesized rRNA to less than 5% of the control level and led to greater than 95% inhibition of transcription termination at a region 355 to 362 nucleotides downstream of the 3′ end of 28S rRNA, with readthrough continuing into the next transcription unit. Heating of cells to 42°C led to 60% inhibition of termination at this site; 50% of transcripts that extended into the nontranscribed spacer ended in a region 200 to 210 nucleotides upstream of the polymerase I (Pol I) initiation site. This is presumed to be the human upstream transcription termination site because of the absence of RNAs with a 5′ end corresponding to this region, the location relative to the Pol I initiation site (which is similar to the location of upstream terminators in other species), and the fact that it is 15 to 25 nucleotides upstream of the sequence GGGTTGACC, which has an 8-of-9 base identity with the sequence 3′ of the downstream termination site. Surprisingly, treatment of cells with sodium arsenite, which also leads to the induction of a stress response, did not inhibit termination. Pol I initiation was decreased to the same extent as termination, which lends support to the hypothesis that termination and initiation are coupled. Although termination was almost completely inhibited at 43°C, the majority of the recently synthesized rRNAs were processed to have the correct 3′ end of 28S. This finding suggests that 3′-end formation can involve an endonucleolytic cut and is not solely dependent on exonucleolytic trimming of correctly terminated rRNAs.

The control of rRNA transcription initiation, termination, and processing is of considerable biological importance. There are approximately 200 rDNA genes per haploid mammalian genome, which are located in tandem arrays in five chromosomal locations. The 18S, 5.8S, and 28S species are cotranscribed as a single rRNA precursor which ranges in size from 13.5 kilobases (kb) (47S) in mammalian cells to 8 kb (37S) in yeast cells (reviewed by Hadjiolov [21]). Its 5′ end is formed by transcription initiation, and its 3′ end is formed by either termination or processing 210 to 565 nucleotides downstream of the 3′ end of the 28S species. Each transcription unit is separated from the next by a 2- to 30-kb region known as the nontranscribed spacer (NTS), although this region may in fact be transcribed to give highly unstable transcripts.

The sequences required for polymerase I (Pol I) initiation have been extensively studied in vivo and in vitro (reviewed by Sollner-Webb and Tower [43]). The question of the site of termination is more controversial. Grummt et al. (18) postulate that an 18-nucleotide sequence (the *Sal* box) located 589 nucleotides downstream of the 3′ end of 28S rRNA in mice is sufficient to cause transcription termination 24 nucleotides upstream. We will refer to the event that forms ends in this vicinity in human cells as downstream termination both for the sake of simplicity and because in the most extensively studied mammalian species, the mouse, it appears to be a termination event. However, it should be kept in mind that in *Xenopus* (12, 28) and *Drosophila* (45) species, investigators believe that there is no true terminator immediately downstream of, or at, the 3′ end of 28S rRNA and that any 3′ ends in this region are formed by rRNA processing.

Although the nature of the event (termination or processing) a few hundred nucleotides 3′ to 28S rRNA is unclear, most investigators agree that there are Pol I transcription terminators located a few hundred nucleotides upstream of the Pol I initiation site (16, 22, 28, 32, 37). These lead to 3′-end formation of mouse or *Xenopus* mini-rRNA genes (16, 22, 28, 37) or of the endogenous *Xenopus* rRNA gene (28, 37). It has been hypothesized that these upstream terminators function to deliver Pol I molecules to the nearby initiation site and thus stimulate the rate of initiation by increasing the local concentration of free Pol I molecules (1, 12, 28, 36, 37). An alternative (but not mutually exclusive) theory for the function of upstream terminators was recently proposed by Bateman and Paule (4), who showed that transcription through a Pol I promoter dissociates initiation factors from their DNA-binding sites; thus, upstream termination would prevent this dissociation, thereby leading to an increased rate of initiation.

There are also several rRNA processing steps which are required to form the mature 18S, 5.8S, and 28S rRNAs. In mammalian cells, two early processing events lead to the formation of a 45S RNA, which is the first stable rRNA processing intermediate (Fig. 1). The first to occur in the mouse (20) is within the external transcribed spacer (ETS),

* Corresponding author.
† Present address: Department of Cellular Biology, Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

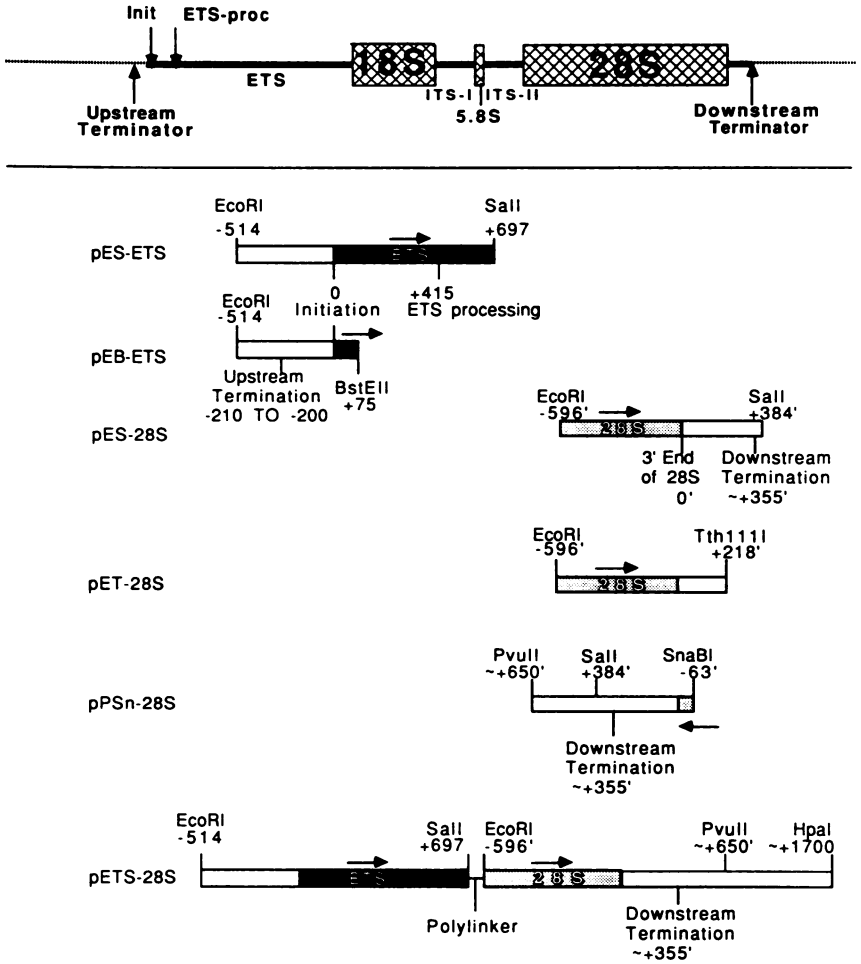

FIG. 1. rRNA constructs. A single transcription unit for rRNA is shown at the top. The 45S precursor is formed from the 47S primary transcript when processing at the ETS site and the 3′ end of 28S rRNA have occurred (20). rRNA constructs were cloned as described in Materials and Methods. Symbols: ■, ETS; ▭, mature 28S rRNA; ▭, NTS sequence. Construct names are shown on the left. Restriction sites of interest are marked above, and numbers refers to distance upstream (−) or downstream (+) of the Pol I initiation site for the ETS-containing clones or the distance upstream (−) or downstream (+) of the 3′ end of 28S rRNA (′) for the 28S rRNA-containing clones. The upstream termination, the ETS processing, and downstream termination sites are marked on the appropriate clones. →, Orientation in pGEM4 such that transcription with SP6 will result in synthesis of a positive-sense (rRNA-like) RNA; ←, orientation such that transcription with T7 polymerase will result in positive-sense RNA.

at a position 650 nucleotides downstream of the initiation site (10, 24, 35); this appears to be an endonucleolytic event that requires a protein factor (10). The second processing event (20) forms the mature 3′ end of 28S rRNA; little is known about this event except that in some species a processing intermediate with 7 to 30 nucleotides of downstream sequence has been identified (5, 19, 26, 46).

It has been previously reported that heat shock in *Xenopus laevis* (27) inhibits downstream termination (or processing) and rRNA processing steps but does not effect initiation or elongation. This phenomenon provides a unique system for studying rRNA synthesis in that an alteration in the normal pathway can be imposed at a particular time, and the effect on various steps of rRNA biogenesis can be studied shortly thereafter. We used this system to analyze rRNA transcription initiation and termination in the human HeLa cell line. By determining conditions under which downstream termination is partially inhibited in vivo, we demonstrate for the first time that mammalian upstream terminator sequences are able to function in their chromosomal locations. New initiation by Pol I decreased in parallel with inhibition of termination, lending support to the theories presented above that termination facilitates initiation in vivo.

MATERIALS AND METHODS

Heat shock and sodium arsenite treatment. (i) Preparation of unlabeled RNA. HeLa cell stocks were maintained in RPMI 1640 medium with 10% fetal calf serum, glutamine, penicillin, and streptomycin. A total of 4×10^6 cells in 20 ml were stressed 4 to 16 h after passaging. Cells were placed in water baths at the designated temperatures, with manual agitation every 5 min; sodium arsenite was added to a final concentration of 100 μM and incubated with cells at 37°C. After 1 to 3 h, cells were spun for 5 min in a clinical centrifuge at 500 × *g*, washed with 10 ml of cold phosphate-buffered saline, and suspended in 10 ml of 150 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA. Nonidet P-40 was added to 0.5% (final concentration), and the cells were vortexed. Nuclei obtained by centrifugation for 10 min at 3,000 × *g* in an SS34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) were

suspended in 10 ml of RNA extraction buffer (0.5 M NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol). Nuclear and cytoplasmic (supernatant) samples received 1% sodium dodecyl sulfate and 200 μg of proteinase K per ml (final concentrations) and were digested for 15 min at 37°C. In some instances, nuclear samples were sonicated to shear DNA. Samples were then extracted twice with an equal volume of phenol and once with chloroform and ethanol precipitated. Nuclear RNA was resuspended in 300 μl of 50 mM Tris hydrochloride (pH 7.5)–10 mM $MgSO_4$–1 mM dithiothreitol and digested for 15 min at 37°C with 230 U of RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), phenol extracted, chloroform extracted, and ethanol precipitated. RNA was quantitated by measuring optical density at 260 nm.

(ii) In vivo labeling. A total of 4×10^6 cells were spun for 5 min at $500 \times g$ in a clinical centrifuge and then suspended in 20 ml of phosphate-minus complete medium preheated at the designated temperature or containing 100 μM sodium arsenite at 37°C in parallel with the unlabeled samples described above. After 15 min, 1.25 mCi of [^{32}P]PO_4 was added, and the cells were incubated for an additional 2 h. The cells were washed once in cold phosphate-buffered saline, and total RNA was isolated by suspension of cells in RNA extraction buffer and digestion with proteinase K followed by DNase I as described above. RNA was quantitated by comparison with cold total cellular RNA of known concentration on an ethidium-stained formaldehyde-agarose gel.

Plasmid constructs. The human rDNA subclones depicted in Fig. 1 were generated by using gel-purified fragments and standard cloning techniques (30). pES-ETS (which contains nucleotides −514 to +697 relative to the transcription initiation site) and pES-28S (which contains 596 nucleotides of 28S rRNA and 384 nucleotides of downstream sequence) were constructed as previously described (38), using clones generously provided by N. Arnheim (34) and R. Schmickel (14). To generate pEB-ETS, pES-ETS was cleaved with *Bst*EII (which cleaves at position +75 relative to the initiation site) and *Hin*dIII, filled in with Klenow fragment, and ligated. To generate pET-28S, pES-28S was cleaved with *Tth*111I (which cleaves at a position 218 nucleotides downstream of the 3′ end of 28S rRNA) and *Hin*dIII, filled in with Klenow fragment, and ligated. The 700-nucleotide *Sna*BI-*Pvu*II fragment of pAD_{BB} (generously provided by R. Schmickel [14]), containing 63 nucleotides of 28S rRNA and 650 nucleotides of downstream sequence, was blunt end ligated into the *Sma*I site of pGEM4 to generate pPSn-28S (note that this orientation is opposite, relative to the SP6 and T7 transcription initiation sequences, that of the other clones). A final construct, containing both ETS and 28S sequences and designated pETS-28S, was obtained by cleaving pES-ETS with *Hin*dIII, filling in with Klenow fragment, and inserting the blunt-ended 2.3-kb *Eco*RI-*Hpa*I fragment of pAD_{BB} (containing 596 nucleotides of 28S rRNA and 1.7 kb of downstream sequence) in the same orientation.

S1 nuclease maps. S1 nuclease probes were 3′ end labeled with the appropriate α-^{32}P-labeled deoxynucleotide triphosphate and with Klenow fragment or T4 DNA polymerase or were 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase to a specific activity of 1×10^6 to 10×10^6 cpm/μg, and the coding strand was purified on a strand-separating gel (30). S1 nuclease maps were generated by the method of Berk and Sharp (7). For all cases, the probe was determined to be in excess of cellular RNA, and hybridization temperature and digestion conditions were maximized by using 20 ng of a positive-sense (rRNA-like) RNA generated by SP6 polymerase. Cellular RNA (0.1 to 10 μg) was mixed with the appropriate amount of yeast carrier RNA (cRNA) to give a total of 10 μg of RNA, dried with 0.06 pmol of probe, and suspended in 30 μl of 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–10 mM EDTA–400 mM NaCl. Samples were mixed and heated to 90°C for 5 min and then at the hybridization temperature for 3 h. A 300-μl sample of S1 digestion buffer (30 mM sodium acetate [pH 4.5], 1 mM $ZnSO_4$, 250 mM NaCl, 5% glycerol) containing S1 nuclease (Pharmacia, Inc., Piscataway, N.J.) at the designated concentration and temperature was added for 1 h; the samples were then ethanol precipitated and analyzed on a denaturing polyacrylamide gel.

In vitro synthesis of RNA. (i) Unlabeled RNA. Positive-sense (rRNA-like) or antisense RNA was transcribed from 10 μg of plasmid template cut with the designated restriction enzyme, using SP6 or T7 RNA polymerase in a reaction volume of 100 μl as described by Melton et al. (33) except that all ribonucleotides were present at 0.5 mM and 0.5 mM GpppG cap (Pharmacia) was added. A tracer amount of [α-^{32}P]GTP (10^4 dpm) was added to enable the transcripts to be quantitated and gel purified, but as the final specific activity was only 82.5 cpm/μg, this label did not interfere with subsequent experiments.

(ii) High-specific-activity RNA. High-specific-activity RNAs were transcribed as described above except that 1 μg of linearized DNA was transcribed in a 20-μl reaction volume without exogenous unlabeled GTP, and 50 μCi of [α-^{32}P]GTP (specific activity, 400 Ci/mmol) was added to generate RNAs with a specific activity of 3.3×10^8 cpm/μg.

RNase mapping. RNase mapping was done by a modification of the method of Melton et al. (33). To study the initiation and ETS processing sites, approximately 5 μg of in vivo-labeled cellular RNA and 5 μg of cRNA were mixed with 1 pmol (20 to 40 ng) of unlabeled antisense RNA, dried in a Speed-Vac, and suspended in 30 μl of RNase hybridization buffer (80% deionized formamide, 40 mM PIPES [pH 6.7], 400 mM NaCl, 1 mM EDTA). To study the 3′ end of 28S rRNA, 1 μg of in vivo-labeled RNA and 9 μg of cRNA were mixed with 5 pmol of antisense RNA. After denaturation at 90°C for 5 min, samples were incubated at 50°C for 3 h; then 300 μl of digestion buffer (300 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA) containing RNase T_1 (10 U/ml; 2,941 U/mg; Calbiochem-Behring, La Jolla, Calif.) and RNase A (pancreatic RNase; 5 μg/ml; 5,561 U/mg; Organon Teknika, Malvern, Pa.) was added. The samples were incubated for 60 min at 37°C, extracted with 300 μl of phenol-chloroform-isoamyl alcohol (50:49:1), precipitated with ethanol, suspended in sample buffer, and analyzed on a polyacrylamide–8 M urea gel.

Primer extension. The oligodeoxynucleotide CGTCGTGC TCTCCCGGGCCGGG, complementary to the nucleotides at positions 436 to 457 downstream of the initiation site, was kindly synthesized by J. Flory, Yale University, New Haven, Conn. Primer extension and dideoxynucleotide sequencing were carried out as described previously (39), using 10 μg of cellular RNA with hybridization for 60 min at 42°C.

Gel electrophoresis. Polyacrylamide gels contained 8 M urea, 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA), and 8% polyacrylamide, with an acrylamide-bis acrylamide ratio of 19:1 unless otherwise specified.

RESULTS

S1 nuclease map of the downstream terminator of Pol I transcription. The effect of heat shock on rRNA termination was studied by performing S1 nuclease mapping on unlabeled RNA derived from cells that had been subjected to heat shock or sodium arsenite treatment. Conditions for heat shock that were known to induce the heat shock proteins (HSPs) in human cells (42) were chosen, and efficacy of the stress response was confirmed by identifying induction of HSP70 protein or HSP27 mRNA (data not shown).

Bartsch et al. (2) previously identified a human rRNA termination site approximately 350 nucleotides downstream of the 3′ end of 28S rRNA. A DNA probe was obtained by cutting pPSn-28S (Materials and Methods; Fig. 2A) within the rDNA sequence with *Tth*111I (which cuts at +218 relative to the 3′ end of 28S rRNA) and within the vector sequence with *Nhe*I, labeling at the 3′ end with Klenow fragment, and purifying the coding strand. This probe was hybridized with the RNA samples, digested with S1 nuclease, and electrophoresed on a denaturing polyacrylamide gel. In Fig. 2C, a positive-sense RNA sample (lane 3) provides a marker for the extent of probe protection obtained if transcription reads through; this is clearly distinguishable from the uncut probe shown in lane 1. In the nuclear RNA of cells that were treated at 37°C (lane 5) and 41°C (lane 7), there was a major band at approximately position +355 (relative to the 3′ end of 28S rRNA), with little or no readthrough visible. At 43°C (lane 11), however, >95% of the RNA represented readthrough transcription. At 42°C (lane 9), 40% of the RNA (as determined by densitometry) terminated at the correct site, with the remainder extending to at least +650, which was the extent of complementarity with the probe.

The effect of another stress, sodium arsenite treatment, was also examined. In this case, treatment with 100 μM sodium arsenite (lane 13) did not result in readthrough, although the HSP70 protein was induced to the same extent as were the heat-shocked samples (data not shown). The amount of correctly terminated RNA in the arsenite-treated samples was generally 50% that of the 37°C control in repeat experiments; the low intensity of this band in Fig. 2C was due to partial loss of the sample.

To identify the termination site more precisely, samples were mapped with the probe shown in Fig. 2B and run alongside a Maxam-Gilbert G-reaction DNA sequencing ladder (31). In this case, pES-28S was cut with *Tth*111I and *Pvu*II and then 3′ end labeled to generate a probe for which the readthrough band was only 20 nucleotides longer than the correctly terminated RNA, which allowed conditions to be maximized. Figure 2D shows four S1 nuclease digestion conditions. Although the control SP6 transcript (not shown) and the 43°C sample (lanes 1 and 3) generated a doublet at the expected position when 200 U of S1 nuclease was added, the 37°C samples (lanes 5 and 7) had heterogeneous ends, with the major bands corresponding to RNAs that ended 355, 359, or 362 nucleotides downstream of the 3′ end of 28S rRNA. A variety of other conditions always generated heterogenous ends at the termination site (data not shown), leading us to conclude that the termination either is heterogeneous or is rapidly followed by exonucleolytic trimming.

S1 nuclease map of the upstream terminator of Pol I transcription. The effect of heat on downstream termination was used to try to identify the upstream terminator of human Pol I transcription. Normally, in mammalian cells there is very little transcription through the NTS, making it difficult to identify a site that could act as a terminator if Pol I were presented to it. We reasoned that treatment at 42°C (which inhibited termination 60% at the downstream terminator) would allow significant amounts of Pol I to traverse the NTS but would still allow termination to occur at the upstream terminator if transcription and termination were indeed related events.

A DNA probe complementary to the NTS region just upstream of the initiation site was 3′ end labeled at the *Dde*I site (Fig. 2E). Figure 2F shows the result of an S1 nuclease mapping experiment with this probe and the samples used in Fig. 2C. As expected, there was no signal from the 37°C nuclear sample (lane 5). The 42°C nuclear sample (lane 9), however, contained some RNA that gave full-length protection and some that gave a very heterogeneous set of bands approximately 15 to 25 and 35 to 45 nucleotides shorter. The shorter bands were clearly not due to S1 nuclease artifacts because they were absent in the positive-sense RNA control (lane 3) and, more important, in the nuclear RNA sample that was derived from cells treated at 43°C (lane 11), where full-length protection was even greater. If these ends were generated by RNA processing rather than termination, an RNA corresponding to the downstream fragment might be identified (if it were sufficiently stable). However, no RNAs with a 5′ end corresponding to this region were identified by S1 nuclease mapping with a 5′-end-labeled *Eco*RI-*Bst*EII probe obtained from pES-ETS (not shown). On the basis of these results and sequence homology (see Discussion), we conclude that the ends seen in the 42°C sample most likely represent termination at the upstream terminator. These ends were more precisely mapped by running the samples alongside Maxam-Gilbert sequencing lanes (data not shown). Since readthrough from the downstream terminator was not observed in the sodium arsenite-treated sample (Fig. 2C), no transcription was apparent in this region (data not shown).

Initiation site. As it has been proposed that termination and reinitiation of Pol I transcription are intimately coupled, we tested whether correct initiation would be inhibited to the same extent as termination after heat shock. By using S1 nuclease mapping, we found that the steady-state levels of correctly initiated RNA were equal in the control, heat-shocked, and sodium arsenite-treated samples, although the heat-shocked samples had only a small amount of read-in transcription, which represented about 2% the amount of correctly initiated RNA (data not shown).

In vivo-labeled RNA. The S1 nuclease mapping experiments described above cannot distinguish whether the correctly initiated RNAs were formed before or after the stress was induced. If stress were to stabilize previously synthesized pre-rRNAs, the amount of correctly initiated RNA would not change even if new initiation events were, in fact, inhibited.

To determine whether newly synthesized RNAs were correctly initiated or processed, stressed or control cells were labeled in vivo with [^{32}P]PO_4 for 2 h in parallel with (i.e., the same source of cells in the same bath) the unlabeled cells whose RNA was used in Fig. 2. Total cellular RNA was isolated and fractionated on a formaldehyde-agarose gel (Fig. 3). 45S, 32S, 28S, and 18S rRNAs were identified by staining the gel with ethidium bromide before drying; the high-molecular-weight signal disappeared with DNase I treatment. At 42°C, the signals for the 45S and 32S species were approximately 25 and 10%, respectively, of the control value; at 43°C, each was less than 5% of the control value (although this experiment cannot differentiate between a

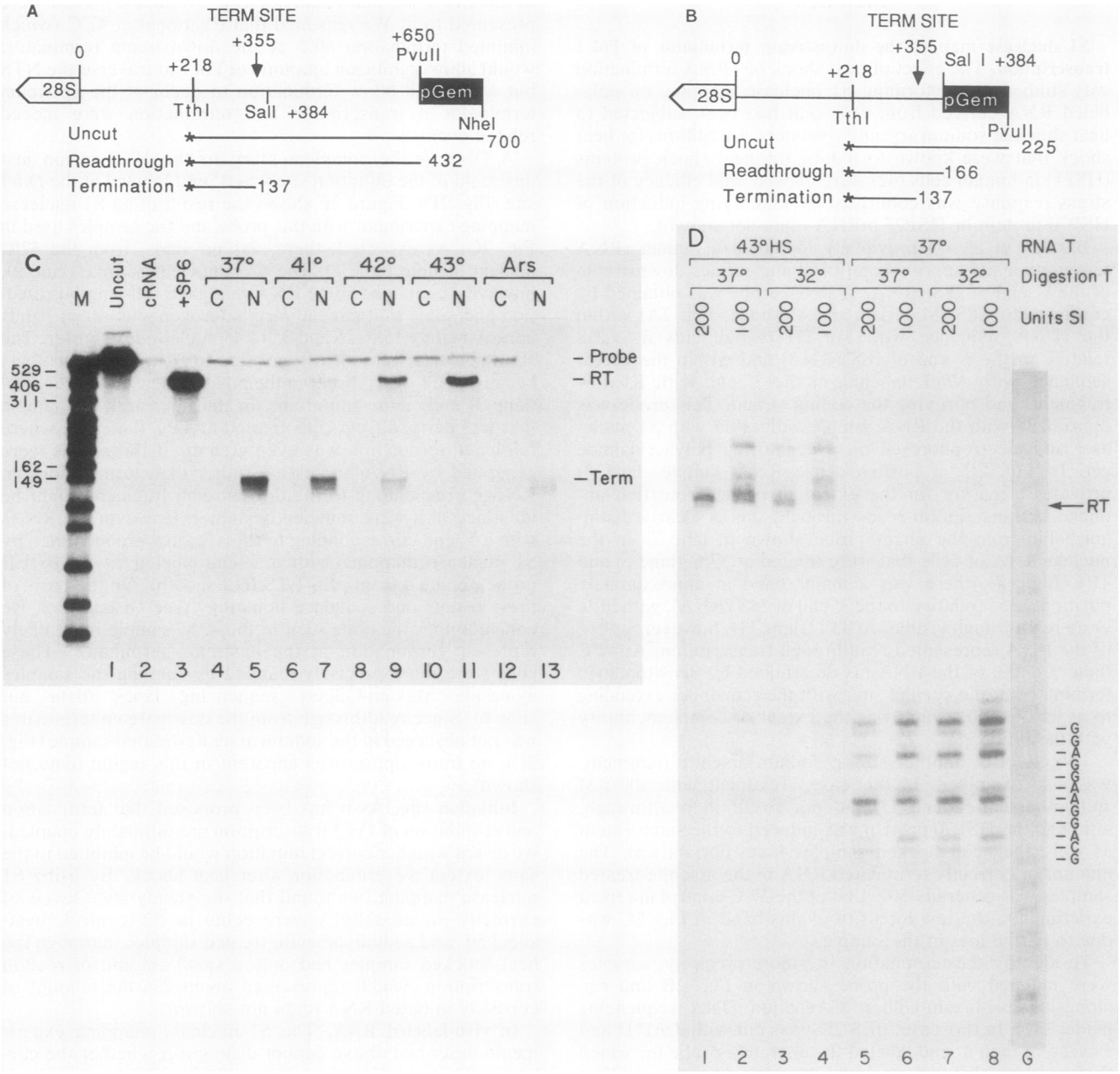

FIG. 2. S1 nuclease mapping of the downstream and upstream transcription terminators. (A and B) Probes used to map the downstream terminator. A *Tth*111I-*Nhe*I fragment of pPSn-28S (probe A) and a *Tth*111I-*Pvu*II fragment of pES-28S (probe B) were 3′ end labeled, and the coding strand was isolated. *, Location of label; sizes of predicted products are shown on the right. Note that probes A and B contain 268 and 59 nucleotides, respectively, of pGEM4 vector sequence that has no complementarity to cellular pre-rRNA. (C) Results for nuclear (N; lanes 5, 7, 9, 11, and 13) and cytoplasmic (C; lanes 4, 6, 8, 10, and 12) RNAs isolated from HeLa cells that had been incubated for 2 h at the designated temperature or at 37°C with 100 μM sodium arsenite (Ars). A 10-μg sample of cellular RNA, 10 μg of cRNA (lane 2), or 10 μg of cRNA and 20 ng of an unlabeled positive-sense RNA (lane 3; made from SP6 polymerase transcription of pETS-28S linearized at the *Pvu*II site [see Fig. 1]) was hybridized with probe A at 60°C as described in Materials and Methods and then digested at 33°C for 60 min, using 200 U of S1 nuclease per reaction; the surviving DNA was analyzed on a denaturing polyacrylamide gel. RT, RNAs that extended to the end of complementarity with the probe; Term, transcripts that ended at the Pol I termination site. Lane 1, Uncut probe; lane M, DNA markers obtained by filling in an *Hpa*II digest of pBR322. (D) Results of an assay to map the termination site more precisely. Probe B was hybridized with nuclear RNA from control (37°C; lanes 5 to 8) or heat-shocked (43°C; lanes 1 to 4) samples and treated with various concentrations (Units S1) and temperatures (Digestion T) of S1 nuclease. Lane 9 contains a Maxam-Gilbert G-reaction sequencing ladder (31) of the coding strand, with the nucleotide position identified on the right (top base is complementary to +C362 relative to the 3′ end of 28S rRNA; bottom base is complementary to +C351). The fragments generated by S1 nuclease migrated 1 nucleotide slower than the corresponding DNA in the sequencing lane. (E) Mapping of the upstream terminator with a 3′-end-labeled *Dde*I-*Hin*cII fragment of pES-ETS. (F) Samples (10 μg) of the RNA shown in panel C that were hybridized at 62°C with probe E, digested with 200 U of S1 nuclease at 30°C for 60 min, and analyzed in the same way.

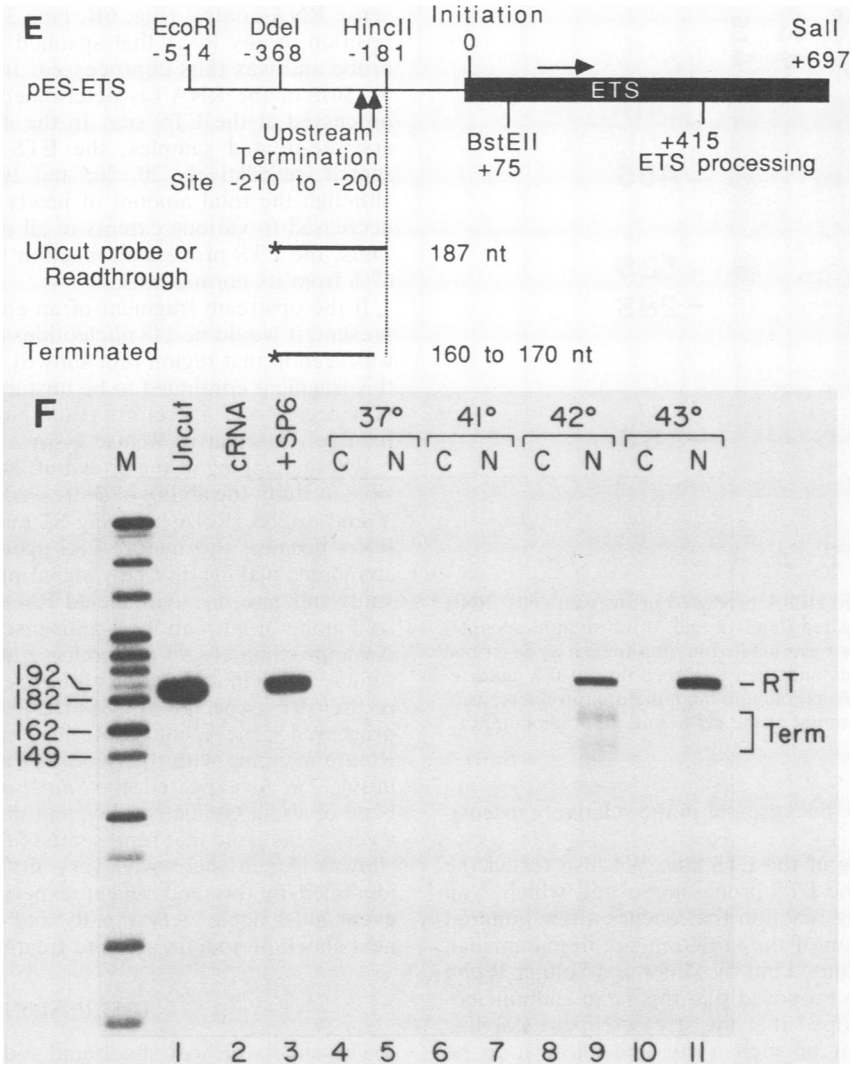

FIG. 2—*Continued*

decreased rate of synthesis and instability of recently synthesized rRNA). Although the signal for the 45S and 32S species was decreased to 50% of the control value in the sodium arsenite-treated sample, the 18S and 28S products were just barely detectable in this and the heat-shocked samples. Similar results were seen when cells were labeled with [^{3}H]uridine (data not shown).

RNase mapping of upstream termination and initiation sites. To define more precisely which events were inhibited by stress, RNAs that had been labeled in vivo were mapped by hybridization with cold antisense RNA probes under conditions of probe excess, followed by digestion with the single-strand-specific RNases T_1 and A. Deproteinized RNA was then fractionated on a denaturing polyacrylamide gel. Each sample lane was run alongside the same in vivo-labeled RNA which had been digested in the absence of antisense RNA, and only differences were considered significant. Because the sample lanes consisted primarily of RNA that was not complementary to the probe, there was a diffuse background in all lanes; more extensive digestion conditions decreased this background but led to incorrectly sized bands when a high-specific-activity positive-sense transcript generated by SP6 polymerase was used as a control; therefore, these conditions were avoided.

To study the upstream termination and initiation sites, RNAs that had been labeled in vivo were incubated without probe or with the three unlabeled antisense probes (Fig. 4A). In Fig. 4B, a positive-sense high-specific-activity RNA (lanes 2 to 4) identifies the position where an RNA protected for the full length of the antisense probe would migrate (note that the markers on the left are DNA and therefore migrated faster than an RNA molecule of the same length). In all cases, properly initiated RNA should give a signal at 75 nucleotides, whereas read-in transcription would give a signal of variable length. In the 37°C samples (lanes 5 to 8) and the sodium arsenite-treated samples (lanes 17 to 20), the only signal seen represented correctly initiated RNA. For the 42 and 43°C samples, properly initiated RNA represented 10 and <1%, respectively, of the amount seen in the control lane, and read-in transcription could be seen clearly. Ends that would correspond to the upstream termination site could not be identified, probably because the ends are very heterogeneous by S1 nuclease mapping and therefore would

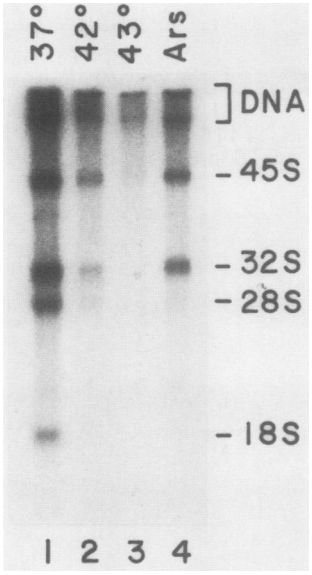

FIG. 3. In vivo-labeled rRNA produced in stressed cells. RNA from control (lane 1), heated (lanes 2 and 3), or sodium arsenite-treated (Ars; lane 4) cells were labeled in vivo for 2 h as described in Materials and Methods and then analyzed on a 1.0% agarose-formaldehyde gel. rRNA precursors and mature products were identified by ethidium staining of the gel before autoradiography.

not be identified above background in the relatively insensitive RNase assay.

S1 nuclease mapping of the ETS site. We also tested the effect of stresses on the ETS processing event, which is an early rRNA processing reaction that occurs a few hundred nucleotides downstream of the initiation site in mammalian species, as was first pointed out by Miller and Sollner-Webb (35). Since it has been proposed that this is an endonucleolytic event (10), we hoped that the stresses might stabilize the upstream fragment of such a cut and allow it to be definitively identified.

In human cells, the ETS processing site has been identified to occur 5′ to nucleotides +414, +415, +422, and +423 (relative to the initiation site [24]). Primer extension mapping of RNA derived from control or stressed cells was performed by using a DNA primer complementary to nucleotides +436 to +457. As with correctly initiated rRNAs, there was no effect of heat shock (Fig. 5, lanes 10 and 12) or sodium arsenite treatment (lanes 14 and 16) on the steady-state levels of nuclear transcripts that were correctly processed at the ETS processing site as compared with an unstressed control (lane 8). We found a slightly different end than was identified by Kass et al. (24), who used S1 nuclease mapping. The major 5′ ends are best identified in lane 1, which is immediately adjacent to the dideoxynucleotide sequencing ladder, and appear to be C416, U417, G422, and A423. However, we were unable to identify an RNA that would correspond to the upstream fragment of an endonucleolytic cut at the ETS site when we performed S1 nuclease mapping with a 3′-end-labeled DNA probe spanning this region, using nuclear RNA from control or stressed cells.

RNase mapping of the ETS site. To determine whether newly synthesized RNA could be processed at the ETS processing site, in vivo-labeled RNA was incubated with or without antisense RNA (obtained by T7 transcription of pES-ETS that had been cut with *Nar*I; Fig. 6A). A positive-sense RNA control (Fig. 6B, lane 3) was used to mark the position of any RNA that spanned the entire length of the probe and was thus unprocessed. In the 37°C sample (lane 5), 30% of the RNA (as determined by densitometry) was processed at the ETS site. In the 42°C, 43°C, and sodium arsenite-treated samples, the ETS processing site represented, respectively 20, 20, and 10% of the total signal, although the total amount of newly synthesized RNA was decreased to various extents in all of the stressed samples. Thus, the ETS processing reaction was decreased by 33 to 67% from its normal rate.

If the upstream fragment of an endonucleolytic cut were present, it would be 148 nucleotides long. Although no band was seen in that region (not shown), it is quite possible that this fragment continued to be unstable even in the presence of stresses and therefore could not be identified in the relatively insensitive RNase assay.

RNase mapping of the 3′ end of 28S rRNA. It was impossible to study the ability of a stressed cell to form the correct 3′ end of 28S rRNA by using S1 nuclease mapping of cold RNA because the mature 28S species is both stable and abundant, making any new signal obscured by the old. To study this site, in vivo-labeled RNA samples were hybridized alone or with an *Acc*I antisense RNA probe (Fig. 7A). An approximately 90-nucleotide RNA was identified in all sample lanes that received antisense RNA (Fig. 7B), which is the size expected if 28S rRNA were to be correctly processed. The identity of this band was confirmed by RNase mapping with the two other antisense probes shown in Fig. 7A. As expected, the *Sna*BI probe generated a major band of about 60 nucleotides, and the *Nar*I probe only gave a very weak band that represented full-length protection (not shown). Since there was very little full-length precursor identified in this and repeat experiments, this processing event must occur very rapidly and must be unaffected by heat shock or sodium arsenite treatment.

DISCUSSION

The effects of heat shock and sodium arsenite treatment on Pol I initiation and termination and on early rRNA processing steps were studied. S1 nuclease mapping of unlabeled rRNA was performed to determine the steady-state levels of various intermediates after the induction of a stress, and antisense RNase mapping of in vivo-labeled RNA was done to determine whether these events occurred in newly synthesized rRNAs.

Others have reported that heat shock primarily inhibits rRNA processing but has little or no effect on the amount of newly synthesized pre-rRNAs in human cells (47) and cells derived from lower organisms (6, 13, 27). When studied, the slight decrease in accumulation has appeared to be due primarily to an increased rate of turnover of these rRNAs (6). Our results differ in that heating human cells to 42 and 43°C led to a more profound decrease (75 and >95%, respectively) in the amount of newly synthesized rRNA (Fig. 3). This finding is consistent with results of the recent study by Sadis et al. (42), who also examined heat shock in HeLa cells; they propose that the decreased accumulation is due to instability of the newly synthesized rRNAs. One possible explanation for the apparent discrepancy in the amounts of newly synthesized rRNA after heat shock in human cells may be related to the fact that both our study and that of Sadis et al. (42) analyzed rRNAs shortly (1 to 4 h) after induction of the heat shock, whereas in the earlier study (47) the stress was inflicted upon the cells for at least 13 h before

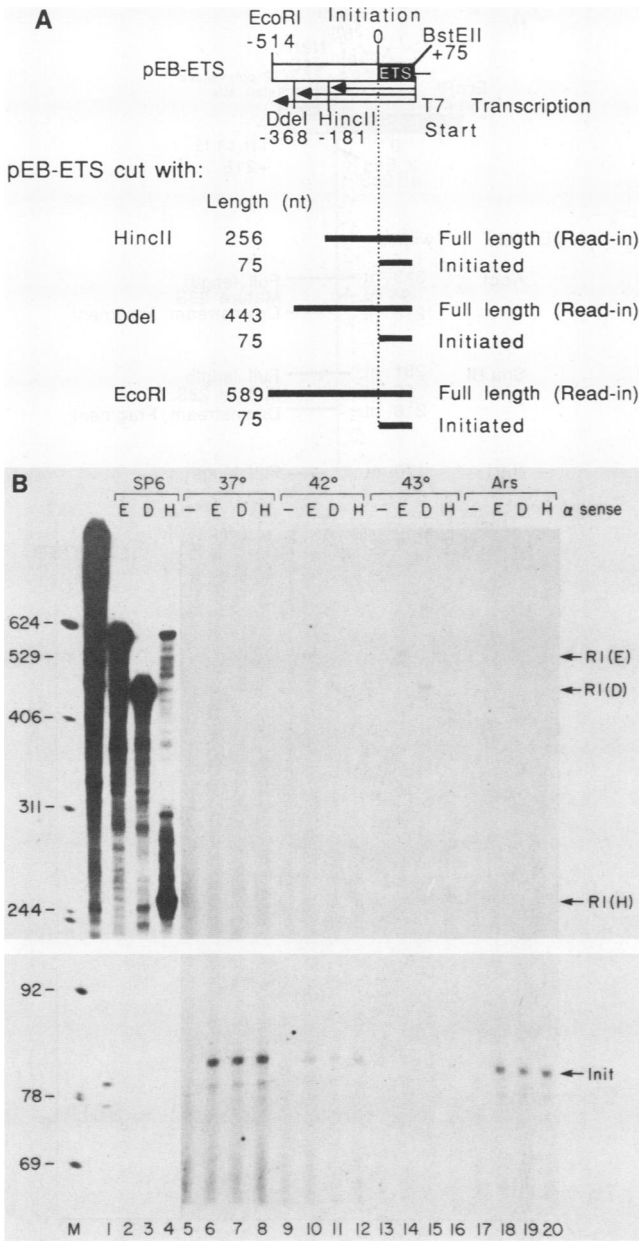

FIG. 4. RNase mapping of in vivo-labeled RNA at the initiation site of Pol I transcription. (A) Anti-sense RNA probes to test initiation site. Unlabeled antisense RNAs of 256, 443, or 589 nucleotides were generated by cleaving pEB-ETS with *Hin*cII, *Dde*I, or *Eco*RI, respectively, and transcribing with T7 RNA polymerase. Correctly initiated RNA should give a signal of 75 nucleotides in all cases; read-in transcription should be of variable length, as indicated (B) Results obtained when approximately 5 μg of total RNA from the control (37°C; lanes 5 to 8), heated (42 and 43°C; lanes 9 to 16), or sodium arsenite-treated (Ars; lanes 17 to 20) samples shown in Fig. 3 or 10 ng of high-specific-activity positive-sense RNA (generated by transcribing *Sal*I-cut pES-ETS with SP6 polymerase [lanes 1 to 4]) was incubated without (–; lanes 1, 5, 9, 13, and 17) or with *Eco*RI antisense probe (E; lanes 2, 6, 10, 14, and 18), *Dde*I antisense probe (D; lanes 3, 7, 11, 15, and 19), or *Hin*cII antisense probe (H; lanes 4, 8, 12, 16, and 20). The samples were digested with RNases T_1 and A, and the surviving RNA was analyzed on a denaturing polyacrylamide gel. Lane 1, Uncut positive-sense RNA; lane M, markers obtained by filling in an *Hpa*II digest of pBR322 (sizes [in nucleotides] of the DNA bands are shown on the left). Bands corresponding to correct initiation (Init) and read-in transcription [RI(E), RI(D), and RI(H) for the *Eco*RI, *Dde*I, and *Hin*cII antisense probes, respectively] are indicated on the right. Although the middle region of the gel was spliced out, identical exposures were used for the top and bottom.

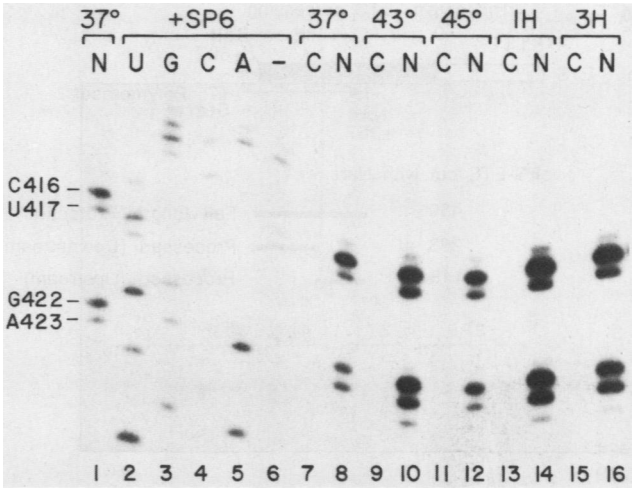

FIG. 5. Primer extension analysis of the ETS processing site. An oligodeoxynucleotide probe complementary to nucleotides +436 to +457 (relative to the initiation site) was hybridized with 10 μg of cytoplasmic (C; lanes 7, 9, 11, 13, and 15) or nuclear (N; lanes 1, 8, 10, 12, 14, and 16) RNA obtained from the stressed or control cells, and then reverse transcription was performed. Control (37°C; lanes 1, 7, and 8) and heat-shocked (43 and 45°C; lanes 9 to 12) cells were incubated at the designated temperatures for 2 h; sodium arsenite-treated cells were incubated for 1 h (lanes 13 and 14) or 3 h (lanes 15 and 16) at 37°C before isolation of RNA. Lanes 2 to 6 show primer extension of an unlabeled positive-sense RNA (obtained by cleaving pETS-28S with *Pvu*II and then transcribing with SP6 polymerase). Sequencing ladders were obtained by adding ddATP (lane 2), ddCTP (lane 3), ddGTP (lane 4), or ddTTP (lane 5) to detect the complementary RNA base designated above the appropriate lane. Extension products were analyzed on a denaturing polyacrylamide gel. The 5′-most bases of RNAs that were processed at the ETS site are identified on the left.

addition of label. Thus, the latter study may have induced some thermotolerance, allowing at least partial resumption of normal cellular processes.

It has recently become clear that different stresses that induce HSPs can vary in their effects. For example, Bond found that heat shock and sodium arsenite treatment had differing effects on pre-mRNA splicing (8). Similarly, we found that although inhibition of formation of mature 18S and 28S rRNAs (Fig. 3) and partial inhibition of the ETS processing reaction occurred with both heat shock and sodium arsenite treatment, Pol I initiation and termination were affected only by heat shock.

Pol I termination at the upstream and downstream sites. It has recently been determined that either termination or rRNA processing occurs several hundred nucleotides downstream of the 3′ end of 28S rRNA in most, if not all, species. As discussed above, we will refer to this event as termination because in the best-studied mammalian species, the mouse, this region appears to act like a termination site (17, 18). Since Labhart and Reeder (27) have reported that the event 265 nucleotides downstream of the 3′ end of 28S rRNA in *X. laevis* (which they believe to be processing) is completely inhibited by heat shock, we tested whether heat or other stresses might have a similar effect in human cells.

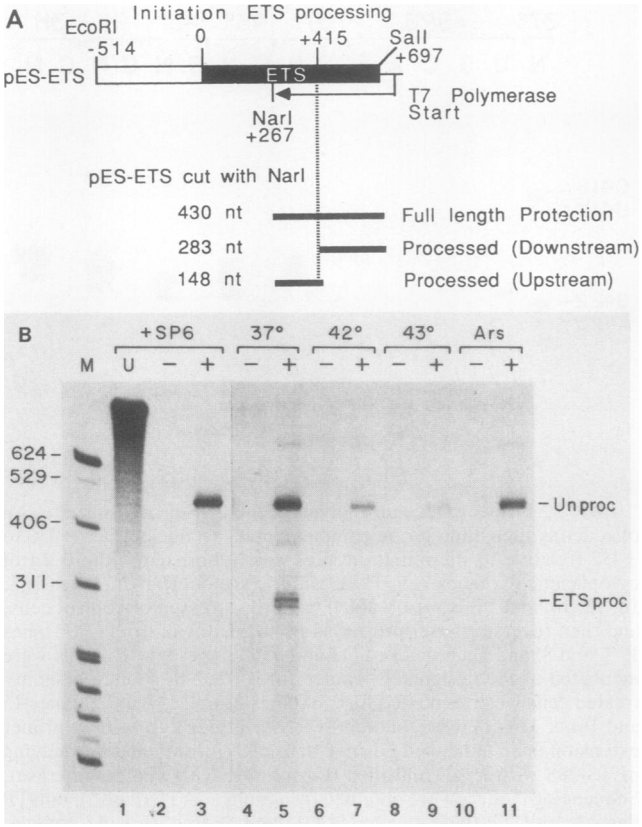

FIG. 6. RNase mapping of in vivo-labeled RNA at the ETS processing site. (A) An unlabeled antisense RNA probe was generated by cleaving pES-ETS with *Nar*I and transcribing with T7 polymerase. The sizes of expected products from RNase mapping are indicated on the left. (B) A 5-μg sample of total RNA obtained from control (37°C; lanes 4 and 5), heated (42 and 43°C; lanes 6 to 9), or sodium arsenite-treated (Ars; lanes 10 and 11) cells or 10 ng of high-specific-activity positive-sense RNA (obtained by transcribing *Sal*I-cut pES-ETS with SP6 polymerase; +SP6, lanes 2 and 3) was hybridized with (+) or without (−) 10 ng of the *Nar*I antisense RNA shown in panel A and digested with RNases A and T_1 as described in Materials and Methods. Lane 1, Uncut positive-sense control RNA (U); lane M, markers obtained by filling in an *Hpa*II digest of pBR322 (sizes [in nucleotides] of the DNA bands are shown on the left). Expected positions of unprocessed (Unproc) RNA or RNA processed at the ETS processing site (ETS proc) are shown on the right.

Bartsch et al. (2) mapped the Pol I termination site in humans to occur approximately 350 nucleotides downstream of the 3′ end of 28S rRNA (note that the sequence they used was from La Volpe et al. [29], whose nucleotide +350 corresponds to +362 on our sequence, which was kindly provided by J. Sylvester and R. Schmickel). Using a probe that extended from +218 to either +384 (Fig. 2B and D) or +650 (Fig. 2A and C; numbers refer to the distance downstream of the 3′ end of 28S rRNA), we found that pre-rRNA from control cells either terminated heterogeneously at positions +355, +359, and +362 or terminated at +362 and were then rapidly trimmed. Heating of cells to 43°C led to a >95% inhibition of termination, with readthrough extending to at least position +650.

We were fortunate to discover an intermediate condition (42°C) under which termination was inhibited by 60%, with the remainder of the RNA extending to at least +650. Even with a longer exposure, no bands were identified that would

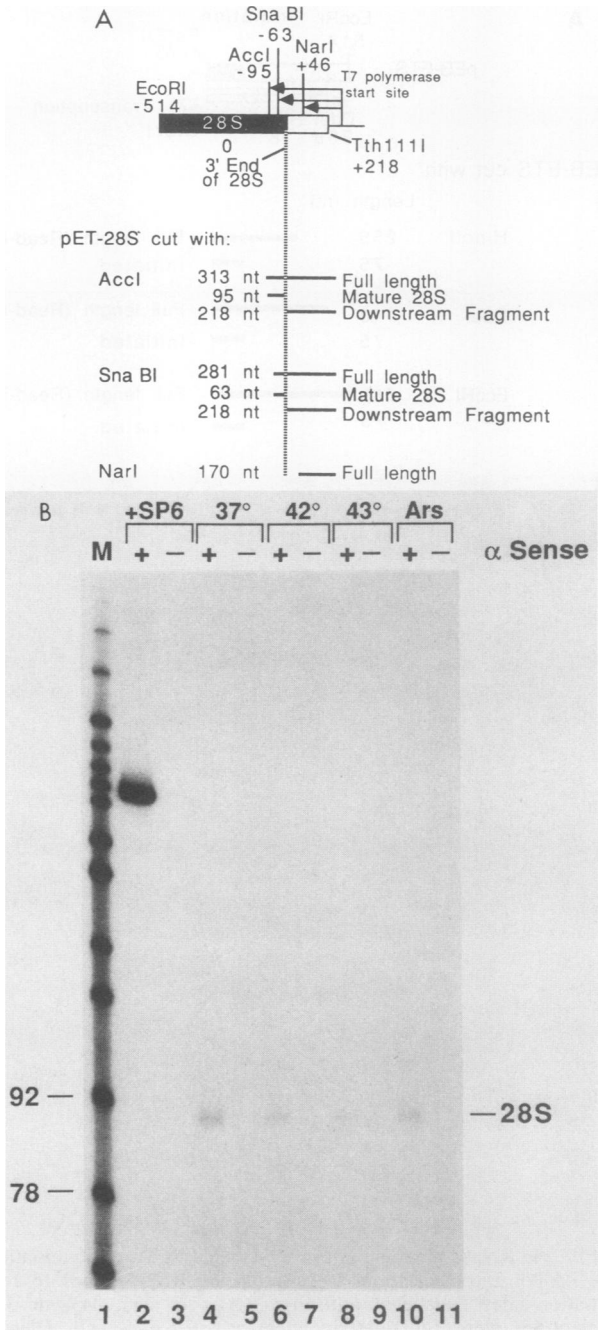

FIG. 7. RNase mapping of in vivo-labeled RNA at the 3′ end of 28S rRNA. (A) Unlabeled antisense RNA probes were generated by cleaving pET-28S with *Acc*I, *Sna*BI, or *Nar*I, respectively, and transcribing with T7 RNA polymerase. The sizes of expected products of RNase mapping are indicated. (B) A 1-μg sample of total RNA obtained from control (37°C; lanes 4 and 5), heated (42 and 43°C; lanes 6 to 9), or sodium arsenite-treated (Ars; lanes 10 and 11) cells or 10 ng of high-specific-activity positive-sense RNA (obtained by transcribing *Sal*I-cut pES-28S with SP6 polymerase; +SP6, lanes 2 and 3) was hybridized with (+) or without (−) 10 ng of the *Acc*I antisense RNA shown in panel A and digested with RNases A and T_1 as described in Materials and Methods. The surviving RNA was electrophoresed on a 10% denaturing polyacrylamide gel and autoradiographed. Lane 1, DNA markers obtained by filling in an *Hpa*II digest of pBR322 (sizes [in nucleotides] are shown on the left). The position expected for mature 28S RNA that is correctly processed is shown on the right.

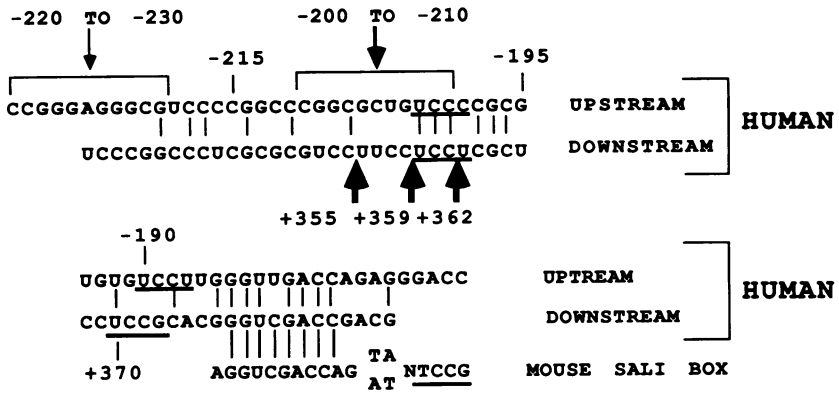

FIG. 8. Sequence comparison of the upstream and downstream terminators. The sequences surrounding the upstream and downstream human Pol I terminators are aligned with each other and with the mouse *Sal*I box, which has been shown to direct termination 24 nucleotides upstream in mouse cells (18). Relative amounts of a terminated RNA are indicated by sizes of the arrows. Numbers represent nucleotides upstream of the Pol I initiation site (upstream terminator) or downstream of the 3′ end of 28S rRNA (downstream terminator). Vertical lines signify conserved bases (as compared with the human downstream sequence); horizontal lines identify the sequence UCCX (where X can be any base), which has been hypothesized by Bartsch et al. (2) to play a role in human Pol I termination. Using Maxam-Gilbert sequencing ladders, we identified an extra base (G) at position −220 that was not present in a different rRNA clone (15) and have altered the sequence in that position accordingly.

correspond to termination near position +471, +526, or +621, all of which contain a sequence related to the human *Sal* box and were predicted by Bartsch et al. (2) to be terminators. The inhibition of termination does not occur when HSPs are induced by treatment with sodium arsenite.

The accumulation of spacer transcripts enabled us to identify what we believe to be the upstream terminator of Pol I transcription. Although we believe that this accumulation is due primarily to the failure to terminate transcription just downstream of 28S rRNA, it is also possible that it results from stabilization of normally unstable transcripts originating from a spacer promoter.

S1 nuclease mapping with a 3′-end-labeled probe complementary to the region upstream of the Pol I initiation site revealed a heterogeneous set of bands corresponding to positions −200 to −210 and −220 to −230 (relative to the initiation site) (Fig. 2F). We believe this to be the human upstream terminator for three reasons: (i) the location is quite similar to that of upstream terminators in other species (16, 22, 28, 32, 37); (ii) there was no evidence found for the existence of a fragment corresponding to the downstream portion of an endonucleolytic cut when we analyzed cold RNA by S1 nuclease mapping (not shown; we cannot, however, rule out instability); and (iii) the major 3′ ends are 15 to 25 nucleotides upstream of a sequence with an 8-of-9 base identity with the sequence in a similar location relative to the downstream termination site (Fig. 8). We note an additional 6-of-7 nucleotide homology at positions −11 to −17 (relative to the first base of the *Sal* box sequence), which may be important in directing the precise site of cleavage.

Initiation. As it has been postulated that upstream terminators enhance initiation at the nearby Pol I promoter, it was of particular interest to study the effects of heat and other stresses on Pol I initiation. S1 nuclease studies of unlabeled RNA from control or stressed cells showed no change in the steady-state levels of correctly initiated RNAs, although a small amount of read-in transcription was visible in the heat-shocked samples only. However, the presence of correctly initiated RNAs appeared to be due to the remarkable stability of previously synthesized rRNAs: there was a 90 to >99% decrease in the amount of correctly initiated RNAs that were recently synthesized, as determined by RNase mapping (Fig. 4B) of samples labeled in vivo with [^{32}P]PO_4. These previously synthesized RNAs were stable even after 2 h at 45°C, which is an extremely high temperature for human cells (23). Labhart and Reeder observed a similar stability of previously synthesized rRNAs in *X. laevis* after heat shock, although they saw no change in the rate of new synthesis of correctly initiated rRNAs (27).

Does initiation by Pol I require upstream termination? One of the goals of this study was to determine whether Pol I initiation from the endogenous rRNA gene is indeed dependent on or greatly facilitated by upstream termination. When mini-RNA genes are transcripted in vivo or in vitro (16, 22, 36), more RNAs are correctly initiated when an upstream terminator is present that when it is absent. Two mechanisms have been proposed to explain why the upstream terminator might enhance the level of Pol I initiation: (i) the terminator could increase the local concentration of Pol I by virtue of the fact that termination occurred within a few hundred nucleotides of the initiation site (1, 12, 28, 36, 37), or (ii) as has been shown, read-in transcription dissociates transcription initiation factors from the Pol I promoter (4). Thus, the upstream terminator would facilitate initiation by allowing the initiation complex to bind more stably.

In samples from cells that were subjected to heat shock, there was a parallel decrease in Pol I transcription termination and initiation (Fig. 4B). Although this result seems to support the theory that initiation depends directly on termination in human-derived cells, there are other potential explanations for this result: (i) some initiation factor could also be sensitive to heat, or (ii) if there are very few Pol I molecules available in the steady state, inhibition of termination would indirectly eliminate new initiation solely because of the inability to release free Pol I molecules. Our result in human cells differs from that of Labhart and Reeder, who found that the rate of Pol I initiation was unaffected by heat shock in *X. laevis*, although both downstream termination (processing) and upstream termination were almost completely inhibited (27).

ETS processing. The ETS processing reaction is the first to occur after the completion of rRNA synthesis (20). We found that heat shock and sodium arsenite treatment partially

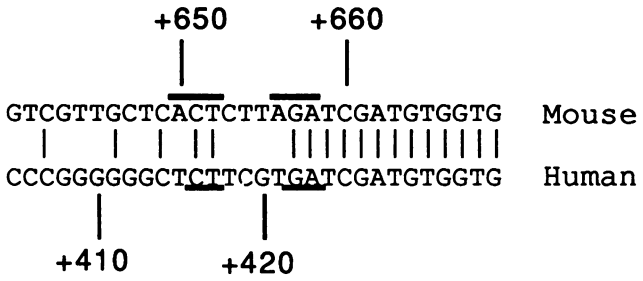

FIG. 9. Alternative alignment of the mouse and human sequences near the ETS processing site. Alignment is as described by Kass et al. (24) except that the cleavage site for the human sequence is slightly different (indicated by underlined bases). This enables the mouse and human ends to be aligned without bulging out 2 bases in this region. Vertical lines indicate identity between the two sequences.

inhibited this reaction to 67 and 33%, respectively, of the rate in control cells. Stresses were unable to stabilize any upstream fragments that have been postulated to exist, although the previously synthesized 45S precursor appeared remarkably stable after the induction of stress, with no differences noted in the relative amounts of unlabeled RNA in heat shock or control cells despite a drastic alteration in the amount of newly synthesized pre-rRNA. We did note a slightly different end than that mapped in human cells by Kass et al. (24) by S1 nuclease mapping. The major 5′ ends map to positions C416 to U417 and G422 to A423 (Fig. 5 and 9). This allows the human ends to be aligned with those of the mouse (identified by using both primer extension and S1 nuclease mapping) without invoking a 2-base-pair insertion in the human gene very close to the cleavage site.

3′ end of 28S rRNA. Almost nothing is known about how the mature end of 28S rRNA is formed. In view of the recent data that Pol I transcription termination occurs several hundred nucleotides downstream of 28S rRNA in all species in which it has been carefully studied, there are two possible ways that the 3′ end could be formed: (i) 3′ exonucleases, which are prevalent in cells, could digest from the end until a stable RNA structure is reached, or (ii) a processing factor could recognize a sequence at or near the 3′ end of 28S rRNA and either directly generate the 3′ end or produce an end that would require only a small amount of exonucleolytic trimming. The fact that in several species there is an intermediate with 10 to 30 nucleotides of sequence downstream of the 3′ end of 28S rRNA (5, 19, 26, 46) supports the latter theory, although it is possible that those ends are merely regions where an exonuclease pauses because of the secondary structure of the RNA.

Our data for the heat shock samples suggest that a specific processing factor can contribute to 3′-end formation of 28S rRNA. Although termination was completely inhibited at 43°C and 60% inhibited at 42°C, most of the transcripts had the correct end of 28S rRNA (Fig. 7B). Since the exonuclease model requires that a 3′ end be generated before it can act, it is highly unlikely that this result would be observed. The site of the putative endonucleolytic cut could not be identified by RNase mapping (Fig. 7B) or by using 5′- or 3′-end-labeled S1 nuclease probes and 45S RNA isolated on a sucrose gradient or total nuclear RNA (data not shown).

However, we cannot rule out the possibility that exonucleolytic trimming is an alternative route whereby the 3′ end of 28S rRNA is formed in non-heat-shocked cells. The region between the 3′ end of 28S rRNA and the termination site is highly unstable in correctly terminated pre-rRNAs. Almost no RNAs that end at the termination site remain 2 h after induction of heat stress, as determined by S1 nuclease mapping of unlabeled RNA. This contrasts sharply with the remarkable stability of pre-rRNAs which are correctly initiated or processed at the ETS site during the same period. It is also possible that the instability of this region is due to a 5′-to-3′ exonucleolytic destruction of RNAs that have been cleaved near the 3′ end of 28S.

The ability to process the 3′ end of 28S rRNA in heat-shocked cells should prevent the accumulation of extremely long RNAs transcribed from two or more transcription units. Indeed, no extremely long RNAs were identified on an agarose gel of in vivo-labeled RNAs (Fig. 3). This finding contrasts with the result of Labhart and Reeder for *Xenopus* rRNA, which showed that mature 28S rRNA 3′-end formation appeared to be inhibited, resulting in the appearance of extremely long transcripts.

Previous studies in yeast cells (25) demonstrated that a region containing nucleotides −36 to +74 relative to the 3′ end of 26S rRNA is sufficient to direct a high efficiency of correct 3′-end formation of a mini-rRNA gene in vivo. Furthermore, a construct containing nucleotides −2 to +41 of rRNA sequence in addition to nonribosomal sequence was able to direct 3′-end formation in *X. laevis* both in vivo and in vitro (28), although in this case at an extremely low efficiency, which implies that auxiliary sequences were required. Once a system for mammalian 3′-end formation of 28S rRNA is developed in vitro, it should be possible to determine whether it is indeed an endonucleolytic event and what sequences are necessary for it to occur.

The effect of heat is a very powerful tool for studying Pol I transcription termination, since heat treatment can quantitatively inhibit this activity. Bartsch et al. have purified a highly labile protein factor that has termination activity in vitro (3). It is possible that heat denaturation of this same protein is responsible for the in vivo effect we observe. This could easily be tested by attempting to complement extracts from heat-treated cells with the purified protein factor.

One of the functions of HSPs might be to restore the activity of this protein factor. The nucleolus accumulates large amounts of HSPs after a stress (44, 48), which may play a role in recovery from heat shock (40). It has been hypothesized that HSPs function in the normal state to unfold proteins for transport through membranes (9, 11) and function in the stressed state to unfold aggregated proteins to allow them to renature into their native states (41). Since an in vitro assay for Pol I transcription termination exists, one could directly test the ability to purified HSPs to restore activity in this system.

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