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Heat Shock Inhibits Pre-rRNA Processing at the Primary Site In Vitro and Alters the Activity of Some rRNA Binding Proteins

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Abstract The effect of heat shock on pre-rRNA processing at the primary site within external transcribed spacer region 1 (ETS1) was studied in S-100 extract derived from mouse lymphosarcoma cells. In vivo labeling with [^{32}P]orthophosphate showed that the synthesis of the rRNA precursor and its processing to 28S and 18S rRNAs were inhibited significantly due to heat shock. The processing activity was reduced by 50% at 1 h and was completely blocked following 2-h exposure of cells at 42°C. Mixing S-100 extracts from the control and heat-treated cells did not affect the processing activity in the control extract, which proves the absence of a nuclease or other inhibitor(s) of processing in the extract from the heat-shocked cells. Heat shock did not affect interaction between pre-rRNA and U3 snoRNA, a prerequisite for the processing at the primary site, but significantly altered RNA–protein interaction. Three polypeptides of 200, 110, and 52 kDa that specifically cross-link to pre-rRNA spanning the primary processing site were inactivated after heat shock. Hyperthermia did not alter 3′ end processing of SV40L pre-mRNA.

Key words: heat shock, pre-rRNA processing, S-100 extract, U3 snoRNA, 3′ processing

Mammalian ribosomal RNA (rRNA) is synthesized as a large precursor molecule of 47S (~ 13.5 kb) that subsequently undergoes a series of site-specific cleavage and trimming to generate the mature rRNA subunits, namely 18S, 5.8S, and 28S rRNAs. The first step in the processing of pre-rRNA is the endonucleolytic cleavage near the 5′ end of the external transcribed spacer region, approximately 4 kb upstream of the 18S rRNA start site. The 5′ end product is rapidly degraded and the 3′ end product undergoes a series of processing reactions to generate the mature rRNA subunits [for review, see Eichler and Craig, 1994].

The molecular mechanism of rDNA transcription has been characterized in great detail [for review, see Paule, 1993; Jacob, 1995]. Unlike the transcription machinery, the *trans*-acting factors involved in pre-rRNA processing in animal cells have not been explored in great detail due to lack of an in vitro system that can accurately and efficiently process pre-rRNA at all the sites, namely ETS1, ETS2, ITS1, and ITS2. Mouse S-100 extract has been shown to process in vitro transcribed pre-rRNA fragment at the primary site within ETS1 [Miller and Sollner-Webb, 1981]. This system was used to demonstrate that the processing takes place at any of the two sites that are few nucleotides apart (at nucleotide 651 or 656 in mouse and at position 415 or 419 in human, with respect to transcription start site). A minimal sequence of 11 nucleotides spanning the processing site, which is conserved among humans, mouse [Mougey et al., 1993], rat [Stroke and Weiner, 1989], and frog [Mougey et al., 1993], is critical for the rRNA processing reaction. An additional 200 nucleotides downstream of this 11 nucleotide is essential for efficient processing [Craig et al., 1991]. This sequence is also highly conserved in species ranging from frog to humans [Mougey et al., 1993]. Like pre-mRNA processing, pre-

Abbreviations: ETS1, external transcribed spacer region 1; ETS2, external transcribed spacer region 2; ITS1, internal transcribed spacer region 1; ITS2, internal transcribed spacer region 2; snoRNA, small ribonucleolar RNA; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; AMT-psoralen, 4′aminoethyl-4,5′,8-trimethyl psoralen.

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rRNA processing involves specific RNP complex formation. Six different polypeptides can be ultraviolet (UV) crosslinked to the pre-rRNA fragment spanning the primary processing site [Kass and Sollner-Webb, 1990]. U3 snoRNA, a major small nucleolar RNA [Kass et al., 1990] and a specific nucleolar endoribonuclease devoid of RNA [Eichler et al., 1993], represent two key factors essential for pre-rRNA processing in animal cells. U3 snoRNA is known to form a duplex with the pre-rRNA surrounding processing site in humans [Maser and Calvet, 1989], rat [Stroke and Weiner, 1989], and mouse [Tyc and Steitz, 1992]. The nucleolar endoribonuclease that cleaves pre-rRNA at the correct sites forms specific RNP complex and cross-links to pre-rRNA [Eichler et al., 1993].

Our laboratory has been interested in elucidation of the mechanisms by which rDNA transcription is regulated [Reichel and Jacob, 1993, Hoff et al., 1994; Liu and Jacob, 1994; Niu and Jacob, 1994; Datta et al., 1995]. Recently, we have turned our attention to identification and characterization of the factors involved in pre-rRNA processing. We have developed an in vitro system from mouse lymphosarcoma cells that can efficiently process rat pre-rRNA [Ghoshal and Jacob, 1994]. We used this cell-free system to demonstrate that an important mode of action of 5-fluorouracil (5-FU), a potent anticancer drug, is via inhibition of pre-rRNA processing. Another means for downregulating rRNA processing is to expose the cells just a few degrees above the physiological temperature for a brief period [Sadis et al., 1988; Parker and Bond, 1989]. The molecular mechanism by which hyperthermia downregulates the processing reaction has not been explored. The availability of a highly reproducible in vitro system from mouse lymphosarcoma cells in our laboratory [Ghoshal and Jacob, 1994] prompted us to investigate whether the inhibition of pre-rRNA processing by heat shock is a specific event. This study showed that elevation of incubation temperature to 42°C for 1–2 h rapidly inactivated pre-rRNA processing machinery without affecting pre-mRNA 3′ end processing activity. The heat shock-induced block in pre-rRNA processing appears to be mediated by inhibition of interaction of three polypeptides known to be involved in pre-rRNA processing.

MATERIALS AND METHODS

Plasmids and RNA Substrates

The construction of pT3(X-S) has been described earlier [Ghoshal and Jacob, 1994]. Linearization of this plasmid with *Apa*LI and transcription by T3 polymerase generates a 541-nucleotide-long RNA that was used for in vitro processing reaction. For UV-cross-linking studies, RNA (385 nucleotides in length) transcribed from *Hae*II-linearized plasmid was used. Human U3 cDNA [Baserga et al., 1991] was subcloned into pBluescript/KS (Stratagene) vector between SacII and *Eco*RI sites, and α-^{32}P-UTP labeled antisense U3 RNA was transcribed by T3 polymerase. The plasmid, pSPSV −58/+70, spanning SV40L polyadenylation site was used for assaying pre-mRNA 3′ end processing activity [Sheets et al., 1990]. Linearization of this plasmid with Dra I and transcription by SP6 polymerase yields a 125-nucleotide-long pre-mRNA that was used as substrate for cleavage and polyadenylation.

Cell Culture, Heat Shock, and Analysis of Protein Synthesis

Mouse lymphosarcoma cell line, P1798, was a generous gift of Aubrey E. Thompson (University of Texas, Galvaston). These cells were grown in a spinner flask in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and subjected to heat shock by incubating at 42°C (5–7.5 × 10^5 cells/ml) for 1–2 h. The control cells were incubated at 37°C for the same time period. Both control and heat-shocked cells were harvested, washed with methionine-free RPMI and resuspended in the same media containing 5% FBS and ^{35}S-Met (10 μCi/ml) at a density of 10^6 cells/ml and incubated at 37°C for 30 min [Watowitch and Morimoto, 1988]. The cells were harvested from 1 ml of culture, proteins were dissolved in Laemmli sample buffer, separated by SDS–PAGE, and analyzed by autoradiography.

Labeling of Pre-rRNA and Its Processed Products, 28S and 18S RNAs In Vivo With [^{32}P]Phosphate

Cells were labeled in vivo following the method of Parker and Bond [1989]. Five ml of growing lymphosarcoma cells (10^6 cells/ml) were harvested, washed with phosphate-free RPMI media and resuspended in 5 ml of the same media prewarmed at 37°C or 42°C. After 15 min of incubation (for control and 1-h heat shock) and

75-min incubation (for 2-h heat shock) at the designated temperature, [^{32}P]PO_4 (1 mCi/ml) was added and the cells were incubated for an additional 45 min at the respective temperatures. The cells were washed with cold PBS, and total RNA (tRNA) was isolated by acid guanidinium thiocyanate phenol method [Chomczynski and Sacci, 1987]. RNA (10 μg) was separated in a formalydehyde–agarose (1%) gel, stained with ethidium bromide, dried, and subjected to autoradiography for 2 h at room temperature.

Preparation of Cell Extracts

S-100 extracts were prepared from control and heat-shocked P1798 cells [Mahajan and Thompson, 1990]. Nuclear extracts were prepared as described [Ghoshal and Jacob, 1991].

Coupled Transcription and Processing at the Primary Site

The coupled transcription and processing of rat rDNA was performed as described [Ghoshal and Jacob, 1994]. The 50-μl reaction mixture contained 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, 0.5 mM each of ATP, GTP, and UTP; 0.03 mM CTP and 5 μCi of (α-^{32}P)CTP (specific activity, 800 Ci/mmol), 100 ng of the linearized template DNA, and 50 μg of S-100 extract. After 30 min of incubation, RNA was extracted from one-half the reaction mixture and ethanol precipitated ("pulse"). To the other half, 0.5 μl of 100 mM CTP was added and incubated at 30°C for another 45 min ("chase"). The reaction was stopped and the precipitated RNA separated by electrophoresis in 4% polyacrylamide–8.33 M urea gel and analyzed by autoradiography.

Uncoupled Processing of Pre-rRNA at the Primary Site

α-^{32}P-labeled pre-rRNA was synthesized from *Apa*LI linearized pT3(X-S) using T3 polymerase for transcription. The in vitro-synthesized RNA was purified by polyacrylamide–urea gel electrophoresis. For processing, 2–5 pmol of the labeled RNA was added to a 25-μl reaction mixture containing 20 μg of S-100 extract, 20 mM Hepes (pH 7.9), 120 mM KCl, 2 mM $MgCl_2$, 1.5 mM ATP, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The reaction mixture was incubated for 60 min at 30°C. The product was analyzed by PAGE under denaturing conditions as described.

3′end Processing of SV40L Pre-mRNA

α-^{32}P-labeled pre-mRNA (25,000 cpm) was incubated with the nuclear extract in the presence of ATP (for polyadenylation reaction) or 3′dATP (to study cleavage reaction) [Ghoshal and Jacob, 1991]. After 60 min of incubation at 30°C, the reaction was stopped and the products were separated in polyacrylamide (6%)–urea gel and analyzed by autoradiography.

Interaction of U3-snoRNA With Pre-rRNA

In vivo psoralen crosslinking assay was used to determine interaction of U3 snoRNA with pre-rRNA. This assay was performed essentially according to the method of Maser and Calvet [1989]. Control or heat-shocked (2-h) lymphosarcoma cells were harvested and washed once with ice-cold PBS and once with TBS. The cell pellet was then resuspended in equal volumes of TBS containing 0.25 μg/ml AMT-psoralen (HRI Associates) and incubated on ice for 10 min. One-half of the cells were kept in the dark on ice, and the other half was transferred to a petri dish (2-cm-diameter) and irradiated for 10 min with 365-nm light from a UV lamp held at a distance of 3 cm from top. The cells were then harvested and RNA isolated by guanidinium thiocyanate–CsCl density-gradient procedure [Sambrook et al., 1989]. Total RNA (25 μg) was separated on formaldehyde–agarose gel, transferred to nitrocellulose membrane and subjected to Northern blot analysis with α-^{32}P-labeled antisense U3 RNA as probe.

Analysis of Proteins That Cross-link to Pre-RNA Spanning the Primary Processing Site

To analyze the proteins that cross-link to pre-rRNA spanning the processing site, α-^{32}P-labeled rRNA was synthesized in which 4S-UMP was incorporated instead of UMP. For this purpose, 4S-UTP was synthesized [Bartholomew et al., 1986], and *Hae*II linearized pT3(X-S) was transcribed with T3 polymerase with 4S-UTP as substrate along with other necessary components. α-^{32}P-labeled RNA was then incubated at 30°C with S-100 extract (25 μg of protein) from control or heat-shocked cells under processing conditions as described [Kass and Sollner-Webb, 1990]. Each reaction mixture was preincubated with tRNA and poly(ACI) (1 μg of each) for 30 min to minimize nonspecific binding. After 50 min of incubation, heparin (0.8 mg/ml) was added and the mixture was incubated for 10 min

at 30°C. Next, the mixture was irradiated with 310 nm UV light for 5 min at room temperature. The mixture was treated with RNase A (1 mg/ml) and RNase T1 (1 U) for 15 min at 30°C. Laemmli loading buffer was added to the mixture, the proteins were denatured by heating in boiling water and separated by SDS–PAGE. The radiolabeled proteins were analyzed by autoradiography of the dried gel.

RESULTS

Exposure of Lymphosarcoma Cells at 42°C Results in Induction of Heat-Shock Response

Because S-100 extract from mouse lymphosarcoma cell line, P1798 can efficiently transcribe and process mouse or rat rRNA gene, we selected these cells to investigate the effect of heat shock on rDNA transcription and processing. It was important to determine first whether these lymphoid derived cells respond to heat shock, as all mammalian cell lines do not show the heat-shock response in culture [Lindquist, 1986]. To test this possibility, induction of heat-shock response at the protein level was studied by in vivo labeling with ^{35}S-methionine. For this purpose, the control and heat-shocked cells (2 h at 42°C) were incubated for 30 min at 37°C in the presence of ^{35}S-Met in methionine-free media (see Methods for details), cells (same number of control and treated) were then harvested, proteins solubilized, separated by PAGE and analyzed by autoradiography. Analysis of the labeled proteins revealed enhanced synthesis of heat-shock proteins of 110, 90, and 70 kDa in the cells incubated at 42°C for 2 h (Fig. 1, cf. lane 2 with lane 1). The apparent molecular weights of the induced proteins were identical to those of the corresponding mammalian heat-shock proteins [Lindquist and Craig, 1988].

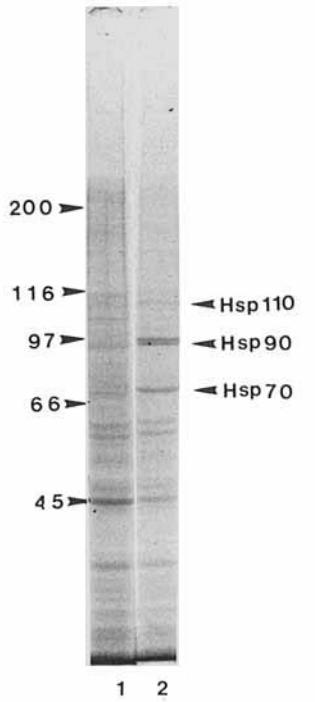


Fig. 1. Induction of heat shock proteins in mouse lymphosarcoma P1798 cells. The cells were incubated at 37°C and 42°C for 2 h (see Methods for details), harvested, and incubated at 37°C in methionine-free RPMI media containing ^{35}S-Met (10 μCi/ml) for 30 min, and the labeled proteins derived from 1 × 10^6 cells were subjected to SDS–PAGE and analyzed by autoradiography. *Lanes 1 and 2,* ^{35}S-labeled protein profiles of cells exposed to 37°C and 42°C, respectively. *Arrows (left),* positions of the molecular-weight marker proteins in kDa.

Heat-Shock Inhibits Synthesis of Pre-rRNA and Its Processed Products, 28S and 18S RNA In Vivo

To demonstrate that heat shock inhibits the synthesis of RNA in vivo, lymphosarcoma cells were either incubated at 42°C for 1 and 2 h or grown at 37°C and incubated with [^{32}P]PO_4 for 45 min. Heat shock at 42°C did not inhibit transport of [^{32}P]PO_4 into the cells (data not shown). Total RNA isolated from these cells was separated on denaturing agarose gel and analyzed by autoradiography. Incubation of lymphosarcoma cells at 42°C for 1 h significantly inhibited synthesis of rRNA precursor (45S) and its processing into intermediate RNAs (41S, 32S, 20S, pre-rRNAs) as well as mature products, 28S and 18S rRNAs (Fig. 2A, cf. lane 2 with lane 1). Exposure of cells for 2 h at 42°C completely blocked the synthesis of rRNAs (Fig. 2A, lane 3). Ethidium bromide staining of the same gel showed that inhibition of rRNA synthesis at elevated temperatures is not due to degradation or unequal loading of RNAs as shown by similar intensities and ratios of 28S to 18S rRNA in every sample (Fig. 2B). These data clearly show that heat shock inhibits rRNA synthesis in mouse lymphosarcoma cells in vivo.

Transcription of rRNA Gene and Its Subsequent Processing at the Primary Site (Coupled Transcription–Processing) Are Inhibited by Heat Shock of Lymphosarcoma Cells

We have shown earlier that S-100 extract prepared from mouse lymphosarcoma cells can transcribe rat rDNA fragment and process the transcript synthesized at the primary site within

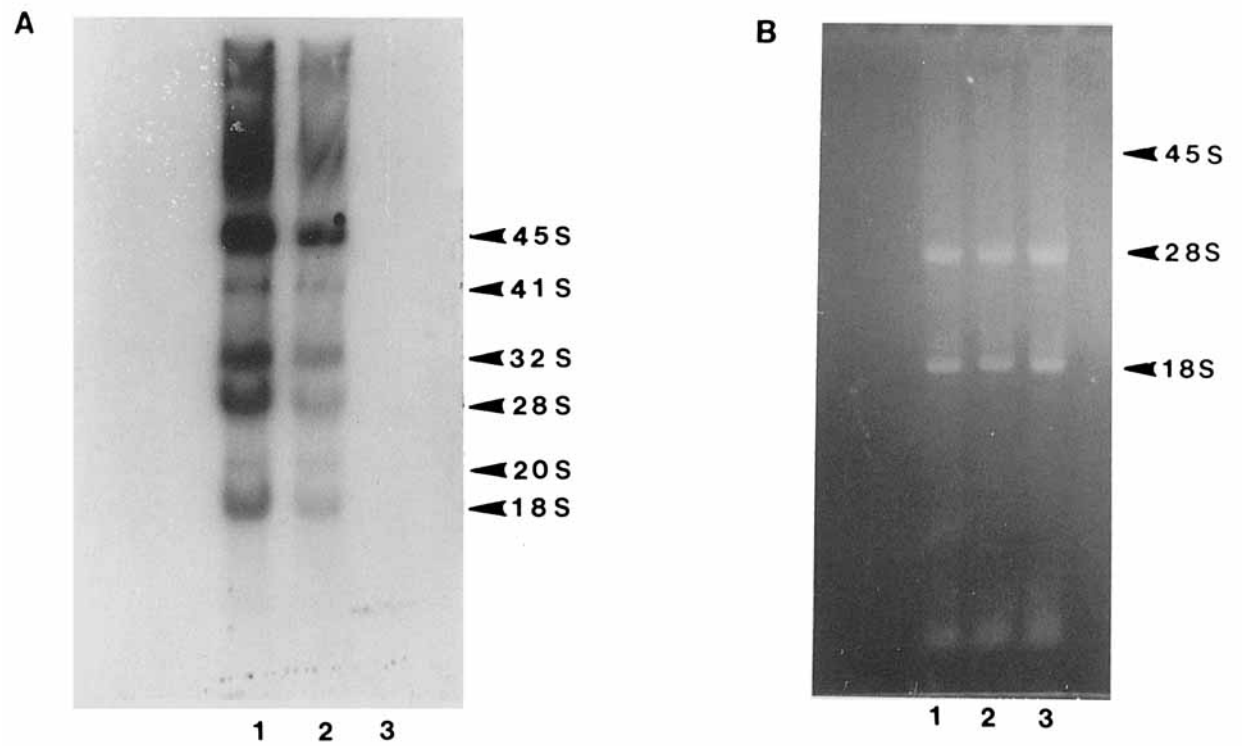


Fig. 2. Effect of heat shock on rRNA synthesis and processing in vivo. The cells were incubated at 37°C or 42°C (1–2 h) and [^{32}P]PO_4 was added to label the cells in vivo (see Methods for details). Total RNA was isolated from these cells, separated on formaldehyde–agarose gel, stained with ethidium bromide, and analyzed by autoradiography. Positions of the stained bands (45S, 28S, and 18S rRNAs) are shown on the right. Positions of the precursor rRNA (45S), processing intermediates (41S, 32S, 20S) and mature products (28S and 18S RNAs) are marked on the right. **A:** Profile of labeled rRNAs of cells exposed to 37°C (*lane 1*), 42°C for 1 h (*lane 2*), and 2 h (*lane 3*). **B:** Photograph of the same gel after eithidium bromide staining.

5′ETS [Ghoshal and Jacob, 1994]. The *Apa*LI-linearized pB7-2.0 yields an α-^{32}P-labeled runoff transcript of 1149 nucleotides (denoted by T; see Fig. 3, lane 1) which, in the presence of 100-fold excess cold CTP, undergoes subsequent processing to generate a 3′ end product of 354 nucleotides (denoted by P) in the control S-100 extract (lane 2). The 5′ end product is rapidly degraded by nucleases present in the extract. When S-100 extract was prepared from cells exposed at 42°C for 2 h was used for this assay, transcription was inhibited by 50% (compare lane 3 with lane 1) and processing was abolished (cf. lane 4 with lane 2).

Uncoupled Processing of Pre-rRNA at the Primary Site Is Inhibited In Vitro by Heat Shock of Lymphosarcoma Cells

To determine whether heat treatment of cells also inhibits rRNA processing independent of transcription (uncoupled reaction), pre-rRNA fragment obtained from *Apa*LI-linearized pT3(X-S) was incubated with an identical amount of protein from S-100 extracts from control and heat-treated cells under processing conditions (see Methods for details). In control S-100 extract, the 541-nucleotide-long substrate RNA undergoes endonucleolytic cleavage at the specific processing site to give a 3′ end product of 354 nucleotides and a rapidly degraded 5′ end product of 187 nucleotides (Fig. 4, lane 2). When identical amounts of S-100 extracts from heat-shocked cells were used in this assay, the processing activity decreased in a time-dependent manner. After 1 h of heat shock, the processing activity decreased by 50% (cf. lane 3 with lane 2) and 2 h exposure resulted in complete loss of processing activity (lane 4). To test whether this loss of processing activity is reversible, cells heat-shocked for 2 h were incubated at the physiological temperature for 6 h and S-100 extract made from these cells was assayed for the in vitro processing reaction. Reincubation at 37°C restored the processing activity in the heat-shocked cells (cf. lane 5 with lane 4). A mixing experiment was performed to rule out the possibility that the inhibition of rRNA processing at the primary site is due to activation of nonspe-

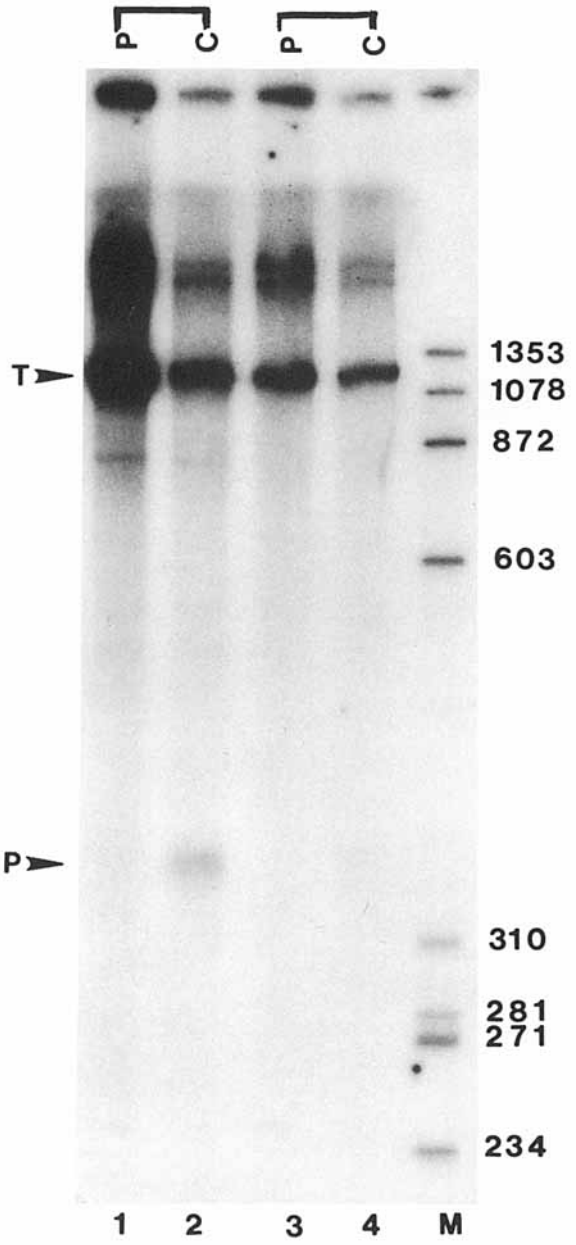


Fig. 3. Effect of heat shock on coupled transcription and processing of pre-rRNA in mouse S-100 extract. Identical amounts (50 μg) of S-100 extract prepared from control (grown at 37°C) and heat-shocked (42°C for 2 h) cells were initially incubated under transcription conditions with (α-^{32}P)CTP. After 45-min incubation at 30°C, one-half the reaction was stopped as indicated by P (pulse). To the other half, 100-fold excess cold CTP was added and the mixture was incubated for another 45 min at 30°C to study processing of the transcribed RNA, as indicated by C (chase). *Lanes 1,2,* transcription and processing in the control S-100 extract; *lanes 3,4,* transcription and processing in the extract prepared from the heat-treated cells. *Lane M,* positions of γ-^{32}P-labeled fragments of ϕX174 DNA digested with *Hae*III (sizes in nucleotides given on the right). T and P on the left indicate the positions of the transcript and the 3′ processed product, respectively.

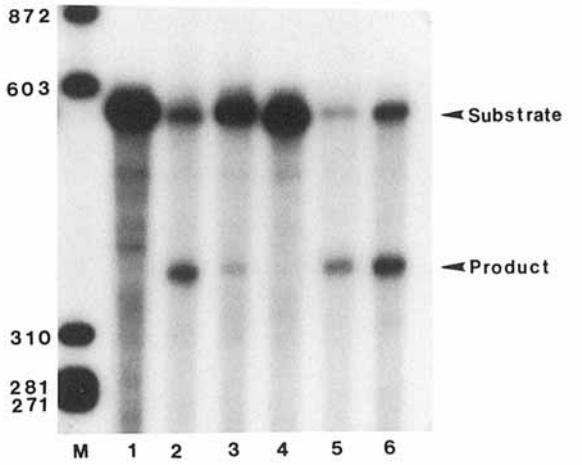


Fig. 4. Effect of heat shock on uncoupled processing of pre-rRNA fragment in vitro. Uncoupled processing of pre-rRNA transcript in S-100 extract prepared from control, heat shocked and cells reincubated at 37°C for 6 h after exposed to 42°C for 2 h. α-^{32}P-Labeled precursor rRNA fragment spanning the primary processing site was obtained by transcription of *Apa*LI-linearized pT3 (X-S). This RNA was incubated under processing condition with 25 μg of proteins from extract of control (*lane 2*) cells and cells heat-shocked for 1 h (*lane 3*) and 2 h (*lane 4*). *Lane 5,* processing activity of identical amounts of extract prepared from cells reincubated at 37°C after exposure at 42°C for 2 h. *Lane 6,* processing activity using 12 μg each of the extract from control cells and cells exposed to heat for 2 h. *Lane 1,* substrate RNA incubated without extract. *Lane M,* positions of γ-^{32}P-labeled fragments of ϕX174 DNA digested with *Hae*III. Their sizes in nucleotides are given on the left.

cific nuclease activity. When half of the control extract was mixed with equal amount of extract prepared from 2-h heat-treated cells, the processing reaction continued unabated (lane 6). These data indicate that heat shock neither induces a nonspecific nuclease nor causes irreversible inactivation of the processing machinery.

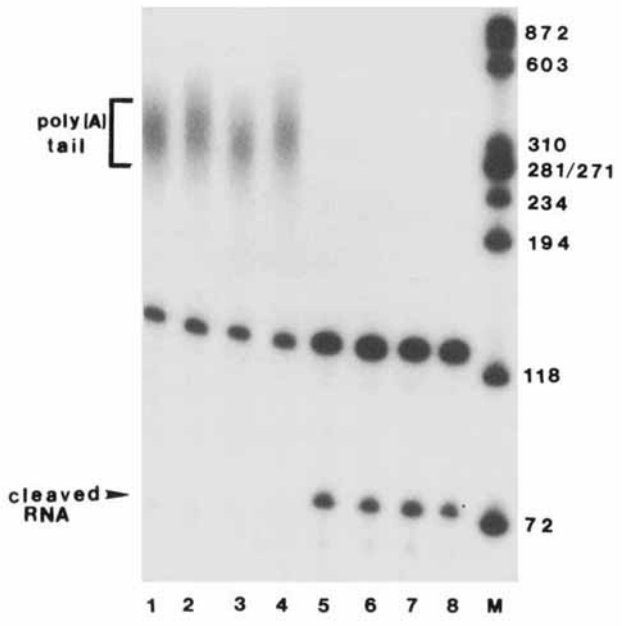
The 3′ End Processing of SV40 Late Pre-mRNA in the Nuclear Extract Is Not Affected by Heat Shock of Lymphosarcoma Cells

To prove that the heat shock-induced inhibition of pre-rRNA processing is a specific effect, we investigated whether other processing reactions are affected under this condition. We have previously shown that nuclear extract prepared from lymphosarcoma cells can cleave α-^{32}P-labeled pre-mRNA at the poly(A) site followed by the addition of a poly(A) tail of ~250 adenylate residues [Ghoshal and Jacob, 1994]. The cleavage and polyadenylation reactions are tightly coupled in vivo. In the presence of cordycepin triphosphate, an RNA chain terminator, or EDTA, a Mg^{2+} chelator, the 5′ cleaved prod-

uct could be detected in vitro. The 3′ end product was rapidly degraded in the nuclear extract. In the present study, we used SV40 late pre-mRNA as substrate for the 3′ end processing activity [Sheets et al., 1990]. When this mRNA was incubated in the nuclear extract under the cleavage condition, a 5′ product of 70 nucleotide was generated (Fig. 5, lanes 5 and 6). Cleavage activity of the nuclear extract prepared from the cells exposed to 42°C for 2 h was unaffected (lanes 7 and 8). Similarly, when identical amounts of protein from the control and heat-treated cells were incubated with labeled pre-mRNA fragment along with ATP and Mg^{2+}, the efficiency of poly(A) tail addition remained unaffected (compare lanes 1 and 2 with lanes 3 and 4 respectively). It is evident from these data that, unlike pre-rRNA processing activity, the 3′ end processing activity is not inhibited by heat shock. Therefore, heat shock does not inhibit all cellular activities nonspecifically in lymphosarcoma cells.

In Vivo Cross-linking of U3 snoRNA to Pre-rRNA Remains Unaffected by Heat Shock of Lymphosarcoma Cells

Earlier studies have shown that U3 snoRNP particle is involved in the processing of pre-rRNA at the primary site [Kass et al., 1990]. U3 snoRNA also cross-links in vivo to human [Maser and Calvet, 1989] and rat [Stroke and Weiner, 1989] pre-rRNA, spanning the primary processing site. It was logical to study the potential effect of heat shock on the interaction between these two RNA species. When lymphosarcoma cells were treated with AMT-psoralen, exposed to 365 nm UV light and the RNA isolated from these cells was subjected to Northern blot analysis with antisense U3 RNA as probe, several crosslinked high molecular weight bands, e.g., 47S, 35S, 27S, and 24S were detected (Fig. 6, lane 3), which did not appear in RNA from the non-irradiated cells (lanes 1 and 2). In both cases, antisense U3 probe detected U3 RNA. These crosslinked bands were shown to be RNAs, as DNase treatment had no effect (data not shown). When the same amounts of total RNA from the control and heat-shocked cells were analyzed by this procedure, similar crosslinking pattern was obtained (cf. lane 3 with 4). This result shows that heat shock does not affect hybridization of U3 RNA to pre-rRNA, a step essential for pre-rRNA processing [Eichler and Craig, 1994].



SV40L pre-mRNA in vitro. Identical amounts (25 μg) of nuclear extract from control cells (*lanes 1,2,5,6*) and cells heat-shocked for 2 h (*lanes 3,4,7,8*) were incubated with α-^{32}P-labeled pre-mRNA (125 nucleotides) under polyadenylation (*lanes 1–4*) or cleavage (*lanes 5–8*) conditions (see Methods for details of polyadenylation and cleavage reaction). *Lane M*, positions of molecular-weight markers (*Hae*III digests of ϕX174 DNA) whose sizes in nucleotides are shown on the right.

UV-Cross-linking of Some RNA Binding Proteins to Pre-rRNA Spanning the Primary Processing Site Is Inhibited by Heat Shock of Lymphosarcoma Cells

For cross-linking studies, we used thiouridine incorporated pre-rRNA fragment, as cross-linking is significantly enhanced in the presence of this modified nucleotide [Kass and Sollner-Webb, 1990]. When 4-SUMP incorporated pre-rRNA was incubated with S-100 extract under processing conditions, irradiated with 310 nm of UV light and subjected to SDS–PAGE after RNase treatment, several polypeptides with approximate molecular masses of 200, 110, 75, 60, 52, and 40 kDa were detected (Fig. 7, lane 1). When cold pre-rRNA was used during preincubation, these bands could be competed out (lane 3), suggesting that the crosslinked proteins are specific for pre-rRNA spanning the processing site. When the extract was preincubated with proteinase K, the labeled bands disappeared, which proves that the cross-linked entities are polypeptides (data not shown). When identical amounts of proteins from the 2-h heat-shocked cells were used for cross-linking studies, polypeptides of 200, 110, and 52 kDa were significantly reduced (cf. lane 2 with lane 1). Hyperthermia also re-

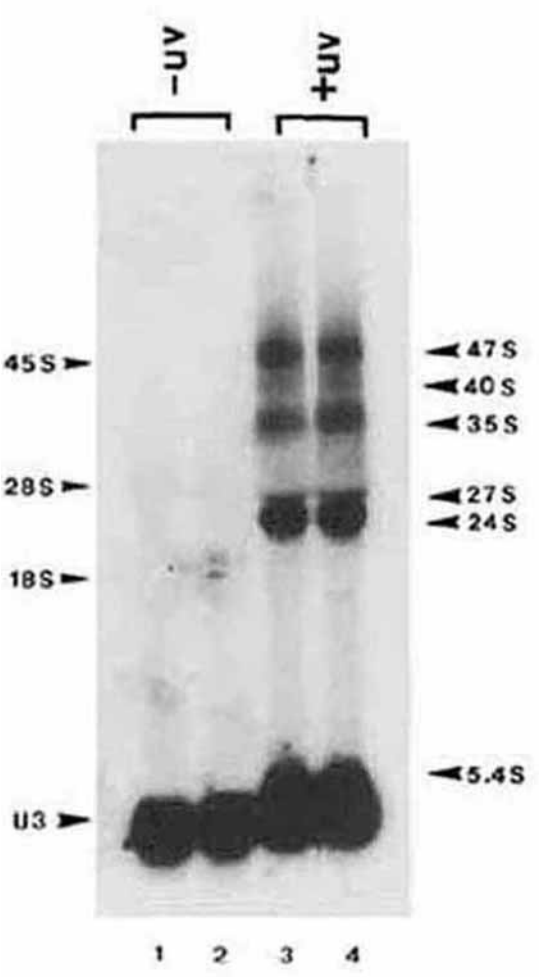


Fig. 6. Effect of heat shock on in vivo cross-linking of U3 snoRNA to pre-rRNA. Lymphosarcoma cells grown at 37°C or heat shocked for 2 h at 42°C were incubated with equal packed cell volume of AMT-psoralen (250 μg/ml) for 10 min on ice. One-half the cells were then irradiated with 365-nm UV light, and the other half was kept in the dark (see Methods for details). Total RNA (25 μg) isolated from these cells was subjected to Northern blot analysis with antisense α-^{32}P-labeled U3 RNA as probe. *Lanes 3,4,* U3 cross-linking profile of RNA isolated from 37°C and 42°C cells, respectively; *lanes 1,2,* unirradiated controls. Positions of marker RNAs as revealed from ethidium bromide profile of the same blot are shown on the left. The sizes of cross-linked RNA bands are shown on the right.

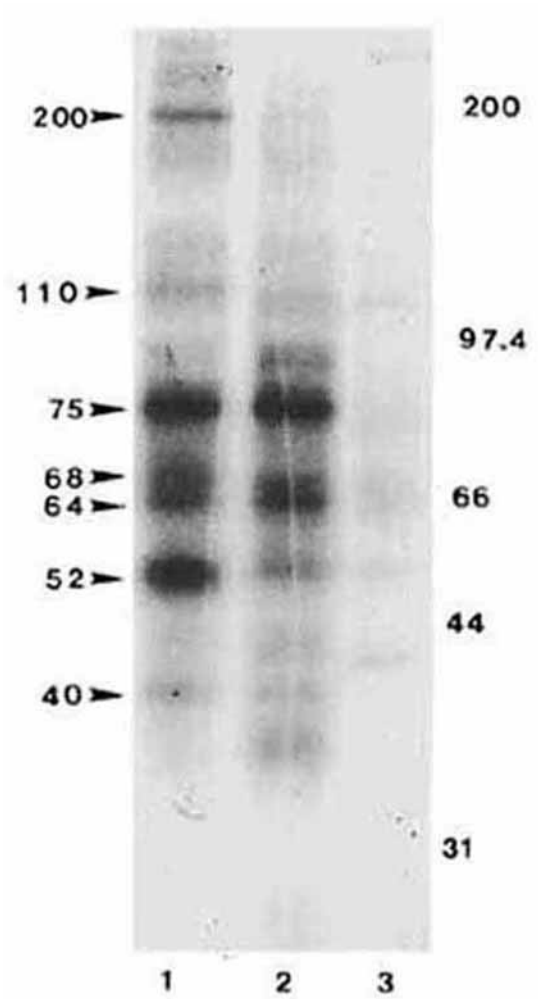


Fig. 7. Effect of heat shock on cross-linking of proteins in S-100 extract to pre-rRNA fragment. Fifty μg of protein from S-100 extract prepared from control and 2-h heat-shocked cells was incubated under processing conditions with 4-SUMP-incorporated α-^{32}P-CMP-labeled pre-rRNA fragment obtained from *Hae*II-linearized pT3(X-S) (see Methods for details). The mixture was then irradiated with 310-nm UV light, digested with RNase, subjected to SDS–PAGE, and analyzed by autoradiography (see Methods for details). *Lanes 1,2,* cross-linked protein bands from control and treated extracts respectively; *lane 3,* cross-linking profile of control S-100 extract preincubated with cold RNA. Numbers on the right side of the figure represent positions of Rainbow (Amersham) molecular-weight marker proteins and those on the left side represent size of the cross-linked polypeptides.

sulted in the emergence of three RNA binding proteins with approximate molecular masses of 90, 44, and 30 kDa that were not detected in the control extract (cf. lane 2 with lane 1). It is not known whether these polypeptides have any significant role in pre-rRNA processing.

DISCUSSION

Transcription of ribosomal RNA gene and pre-rRNA processing are regulated by various physiological means. Both are upregulated by cell growth and serum enrichment [Isfort and Cody, 1992] and downregulated by heat shock (Sadis et al., 1988, Parker and Bond, 1989). rRNA synthesis and processing are not always regu-

lated coordinately. For example, differentiation of myoblasts to myotubes leads to reduction in rRNA synthesis without affecting rRNA processing [Bowman, 1987]. On the other hand, rRNA synthesis proceeds unabated following treatment of cells with 5-FU [Wilkinson and Pitot, 1973] or arsenite [Parker and Bond, 1989], whereas these treatments severely impair the processing.

Heat shock can also inhibit transcription of rDNA in vitro, albeit at a slower rate than the processing reaction by altering the activity/amount of the DNA-binding protein, E_1BF/Ku [Ghoshal and Jacob, in press]. The present study was undertaken to determine whether a cell-free system can mimic the downregulation of pre-rRNA processing by heat shock in vitro. Such a system would facilitate elucidation of the factors responsible for the modulation of the processing reaction by hyperthermia. Indeed, S-100 extract prepared from the mouse lymphosarcoma cells heat-shocked for 2 h was incapable of promoting pre-rRNA processing. The specificity of the heat shock-induced inhibition of pre-rRNA processing was demonstrated by the persistent 3′ end processing of pre-mRNA under this condition. Heat shock does not appear to activate an inhibitor of rRNA processing such as a potent nonspecific nuclease, as mixing the extracts from the control and heat-treated cells does not inhibit the processing reaction in the control extract.

U3 small nucleolar RNA is an important component of pre-rRNA processing machinery. This RNA is known to form a specific hybrid with the pre-rRNA primary processing site and that this reaction is essential for the processing reaction [Eichler and Craig, 1994]. It was, therefore, logical to investigate whether interaction between U3 snoRNA and pre-rRNA is affected by heat shock. We did not observe any significant alteraction in RNA-RNA hybrid formation. This is not an unexpected finding, as a slight elevation in temperature may not perturb RNA:RNA hybridization.

Further exploration of the nature of the factors constituting the specific complex revealed at least six polypeptides of 110, 75, 60, 52, and 42 kDa (Fig. 6). These polypeptides are similar to those observed as part of pre-rRNA processing machinery in another mouse system [Kass and Sollner-Webb, 1990]. The minor differences in the molecular sizes of the polypeptides in the two extracts may be due to different cell types or to variation in the number of labeled nucleotide moieties that remained bound to the polypeptides after RNase digestion. The noteworthy finding was that only three polypeptides that cross-linked to pre-rRNA processing site were reduced after heat shock. Two of these polypeptides correspond to nucleolin (110 kDa) and the nucleolar endoribonuclease (52 kDa), at least in molecular size. The purified endoribonuclease can also cross-link to pre-rRNA spanning the processing site [Eichler et al., 1993]. It is likely that inhibition of processing activity at the primary site is due to inactivation of this enzyme. The induction of three RNA-binding polypeptides of 90, 44, and 30 kDa in response to heat shock is of interest. We cannot rule out the possibility that these are proteolytic degradation products of high-molecular-weight proteins. We are currently investigating whether these proteins are indeed involved in the heat-shock-mediated downregulation of pre-rRNA processing.

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