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Heat shock stabilizes highly unstable transcripts of the *Xenopus* ribosomal gene spacer

(RNA polymerase I/transcription termination/RNA processing)

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ABSTRACT **We have shown recently that, in *Xenopus laevis* oocytes, the 3′ end of the longest detectable ribosomal precursor RNA is not formed by transcription termination but by RNA processing and that RNA polymerase I continues to transcribe through the intergenic spacer region. In oocytes, these spacer transcripts are turned over rapidly, and the only apparent transcription termination site is located 215 base pairs upstream of the 5′ end of the next transcription unit. In this paper we show that, at heat shock temperature (34°C), processing at the 3′ end of the precursor, rapid turnover of spacer transcripts, and termination are all severely impaired. In contrast, transcription initiation and chain elongation are not significantly affected by heat shock. This results in the appearance of large RNA in the range of 10–20 kilobases and longer.**

Our view of the transcription of the ribosomal genes of *Xenopus laevis* has been revised by the finding (1, 2) that the only site in each repeating unit with characteristics of a terminator is located just 215 base pairs (bp) upstream of the initiation site for the 40S precursor. The 40S precursor contains the coding regions for the mature 18S, 5.8S, and 28S RNAs of ribosomes and was initially thought (3, 4) to terminate at a *Hin*dIII site at the 3′ end of the 28S coding sequence (site T1 in Fig. 1). Site T1 is now known to be an RNA-processing site, and the 3′ end of the longest detectable precursor is found 235 nucleotides further downstream at site T2 (1). Since this precursor is rapidly processed at T1, possibly even during transcription, the 40S fraction contains a mixture of two types of RNA molecules with 3′ ends at either T1 or T2, differing in length only by 235 nucleotides. At least two events happen at site T2: the transcript is quantitatively cleaved to yield 3′ ends, and the stability of the transcript changes from a half-life of 45 min or more to a half-life of <1 min. Transcription of highly unstable RNA continues from T2 across the entire intergenic spacer [≈4 kilobases (kb)] until site T3 is reached at a position 215 bp upstream of the 5′ end of the next 40S precursor coding region. Spacer transcripts arising from initiation at the spacer promoters also have been shown to terminate at the −215 site (5). Site T3 has many of the properties we would expect of a true terminator and appears able to stop polymerase travel (1). Because of the proximity of T3 to the adjacent gene promoter, we have speculated that a mechanism exists for passing the polymerase from T3 to the promoter without letting it enter the free pool. A similar interaction between terminator and promoter has been proposed by Moss and co-workers as part of their model of promoter enhancement by spacer transcription (5, 6).

In *Drosophila*, heat shock causes reversible alterations in the morphology of the nucleolus at the cytological level (7, 8). At the molecular level, those changes appear to correlate with a block in the processing of the ribosomal RNA precursor (9, 10). Furthermore, it has been reported that heat shock causes some of the major heat shock proteins to migrate into the nucleolus and concentrate there (11, 12). Pelham (8) has shown that preloading a nucleolus with the *Drosophila* 70-kDa protein alleviates some of the morphological effects of heat shock and causes the normal morphology to recover more quickly when heat shock is removed. This would imply that the heat shock protein itself does not cause the block in ribosomal RNA processing but rather has a protective function. This surmise agrees with the results of Yost and Lindquist (13), who showed by temperature-shift experiments that induction of heat shock proteins at intermediate temperatures protects against the block in mRNA splicing that normally occurs at high temperatures.

The knowledge that heat shock alters ribosomal RNA processing prompted us to examine what effect heat shock would have on the unstable ribosomal gene spacer transcripts described above. In this paper we show that heat shock greatly stabilizes the highly unstable spacer transcripts, resulting in the synthesis of long transcripts that must span several repeating units of ribosomal DNA. Transcription initiation, in contrast, is relatively unaffected by heat shock.

EXPERIMENTAL PROCEDURES

All methods used in this paper have been described in detail (1).

RESULTS

To begin the examination of the effect of heat shock on ribosomal RNA synthesis, mature stage VI oocytes of *X. laevis* were placed at 34°C for 1 hr, injected with [α-^{32}P]CTP while the temperature was maintained at 34°C, and incubated at the same temperature for an additional 4 hr. [We will show later that a temperature of 34°C gives the maximum heat shock effect, in agreement with the previous results of Bienz and Gurdon (14).] As controls, oocytes were treated the same way at room temperature (19°C). Then total RNA was extracted from each sample of oocytes and electrophoresed on denaturing agarose gels. Control oocytes showed a single major band after a 4-hr pulse label that migrated at the position expected for the 40S precursor molecule (Fig. 2, lane 1). By 4 hr, only a minor amount of the label had been processed to smaller species that migrated with 28S and 18S ribosomal RNA, as well as some processing intermediates not further identified. The major effect of heat shock (Fig. 2, lane 2) was to eliminate almost completely the 40S band. Instead, heat shock shifted most of the radioactivity to large RNAs that extended up to the limiting mobility of the gel (20 kb and greater). We also noted changes in the labeling of some processing intermediates, whereas the low amounts of

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Abbreviations: bp, base pair(s); kb, kilobase(s).

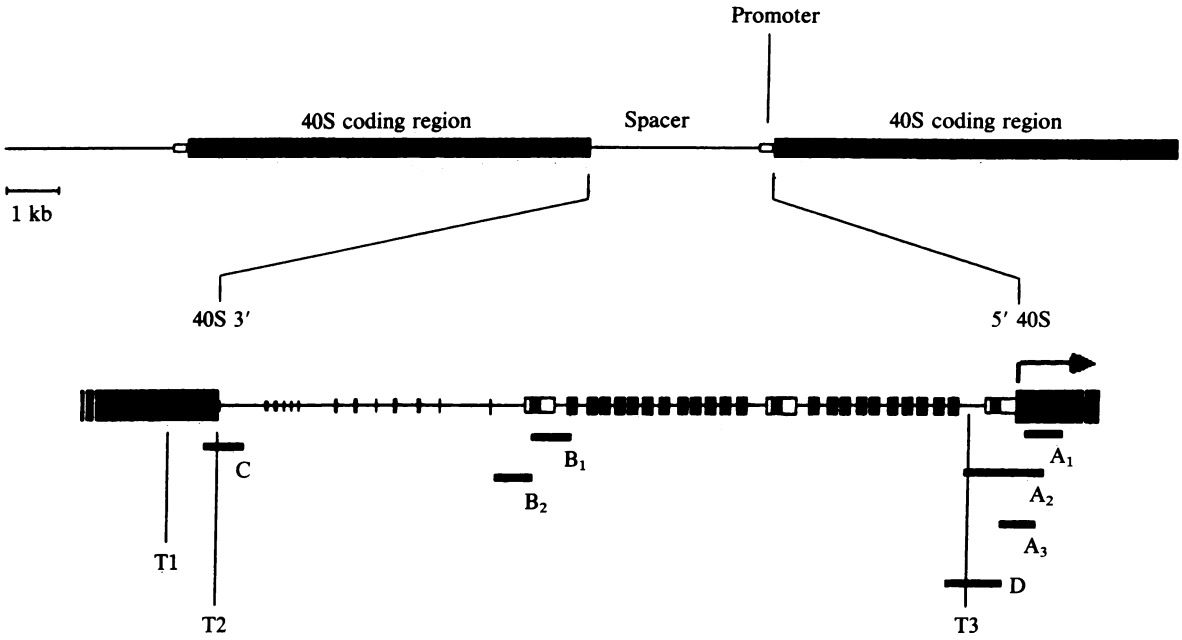

FIG. 1. (*Upper*) Diagram of two repeat units of *Xenopus laevis* ribosomal DNA. (*Lower*) Enlargement of the spacer and flanking regions, showing the sites of transcription initiation (5′ 40S), RNA processing (T1 and T2), and termination (T3). The solid bar indicates the gene region; open boxes, promoter sequences, and small black boxes, region of homology between promoter and enhancer regions. The coordinates of the probes used for RNA blot hybridization, S1 nuclease analysis, and RNase-protection assay are as follows: A_1, +14 to +108 relative to the transcription start site; A_2, −245 to +93; A_3, −34 to +34; B_1, −123 to +47 relative to the transcription start site at the spacer promoter; B_2, −123 to −254; C, +160 to +284 relative to T1; and D, −295 to −123 relative to the transcription start site at the gene promoter. S1 probes were labeled at the 5′ end (A_2 and B_1) or at the 3′ end (C and D).

18S and 28S ribosomal RNA formed during this 4-hr pulse appeared to be the same at both temperatures. Because we were mainly concerned in this study with the events in the intergenic spacer and at the 5′ and 3′ end of the precursor, we did not investigate the effect of heat shock on the production of 18S and 28S ribosomal RNA any further. Overall, this result suggests that the majority of all processing and termination events are blocked or disturbed by heat shock.

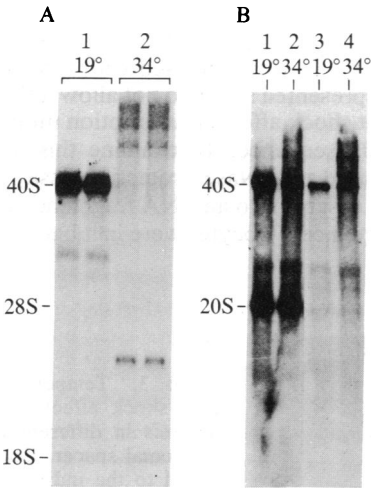

FIG. 2. Effect of heat shock on synthesis and steady-state levels of ribosomal RNA. (*A*) RNA was directly labeled for 4 hr by injection of [α-^{32}P]CTP into oocyte nuclei, either at room temperature (lanes 1) or at 34°C (lanes 2). RNA was analyzed on 1% agarose gels containing 6% formaldehyde. (*B*) RNA of control oocytes (lanes 1 and 3) or of oocytes incubated at 34°C for 6 hr (lanes 2 and 4) was fractionated on denaturing agarose gels as in *A*. After transfer to filters, RNA was hybridized to either probe A_1 (lanes 1 and 2) or probe C (lanes 3 and 4). RNA blot-hybridization analysis was performed as described (1). The migration of the 18S and 28S ribosomal RNA was determined by ethidium bromide staining of the gels. "20S" denotes a processing intermediate that was not further characterized (1, 5).

Another way to survey the effects of heat shock is to use blot-hybridization analysis to examine how it affects the steady-state distribution of ribosomal RNA precursors. Use of probe A_1 to the 5′ end of the 40S region (see Fig. 1 for probe coordinates) showed that the amount of 40S precursor molecules decreased only slightly after 6 hr of heat shock (Fig. 2*B*, lanes 1 and 2). This probe detected a mixture of precursor molecules that extended either to sites T1 or to T2 (see Introduction) and also detected a processing intermediate of about 20 S (see also ref. 5). A similar result was obtained by probing the blot hybridization with probe C, which covers the T2 region at the 3′ end of the 40S precursor (Fig. 2*B*, lanes 3 and 4). From Fig. 2*A* we know that no new 40S molecules were created during heat shock. Therefore, the result with blot hybridization in Fig. 2*B* must mean that heat shock considerably slows down processing of preexisting precursors [at room temperature, the half-life of the precursors detected by the 5′ probe is about 45 min; the precursors detected by the 3′ probe are even more rapidly turned over (1)]. In addition to these preexisting molecules that were stabilized, both probes showed that heat shock caused the appearance of a smear and some bands of large RNAs extending further up the gel, consistent with the pulse-label experiment of Fig. 2*A*. The simplest interpretation of Fig. 2 is that heat shock suppresses multiple processing events on polymerase I transcripts but does not eliminate chain elongation. This results in production of extremely long transcripts. In the remainder of this paper, we examine in more detail how heat shock affects specific events, such as initiation at promoters, processing at T2, stability of the spacer transcripts, and termination at T3.

To determine the optimum temperature for the heat shock response, groups of oocytes were incubated at various temperatures for 3 hr, their total RNA was extracted, and the RNA was probed with end-labeled, single-stranded DNA probes specific for various parts of the ribosomal gene repeat

(the exact coordinates of each probe are given in the legend to Fig. 1). Each end-labeled probe was hybridized with an RNA sample and treated with S1 nuclease to destroy unpaired nucleic acid, and the protected fragments were electrophoresed on a high-resolution polyacrylamide gel. Autoradiographs of the gels are shown in Fig. 3.

The results obtained with probe A_2, which overlaps the 5′ end of the gene region, are shown in Fig. 3*A*. At the non-heat-shock temperature of 19°C, all that the probe detected was a strong band corresponding to the correct initiation of transcription at nucleotide +1. Raising the temperature in successive steps to a maximum of 37°C had little effect on the steady-state amount of this initiation signal. This agrees with the finding (shown in Fig. 2*B*) that heat shock also had little effect on the steady-state amount of the 40S precursor. We do note, though, that at 32°C and 34°C a significant amount of full-length probe protection was observed, suggesting that at these temperatures some read-through transcription was occurring from upstream of the promoter. Since this probe only measures steady-state levels of RNA, we cannot draw any conclusions concerning initiation rates from this experiment (but see below).

A dramatic effect of heat shock was revealed by probe B_1 (Fig. 3*B*). This probe overlaps the potential initiation site of one of the duplicated promoters present in the middle of the spacer. At 19°C this probe detected nothing except for a small amount of full-length protection indicative of read-through transcription across the entire promoter. This agrees with our previous report (1) that normally in oocytes the RNA transcribed from this part of the spacer comes from polymerase that is traversing across the entire spacer. These transcripts are highly unstable so that the steady-state amount is low. At optimum heat shock temperatures (32–34°C), the amount of read-through full-length protection increased dramatically, indicating that this unstable transcript had been stabilized. There was only a slight increase in the S1 band indicating correct 5′-end formation at the spacer promoters.

Probe C overlaps site T2 and was designed to detect the 3′ termini that are normally formed at T2. At 19°C there was almost quantitative cleavage of the transcript at T2, with only a trace amount of read-through RNA detectable (Fig. 3*C*). With heat shock the situation reversed and 3′-end formation at T2 was almost completely suppressed, while read-through became abundant. Note that this result shows that the 3′ ends formed at T2 before heat shock must turn over during heat shock. This is not necessarily in conflict with the blot-hybridization result (Fig. 2*B*, lanes 3 and 4), since most of the 3′ ends detected by the S1 probe are on RNA fragments that are already separated from the precursor molecule (1).

Probe D overlaps T3, the apparent termination site. At normal temperatures this probe detected almost nothing because the transcripts in this region were also highly unstable (Fig. 3*D*). With heat shock a number of bands appeared in the T3 region that were variously due to full-length protection of read-through RNA, artifactual cleavage of read-through RNA, and authentic 3′ ends of RNA. To distinguish bands arising from read-through versus termination, a control was included in which probe D was hybridized to RNA made by SP6 polymerase *in vitro*. Bands arising in the SP6 control could be identified as due to (*i*) full-length protection of the probe; (*ii*) cleavage at a cluster of six T residues, which form a hybrid that is sensitive to S1 nuclease; and (*iii*) a repetitive part near the 3′ end of the probe (Fig. 3*D*, lane SP6). When the bands due to the T cluster and the repetitive region were subtracted from the lanes of *in vivo* RNA (Fig. 3*D*) we observed that two things happened at T3 upon heat shock. First, the amount of full-length, protected, read-through RNA increased dramatically, suggesting that heat shock damages termination. Second, some of this transcript appeared to terminate at T3, but the ratio of read-through to terminated transcripts increased as heat shock became more severe (see also Fig. 4*B*).

We conclude from Fig. 3 that the maximum heat shock effect on ribosomal gene expression is obtained at 32–34°C, the same temperatures that have been found to be optimal for inducing translation of heat shock mRNA in *Xenopus* oocytes (14). At this temperature, 3′-end formation at T2, termination at T3, and turnover of the unstable spacer transcripts were all greatly inhibited. These results readily account for the appearance of the large RNA molecules seen in Fig. 2*A*, lane 2. None of the increased level of spacer transcripts could be attributed to the spacer promoters, since they remained virtually silent before and during heat shock.

We next examined how long it took to achieve the heat shock response, using some of the same probes used in Fig. 3. Heat shock effects showed up without a lag and continued to increase for at least the first 2 hr (Fig. 4). Note that under heat shock at both sites T2 and T3, the ratio of read-through signal to specific 3′ ends increased with time.

The results presented so far do not allow a firm conclusion as to how heat shock affects transcription initiation at the 5′ end of the 40S sequence. To examine this question more directly, we used an RNase protection assay to study the accumulation of label into an RNA fragment from the 5′ end of the 40S sequences. Oocytes were incubated at either 19°C

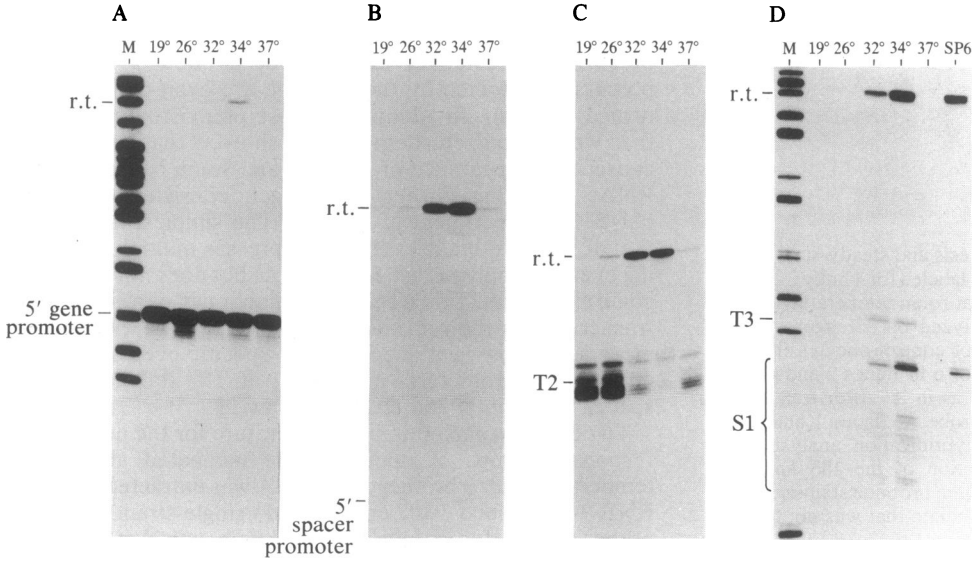

FIG. 3. Temperature optimum of heat shock effect and S1 nuclease analysis in different regions of the ribosomal spacer. Oocytes were exposed to the indicated Celsius temperatures for 3 hr, and total RNA was extracted and analyzed by S1 nuclease protection assay using the probes A_2 (*A*), B_1 (*B*), C (*C*), and D (*D*). Details of the experimental procedures have been described (1). The specific activities of the four probes are not identical. r.t., Full-length protected probe indicating read-through transcription; S1, artifactual bands (see text); SP6, control lane using RNA synthesized *in vitro* by SP6 RNA polymerase; M, end-labeled *Hpa* II digest of pBR322.

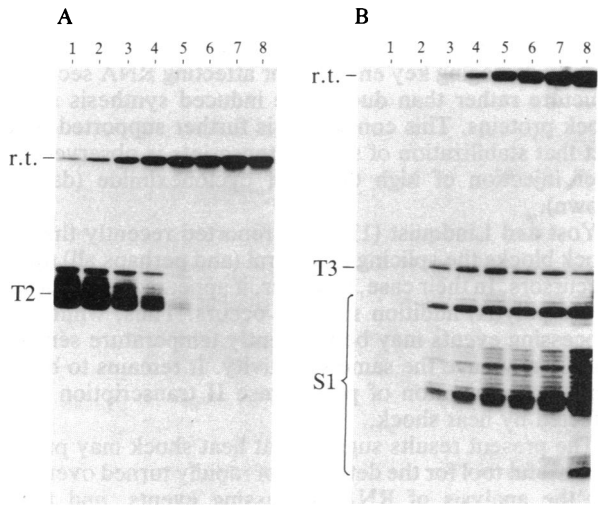

FIG. 4. Time course of heat shock effect on sites T2 and T3. Oocytes were incubated at 34°C for different times, and RNA was analyzed by S1 nuclease protection assay using the probes C (*A*) and D (*B*). For explanation of r.t. and S1, see the legend to Fig. 3. Time of incubation: 0 min (lanes 1), 5 min (lanes 2), 15 min (lanes 3), 30 min (lanes 4), 1 hr (lanes 5), 2 hr (lanes 6), 3 hr (lanes 7), 6 hr (lanes 8).

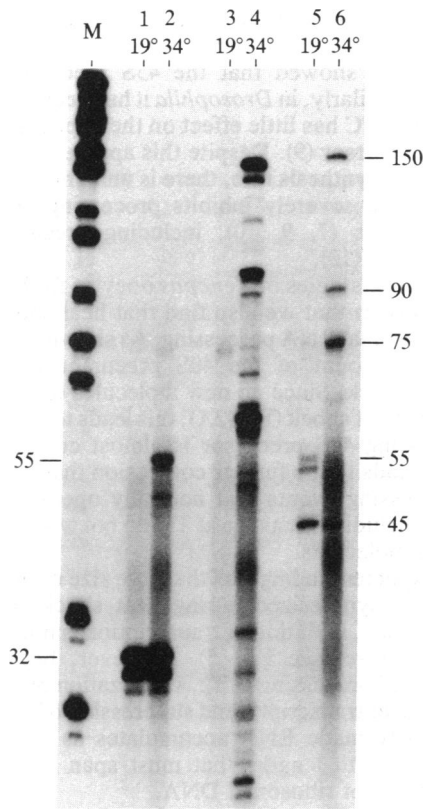

FIG. 5. Effect of heat shock on transcription initiation. RNA was labeled either at room temperature (19°C) or at 34°C exactly as in Fig. 2*A*. The labeled RNA was analyzed by the RNase protection assay as described (1). The probes used were A_3 (lanes 1 and 2), B_2 (lanes 3 and 4), and C (lanes 5 and 6). The approximate sizes of the major protected fragments are indicated. Note that the incorporation of label into about the first 32 nucleotides of the 40S precursor (band at 32) is the same at both temperatures. Because of the RNase treatment, the size of the protected fragments is in most cases shorter than the size expected from the length of the probe (as discussed in ref. 1). M, as in Fig. 3.

or 34°C for 1 hr, injected with [α-^{32}P]CTP, and incubated a further 4 hr at the same temperature before extraction of the RNA (same labeling protocol that was used in Fig. 2*A*). Aliquots of the labeled RNA were then hybridized with different unlabeled, single-stranded DNA probes and treated with RNase to destroy unpaired RNA, and the protected RNA was electrophoresed on a gel and autoradiographed. The results are shown in Fig. 5. Probe A_3 overlaps the 5′ end of the 40S coding region and protects a short piece of RNA about 32 nucleotides long. The amount of label that accumulated in this protected 32-nucleotide fragment was essentially the same whether the labeling was done at 19°C (Fig. 5, lane 1) or at 34°C (Fig. 5, lane 2). Thus, we could detect no significant effect on the transcription initiation rate. However, as expected from the S1 nuclease analysis shown in Fig. 3*A*, a considerable amount of label was incorporated into read-through RNA (due to failure of termination at T3), giving rise to an additional protected RNA fragment ≈55 nucleotides long (Fig. 5, lane 2). [The probe used would be expected to protect a fragment of 68 nucleotides for read-through RNA. The shortening is probably due to the excessive RNase treatment required to reduce the background (as discussed in ref. 1), since the same 55-nucleotide band was seen in controls using read-through RNA made from SP6 vectors.] For comparison with probe A_3, probes to two other regions of the ribosomal DNA repeat were used to measure RNA accumulation by the same RNase protection technique. Probe B_2 detects RNA from a region upstream of the first spacer promoter (see Fig. 1 for the location of the probe). In the absence of heat shock, almost no labeled RNA was detected by this probe, whereas in the presence of heat shock, the probe detected large amounts of labeled RNA (Fig. 5, lanes 3 and 4). This result directly shows that, under heat shock conditions, the accumulation of spacer transcripts becomes similar to the accumulation of the 5′ end of the precursor (compare lanes 2 and 4). Lanes 5 and 6 in Fig. 5 show the analysis of the same labeled RNA using a probe spanning site T2 (probe C). Consistent with the nuclease S1 analysis shown in Figs. 3*C* and 4*A*, at room temperature (Fig. 5, lane 5) the label was incorporated into protected RNA fragments shorter than expected for read-through RNA (at about 45 and 55 nucleotides), indicating 3′-end formation at T2. At heat shock temperature, however, the label was incorporated into longer RNA fragments (75, 90, and 150 nucleotides), indicating read-through transcription. (In fact, the same three longer fragments were found with a control using SP6 RNA; see ref. 1).

We also investigated how fast ribosomal gene transcription would recover from heat shock. Oocytes were pretreated at 34°C for 3 hr and then injected with [α-^{32}P]CTP at room temperature. After 3 hr of labeling at room temperature, the RNA was analyzed as in Fig. 5. The same result was obtained with the pretreated oocytes as with control oocytes (not shown). This indicates that ribosomal gene transcription recovered very rapidly from heat shock, possibly immediately. However, when steady-state RNA of heat-treated oocytes was analyzed by nuclease S1 assay at different times after the return to room temperature, it was found that the heat-stabilized spacer transcripts disappeared only slowly. Even after 20 hr, the level of spacer transcripts was still ≈20% of the level immediately after heat shock (data not shown). Thus, once RNA has been stabilized by heat shock, it is very resistant to further degradation, even when the temperature is lowered.

DISCUSSION

Previous studies of heat shock influence on ribosomal RNA synthesis have reported effects of varying severity. For example, in ascites cells it has been reported that exposure to 43°C for 1 hr results in a 90% loss of autoradiographic grains

incorporated over the nucleolus and a similar loss of label in RNA sedimenting at 45S (7). In contrast, studies of Hela cells treated at 42°C showed that the 45S precursor was still formed (15). Similarly, in *Drosophila* it has been reported that heat shock at 37°C has little effect on the appearance of label in the 38S precursor (9). Despite this apparent variability in effect on RNA synthesis rate, there is unanimity in reporting that heat shock severely inhibits processing of ribosomal RNA precursors (7, 9, 10), including precursors to 5S ribosomal RNA (10).

In general our studies of *Xenopus* oocytes fit the pattern of previous work in that we also find that heat shock strongly disturbs ribosomal RNA processing. As shown in Fig. 2*B*, the steady-state amount of the 40S precursor declines little during heat shock. Since no new molecules of 40S RNA are made during heat shock (Fig. 2*A*), this leads to the conclusion that preexisting 40S precursor is almost completely stabilized. This leads to the further conclusion that essentially all of the processing events that normally operate on the 40S precursor, including cleavage at T1, do not work anymore on preexisting molecules.

The unexpected finding was the large size of the ribosomal RNA that is synthesized during heat shock. In *Xenopus* oocytes, at least, initiation of transcription is not impaired by high temperature (see Fig. 5). However, because of the suppression of processing at T2, stabilization of the normally unstable spacer transcripts, and suppression of termination at T3, the newly made RNA accumulates as high molecular weight RNA with lengths that must span at least several repeating units of ribosomal DNA.

The blocking effect of heat shock on RNA processing and turnover is not absolute. We note, for example, that in Fig. 2*A*, lane 2, the smear of high molecular weight that accumulates during heat shock shows reasonably discrete bands that plausibly could be multiples of one repeat length. The implication is that, as the polymerases pass each T3 region, a fraction terminates while another fraction continues onward. Similarly, the high molecular weight material in Fig. 2*A*, lane 2, does not reach the same steady-state level as does the 40S precursor in lane 1. Since we can detect no drop in the initiation rate (Fig. 5), this implies that those long RNA molecules are still being processed or turned over, perhaps by a pathway that does not lead to stable RNA.

Heat shock effects on ribosomal RNA processing and termination are clearly detected within minutes after shifting the temperature (Fig. 4) and are rapidly reversed on newly synthesized RNA. This implies that the effect is due to directly damaging key enzymes or affecting RNA secondary structure rather than due to the induced synthesis of heat shock proteins. This conclusion is further supported by the fact that stabilization of spacer transcripts is observed even after injection of high doses of cycloheximide (data not shown).

Yost and Lindquist (13) have reported recently that heat shock blocks the splicing of several (and perhaps all) mRNA precursors. In their case, however, it appears that processing at the poly(A) addition site still occurs. Thus, while many processing events may be inherently temperature sensitive, not all may have the same sensitivity. It remains to be seen whether termination of polymerase II transcription is also affected by heat shock.

The present results suggest that heat shock may prove to be a useful tool for the detection of rapidly turned over RNA, for the analysis of RNA processing events, and for the delineation of transcribable DNA domains.

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