Heat shock selectively inhibits ribosomal RNA gene transcription and down-regulates E₁BF/Ku in mouse lymphosarcoma cells

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The effect of heat shock on RNA polymerase I (pol I)-directed transcription of the rRNA gene was studied in S-100 extract derived from mouse lymphosarcoma cells, and by *in vivo* labelling of rRNA. Exposure of cells to 42 °C for 2 h resulted in complete inhibition of rRNA synthesis *in vivo*. Pol I transcription was inhibited by 50 % within 2 h of heat shock and was abolished after 3 h exposure at 42 °C. Under this condition, the core-promoter-binding activity of the factor (CPBF) that modulates pol I transcription was unaffected. In contrast, the promoter-binding activity of enhancer-1-binding factor, a protein related to the Ku autoantigen, which is involved in pol I transcription initiation, was reduced by 50 and 90 % after 2 and 3 h of heat

shock respectively. Western-blot analysis with antibodies specific for the two subunits of Ku protein showed the absence of p72 subunit after 3 h of heat shock. Under this condition, pol II transcription from the adenovirus major late promoter and pol III transcription of 5 S RNA gene remained unaffected. Mixing experiments ruled out the possibility that the inhibition of transcription was due to activation of nucleases or other inhibitors. This is the first report to show selective down-regulation of pol I transcription *in vitro* by heat shock and of the potential involvement of a pol I transcription factor in this process.

INTRODUCTION

In mammalian cells, the ribosomal RNA gene (rDNA) is transcribed exclusively by RNA polymerase I (pol I) to generate a transcript of 47 S (approx. 13.5 kb). This transcript subsequently undergoes a series of site-specific cleavage and trimming to generate the mature rRNAs, namely 18 S, 5.8 S and 28 S rRNA. The biochemical steps involved in the synthesis of pre-rRNAs have been characterized in great detail. With the advent of cell-free transcription systems, significant progress has been made in the identification of the *cis*-acting elements and characterization of the *trans*-acting factors (for reviews, see refs. [1–3]. At least four *cis*-acting elements (core promoter, upstream control element, enhancer and terminator sequences) control rDNA transcription. In addition to pol I, several *trans*-acting factors for rDNA transcription have been characterized. The first well-characterized pol I transcription factor is upstream-binding factor (UBF) which interacts with both the upstream control element and core promoter [4]. The key initiation factor called SL1 [5] consists of TATA-binding protein (TBP) and TBP-associated factors (TAFs) [6]. Human and mouse TAFs are comprised of three polypeptides with molecular masses of 110, 63 and 48 kDa [7,8]. Recently all three subunits of human TAFs have been cloned [7] and SL1 activity was reconstituted from recombinant TAFs and TBP [9]. Each of the TAFs can independently bind to TBP and interact with each other [8]. A hallmark of rDNA transcription is the requirement of a homologous factor for efficient and accurate transcription of rDNA from a particular species [10]. The binding of human or frog SL1 to the promoter requires UBF, whereas the rat or mouse SL1 can independently interact with the promoter [3]. UBF appears to facilitate recruitment of TAFs to form the stable initiation complex [6].

Recently, we have characterized two factors that can modulate pol I transcription. One, enhancer-1-binding factor (E₁BF), was initially purified from rat mammary adenocarcinoma cells [11]. Subsequently, we demonstrated its relationship to the human Ku autoantigen with regard to the size of their two subunits and immunological cross-reactivity [12]. It binds to the enhancer elements and the core promoter, and is involved in the initiation of rDNA transcription, particularly in the preinitiation complex-formation [13]. Another protein, designated core-promoting-binding factor (CPBF), interacts specifically with the core promoter element and augments pol I transcription [14]. CPBF interacts with E₁BF and stimulates rDNA transcription in a synergistic manner [15]. We have further shown that CPBF is functionally and immunologically related to the basic helix–loop–helix–zipper DNA-binding protein upstream stimulatory factor (USF) [16].

The rate of rDNA transcription can be altered dramatically in response to a variety of stimuli. Pol I transcription can be up- or down-regulated depending on the conditions. For example, it is up-regulated in response to growth [17], simian virus 40 and polyoma virus infection [18,19] or glucocorticoid treatment of non-lymphoid cells such as rat hepatoma cells [20], whereas it is down-regulated by glucocorticoid in lymphosarcoma cells [21], poliovirus infection [22] or in response to protein synthesis inhibition [23]. Recently, our laboratory has been involved in investigating the molecular mechanism(s) underlying the down-regulation of rDNA transcription [24]. This investigation revealed that E₁BF can be post-translationally modified after serum starvation of rat cells, N1-S1, under conditions that resulted in arrest of cell growth but not cell death. Interestingly, the modified form of E₁BF functioned as a repressor of rDNA transcription [24]. An obvious question is whether the activity and/or level of E₁BF is altered by other means which lead to

Abbreviations used: rDNA, ribosomal RNA gene; pol I, RNA polymerase I; pol II, RNA polymerase II; pol III, RNA polymerase III; E₁BF, enhancer-1-binding factor; CPBF, core-promoting-binding factor; USF, upstream stimulatory factor; UBF, upstream binding factor; TBP, TATA-binding protein; TAF, TBP-associated factor; Hsp, heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AdMLP, adenovirus 2 major late promoter; EMSA, electrophoretic mobility-shift assay; rCP, rat core promoter; MLTF, major late transcription factor; MRE-c', metal regulatory element-c'; MRE-d, metal regulatory element-d; MT, metallothionein.

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down-regulation of rDNA transcription. A relatively direct approach to this problem is to explore the significant inhibition of rDNA transcription observed after subjecting certain cells to heat shock [25,26]. The present study describes the effect of heat shock of rDNA transcriptions in mouse lymphosarcoma cells, particularly with respect to the level/activity of two key factors involved in rDNA transcription. The results show that the inhibitory effect of heat shock on transcription was largely confined to pol I transcription, and that, of the two factors studied, only E₁BF was down-regulated in the early stages of heat shock.

MATERIALS AND METHODS

Plasmids and oligonucleotides

The following plasmids were used for *in vitro* transcription assays: (a) pB7-2.0 containing -167 to +2000 bp of rat rDNA with respect to transcription initiation site, (b) pML (C₂AT) spanning -400 to +10 bp region ligated to 390 bp G-less cassette [27], (c) pTH1 that contains a 2.2 kb fragment including promoter, coding region and terminator sequence of Syrian hamster 5 S rRNA gene [28].

The double-stranded oligonucleotides used as probes for electrophoretic mobility-shift assays (EMSAs) included rat core promoter (rCP), 106 bp metallothionein (MT)-I promoter fragment (-148 to -43 bp sequence with reference to transcription start site) that includes the major late transcription factor (MLTF)-binding site, mouse MT-I metal regulatory element (MRE)-c' and mouse MT-I MRE-d.

Cell culture, heat shock and analysis of protein synthesis

Mouse lymphosarcoma cell line, P1798, was a generous gift from Aubrey E. Thompson, University of Texas, Galveston, TX, U.S.A. These cells were grown in a spinner flask in RPMI 1640 supplemented with 5% fetal bovine serum and subjected to heat shock by incubating at 42 °C (5×10^5 – 7.5×10^5 cells/ml) for 1–3 h. The control cells were incubated at 37 °C for the same time period. Both control and heat-shock cells were harvested, washed with methionine-free RPMI and resuspended in the same medium containing 5% fetal bovine serum and [³⁵S]methionine (10 µCi/ml) at a density of 10^6 cells/ml and incubated at 37 °C for 30 min [29]. The cells were harvested from 1 ml of culture, proteins were dissolved in Laemmli buffer, separated by SDS/PAGE (8% acrylamide) and analysed by autoradiography.

Northern-blot analysis

Polyadenylated RNA was isolated from the control and heat-shocked cells by oligo(dT)-cellulose chromatography. RNA (5 µg) was separated on a formaldehyde-agarose (1%) gel, transferred to Zetaprobe membrane and probed with random-primed cDNAs for heat shock protein (Hsp) 70 [30] and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31] respectively.

In vivo labelling with [³²P]phosphate and analysis of labelled rRNAs

Cells were labelled *in vivo* essentially by the method of Parker and Bond [26]. Growing lymphosarcoma cells (5 ml; 10^6 cells/ml) were harvested, washed with phosphate-free RPMI medium and resuspended in 5 ml of the same medium prewarmed at 37 °C or 42 °C. After 15 min of incubation (for control and 1 h heat-shocked samples) and 75 min incubation (for 2 h heat-shocked samples) at the designated temperatures, [³²P]P_i (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 was isolated by the guanidinium thiocyanate/acid/phenol method [32]. RNA (30 µg) was separated in a formaldehyde-agarose (1%) gel, stained with ethidium bromide, dried and subjected to autoradiography for 2 h.

Preparation of cell extracts

S-100 extracts were prepared from control and heat-shocked P1798 cells [33]. Nuclear extract for transcription of adenovirus 2 major late promoter (AdMLP) was prepared from the isolated nuclei as described [34]. Protein was estimated by using Bio-Rad DC reagent with BSA as standard.

In vitro transcription assay

Rat rDNA plasmid, pB7-2.0, was used as the template for an *in vitro* transcription assay in S-100 extract. Transcription of this plasmid linearized with *Apa*LI should generate a 1149-nucleotide-long run-off transcript. The run-off pol I transcription assay was performed as described [35]. The plasmid containing the AdMLP, pML (C₂AT), was used as a template for pol II transcription and the reaction was performed in the nuclear extract by the method used for the pol I transcription assay with the following modification. GTP in the reaction mixtures was replaced by 3'-O-methylGTP (0.1 mM), and RNase T1 (10 units) was included in the reaction mixture. The length of the correct transcript derived from pML (C₂AT) should be 400 nucleotides [27]. The plasmid pTH1 containing Syrian hamster 5 S RNA gene was used as a template for pol III transcription which should generate a 120-nucleotide-long transcript [28]. The autoradiograms were analysed by densitometric scanning.

EMSA

High-specific-radioactivity oligonucleotide probe containing 58 bp rat rDNA core promoter sequence spanning from -36 to +18 bp relative to the transcription initiation site was end-labelled with [γ -³²P]ATP (specific radioactivity approx. 6000 Ci/mmol, 150 µCi/µl) catalysed by T4 polynucleotide kinase. EMSA was performed with S-100 extract essentially as described in [12] except that incubation was at 4 °C instead of 30 °C to minimize degradation of the labelled probe by phosphatases present in the extract. For competitive EMSA, control S-100 extract (10 µg) was preincubated for 20 min with different cold unlabelled competitor oligonucleotides (5 pmol of each) in addition to 50 ng of poly(dI-dC) as a non-specific competitor. To test the effect of anti-Ku antibodies (specific for p70 subunit), S-100 extract was pre-incubated with 1 µg of immune IgG or control rabbit IgG for 15 min on ice before the addition of labelled probe.

Immunoblot analysis

Identical amounts of protein (100 µg) from S-100 extracts were separated by SDS/PAGE (7.5% acrylamide) and subjected to Western blotting for E₁BF subunits as described [12]. The monoclonal antibody against the p70 subunit of human Ku autoantigen, N3H10, was used to detect the p72 subunit of mouse E₁BF/Ku. Polyclonal antisera against the p85 subunit of human Ku were used to detect p85 subunit of mouse E₁BF/Ku. The immunoblots were analysed by densitometric scanning.

RESULTS

Exposure of lymphosarcoma cells to elevated temperature results in induction of heat-shock response

Because S-100 extract from the mouse lymphosarcoma cell line, P1798, can efficiently transcribe mouse or rat rDNA, we selected these cells to investigate the effect of heat shock on rDNA transcription. Before we embarked on this study, it was important to determine whether these lymphoid-derived cells respond to heat shock, as all mammalian cell lines do not show heat-shock response in culture [36]. Induction of heat-shock response at the protein level was studied by *in vivo* labelling with [35 S]methionine and at the mRNA level by Northern-blot analysis with cDNA for Hsp 70 as probe. First, the control and heat-shocked cells (2 h at 42 °C) were incubated at 37 °C in the presence of [35 S]Met in methionine-free medium (see the Materials and methods section for details). After 30 min, cells (equal number from both samples) were harvested, and proteins were solubilized, separated by PAGE and analysed by autoradiography. Analysis of the labelled proteins revealed augmented synthesis of heat-shock proteins of molecular mass 110, 90 and 70 kDa in the cells incubated at 42 °C for 2 h (Figure 1A; compare lane 2 with lane 1). The apparent molecular masses of the induced proteins are identical with those of the corresponding mammalian heat-shock proteins [37].

Figure 1 Induction of heat-shock response in mouse lymphosarcoma, P1798, cells

(A) Induction of heat-shock proteins at elevated temperature. The cells were incubated at 37 °C and 42 °C for 2 h (see the Materials and methods section for details), harvested and incubated at 37 °C in methionine-free RPMI media containing [35 S]Met (10 μ Ci/ml) for 30 min. The labelled proteins derived from 1×10^6 cells were subjected to SDS/PAGE and analysed by autoradiography. Lanes 1 and 2 represent 35 S-labelled protein profiles of cells exposed to 37 °C and 42 °C respectively. The arrows on the left side indicate position of the molecular-mass marker proteins in kDa. (B) Effect of heat shock on the expression of heat-shock protein, Hsp 70, gene. The cells were incubated at 42 °C for 2 h, and polyadenylated RNA (5 μ g) isolated from the cells incubated at 37 °C (lane 1) and 42 °C (lane 2) were separated on agarose gel, transferred to Zetaprobe membrane and analysed by Northern blot with human Hsp 70 cDNA as probe (upper band). The same blot was stripped off the probe and reprobbed with GAPDH cDNA to show the equal loading of RNA (lower band).

Next, polyadenylated RNA from control and heat-shock cells were subjected to Northern-blot analysis with human Hsp 70 cDNA as probe. Indeed, the steady-state level of Hsp 70 mRNA increased 3-fold in the heat-shocked cells (Figure 1B; compare lane 2 with lane 1). A constitutive expression of the Hsp 70 gene was also observed at 37 °C (lane 1). This increase in the steady-state level is probably due to an increase in expression as well as stabilization of the message [25]. This blot was reprobbed with GAPDH cDNA to prove that this increase in heat-shock gene is specific. Under this condition, there was no detectable increase in GAPDH mRNA, which also rules out unequal loading of RNA for Northern-blot analysis.

Heat shock inhibits synthesis of rRNA *in vivo*

To demonstrate that heat shock inhibits the synthesis of RNA *in vivo*, lymphosarcoma cells were either stressed at 42 °C for 1 and 2 h or grown at 37 °C and incubated with [32 P] P_i for 45 min. Heat shock at 42 °C did not inhibit transport of [32 P] P_i into the cells (results not shown). Total RNA isolated from these cells was separated on denaturing agarose gel and analysed by autoradiography. Incubation of lymphosarcoma cells at 42 °C for 1 h significantly inhibited synthesis of rRNA precursor (45 S), the processing intermediates (41 S, 32 S) as well as the processed 28 S rRNA (Figure 2A; compare lane 2 with lane 1). The band above 45 S probably corresponds to 47 S rRNA precursor. Its processing to 45 S RNA is blocked by heat shock [38]. Exposure of cells for 2 h at 42 °C completely blocked the synthesis of rRNAs (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 28 S to 18 S rRNA in every sample (Figure 2B). These data clearly show that heat shock inhibits rRNA synthesis in mouse lymphosarcoma cells *in vivo*.

Heat shock inhibits pol I transcriptions of rRNA gene *in vitro*

The S-100 extract prepared from growing mouse lymphosarcoma cells can efficiently and accurately transcribe rat rDNA fragment *in vitro* [12,33]. The *Apa*LI-linearized rDNA plasmid yielded a run-off transcript of 1149 nucleotides in the extract prepared from control cells (Figure 3A, lane 1). To study the effect of heat shock on pol I transcription, identical amounts of S-100 extract from control and heat-shocked cells were used for transcription of rat rDNA *in vitro* (see Materials and methods section for details of transcription assay). Exposure of lymphosarcoma cells to 42 °C resulted in inhibition of rDNA transcription in a time-dependent manner (Figure 3A, compare lanes 2 and 3 with lane 1). rDNA transcription was inhibited by approximately 55% within 2 h of heat shock (lane 2), and extending the heat treatment to 3 h abolished the transcription (lane 3). The inhibition of transcription induced by hyperthermia was not due to non-specific inhibitors or nucleases in the 42 °C extract, as mixing the extracts from the control and the heat-treated cells could support efficient transcription of rDNA (Figure 3B; compare lane 2 with lanes 1 and 3).

Heat shock does not affect pol II transcription from AdMLP or pol III transcription of 5 S RNA gene

Next, we investigated whether heat shock can inhibit transcription by pol II and pol III. For this purpose, we selected (AdMLP) (a pol II template) and 5 S RNA gene (a pol III template). For transcription from AdMLP, identical amounts of

Figure 2 Effect of heat shock on rRNA synthesis *in vivo*

The cells were incubated at 37 °C or 42 °C (for 2 h) and [^{32}P]P_i was added to label the cells *in vivo* (see the Materials and methods section for details). Total RNA was isolated from these cells, separated on formaldehyde-agarose gel, stained with ethidium bromide and analysed by autoradiography. (A) Profile of labelled rRNAs of cells grown at 37 °C (lane 1) or exposed to 42 °C for 1 h (lane 2) or 2 h (lane 3). Positions of the precursor rRNAs (45 S), processing intermediates (41 S, 32 S) and mature 28 S rRNAs are shown on the right. Ethidium bromide staining of the same gel. The stained bands are shown on the right.

Figure 3 Effect of heat shock on rDNA transcription *in vitro*

(A) Growing cells were exposed at 42 °C for 2–3 h, and S-100 extracts (25 μg of protein) made from control (37 °C) cells and cells exposed at 42 °C were analysed for rDNA transcription with *Apa*LI-linearized pB7-2.0 (plasmid containing rat rDNA fragment) as template. The reaction products were analysed on 4% acrylamide-urea (8 M) gel followed by autoradiography. Lanes 1–3 represent transcripts obtained from control extract and extracts from cells exposed to 42 °C for 2 h and 3 h respectively. (B) Effect of mixing S-100 extracts of control and 3 h heat-shocked cells on rDNA transcription *in vitro*. Lanes 1–3 represent transcription in S-100 extracts from control, mixed (37 °C + 42 °C) and 42 °C cells respectively. The arrow on the left indicates the position of the transcript.

nuclear extracts from control and heat-treated cells were incubated with the plasmid, pMI (C_{24}AT) along with other necessary components. Transcription of this plasmid generated the correctly initiated 400-nucleotide-long transcript which remained unaffected after 2 h of heat shock at 42 °C (Figure 4A; compare

Figure 4 Effect of heat shock on pol II and III transcription

(A) Nuclear extracts were prepared from control (37 °C) and heat-shocked (42 °C) cells. Identical amounts (25 μg) of proteins from the nuclear extracts were incubated with the template (pMLC₂AT) along with other necessary components (see the Materials and methods section for details) and the product was analysed by electrophoresis in acrylamide-urea gel as described for pol I transcription. Lanes 1–3 represent transcription in control extract, extracts from cells exposed at 42 °C for 2 and 3 h respectively. (B) Pol III transcription of 5S RNA gene was performed in S-100 extracts prepared from control and heat-treated cells (see the Materials and methods section for details). The reaction product was analysed in 10% acrylamide-urea gel followed by autoradiography. Lanes 1–3 represent transcription in extracts from control cells and cells exposed at 42 °C for 2 and 3 h respectively. The arrow on the left indicates the position of the transcript.

lane 2 with lane 1). Interestingly, a small but reproducible increase (about 1.5-fold) in transcription from the AdMLP (Figure 4A, lane 3) was observed after 3 h exposure to 42 °C. The significance of this observation is unclear. The important issue is that unlike pol I transcription, pol II transcription was not down-regulated after heat-shock treatment of lymphosarcoma cells.

To study the effect of heat shock on pol III transcription, transcription of 5 S RNA gene in S-100 extract from the mouse lymphosarcoma cells was examined in response to heat shock. The 5 S RNA gene was transcribed efficiently and accurately in S-100 extract from lymphosarcoma cells to generate a 120-nucleotide-long transcript (Figure 4B, lane 1). Heat shock for 2–3 h did not alter transcription of this gene (Figure 4B; compare lanes 2 and 3 with lane 1). These results indicate that heat shock does not

Figure 5 Effect of heat shock on core-promoter-binding activities of E₁BF and CPBF

(A) Identification of E₁BF and CPBF activities in S-100 extract prepared from lymphosarcoma cells grown at 37 °C. S-100 extract (10 µg) was preincubated with poly(dI-dC) alone or with poly(dI-dC) (50 ng) plus unlabelled competitor oligonucleotides (5 pmol of each) for 20 min on ice, followed by the addition of [γ -³²P]rCP (20 000 c.p.m.). The binding reaction was allowed to proceed for an additional 30 min on ice. The DNA–protein complexes were separated on a native polyacrylamide gel (6% acrylamide). The dried gel was subjected to autoradiography. Lane 1 shows the migration of free labelled rCP alone (position indicated by F). Lane 2 represents complexes formed with S-100 extract preincubated with poly(dI-dC) alone. Lanes 3–6 represent S-100 extract preincubated with unlabelled rCP, 106 bp region of MT-I promoter encompassing MLTF-binding site, MRE-c' and MRE-d respectively. Lanes 7 and 8 represent S-100 extract preincubated for 15 min with control rabbit IgG and polyclonal anti-p70 IgG (1 µg of each) respectively. (B) S-100 extracts were prepared from cells grown at 37 °C and cells exposed to 42 °C for 2 and 3 h (see the Materials and methods section for details). Each extract (10 µg) was preincubated with poly(dI-dC) (50 ng) followed by incubation with labelled rCP (20 000 c.p.m.). Lanes 2–4 represent the DNA–protein complex profile of S-100 extracts from control (37 °C) cells, and cells exposed to 42 °C for 2 and 3 h respectively.

result in general down-regulation of transcriptional activity; rather it specifically blocks pol I transcription of rRNA gene.

Heat shock specifically inhibits DNA-binding activity of the pol I transcription factor, E₁BF/Ku

The specific inhibition of rRNA synthesis induced by heat shock may be due to inactivation of one or more transcription factors involved in pol I-directed transcription. Several factors involved in rRNA transcription have been identified and characterized in different laboratories. E₁BF is one of these factors initially identified as a factor that binds to the non-repetitive enhancer element of rat rDNA as well as core promoter [11] and later found to be immunologically related to human Ku autoantigen [12,13]. This factor is involved in the preinitiation complex-formation and has no detectable effect in RNA chain elongation [13]. CPBF, a protein related to the helix–loop–helix transcription factor USF [16], is another factor involved in the initiation of rDNA transcription.

We studied the effect of heat shock on DNA-binding activity of E₁BF/Ku in EMSA using end-labelled 58 bp rat core promoter oligonucleotide and S-100 extract (Figure 5). On

incubation with S-100 extract, two major complexes were formed (Figure 5A, lane 2). The upper two closely migrating complexes are characteristic of E₁BF/Ku [11,12] as its formation was eliminated by incubation with antibodies specific for the smaller subunit of Ku (lane 8) or by preincubation with unlabelled rCP as a competitor (lane 3). The complex-formation, however, remained unaffected by incubation with rabbit IgG (lane 7) or with non-specific oligonucleotide competitors such as MRE-c' (lane 5) and MRE-d (lane 6). The other major complex formed in S-100 extract is a doublet that migrates faster than the E₁BF–DNA complex. It is characteristic of CPBF/USF (lane 2) as it could be competed out by preincubation with unlabelled rCP (lane 3) and with the 106 bp fragment of mouse MT-I promoter region spanning the MLTF-binding site (lane 4), whereas other MT-I *cis* elements such as MRE-c' (lane 5) or MRE-d (lane 6) had no effect on its binding [16]. The competition between the promoter–CPBF/USF complex and the 106 bp of mouse MT promoter is probably due to the known binding of USF to the MLTF-binding sequence [16].

To determine the effect of heat shock on DNA-binding activity of E₁BF and CPBF, S-100 extracts obtained from control and heat-shocked cells (10 µg each) were incubated with labelled core

Figure 6 Immunoblot analysis of E₁BF in the S-100 extracts prepared from cells grown at 37 °C and cells exposed to 42 °C for 2 and 3 h respectively

Proteins (100 µg) from each fraction were separated by SDS/PAGE, transferred to nitrocellulose and incubated with antibodies specific for p72 and p85 subunits of E₁BF respectively (see the Materials and methods section for details). Immune complexes formed were detected using alkaline phosphatase-conjugated secondary antibodies and colour was developed with Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. (A) Lanes 1, 2 and 3 represent immune complex formed between p72 and the monoclonal antibody, N3H10, in extracts from control, 2 h- and 3 h-exposed cells respectively. (B) Lanes 1–3 indicate the amount of p85 as analysed by Western blotting using extracts from control cells and cells exposed for 2 and 3 h to hyperthermia respectively. Arrow on the left indicates respective immune complexes.

promoter oligonucleotide, and the DNA–protein complexes were analysed in native polyacrylamide gels. Only two major complexes corresponding to E₁BF and CPBF could be detected. When identical amounts of proteins from control and heat-shocked cells were analysed, E₁BF activity decreased in a time-dependent manner in S-100 extract (Figure 5B; compare lanes 3 and 4 with lane 2). These data show approx. 60 % reduction in core-promoter-binding activity of E₁BF within 2 h of exposure to 42 °C and almost complete loss of activity (90 %) after 3 h of heat treatment. During the same period of stress, DNA-binding activity of CPBF remained essentially unaffected (lanes 2–4).

Heat shock specifically decreases the level of E₁BF polypeptides

Next we investigated whether heat shock affects the level of E₁BF/Ku polypeptides as analysed by immunoblotting. For this purpose identical amounts of S-100 extract prepared from the control and heat-shocked cells were subjected to immunoblot analysis with specific antibodies (Figure 6). Immunoblot analysis with anti-p70 antibodies showed that heat shock for 2 h resulted in a significant decrease (55 %) in the level of the smaller subunit of E₁BF/Ku (Figure 6A; compare lane 2 with lane 1). There was no detectable p72 in S-100 extract after 3 h exposure to higher temperature (Figure 6A; compare lane 3 with lane 1). The level of the p85 subunit did not decrease significantly after 2 h of heat shock (Figure 6B; compare lane 2 with lane 1), but was reduced by approx. 50 % after 3 h of heat shock (Figure 6B; compare lane 3 with lane 1). These data suggest that the decreased amount

of E₁BF/Ku may contribute to the heat-shock-induced down-regulation of rDNA transcription.

DISCUSSION

Previous studies have shown that heat shock primarily inhibits rRNA processing [39]. Recent *in vivo* studies [25,26] have, however, demonstrated that the amount of newly synthesized rRNA is decreased dramatically at higher temperatures. The discrepancy among these studies is probably due to the shorter time of exposure of cells at elevated temperatures in the latter studies. Prolonged heat treatment is likely to cause some degree of thermotolerance allowing at least partial recovery of pol I transcription [26].

The present study was undertaken to determine whether the down-regulation of rDNA transcription by heat shock observed *in vivo* [25,26] can be mimicked in an *in vitro* transcription system, whether it is specific to pol I transcription and whether it is caused by alterations in a specific pol I transcription factor. The data presented here were reproduced three times using three different extracts. The results show that the S-100 extract from the heat-shocked cells can inhibit rDNA transcription whereas pol II transcription from the AdMLP or pol III transcription of 5 S RNA gene is not affected under this condition. It appears that only pol I transcription is down-regulated after heat shock. Inhibition of rDNA transcription at an elevated temperature cannot be due to loss of cell viability, as hyperthermia did not inhibit pol II and pol III transcription. Further, microscopic analysis did not indicate enhanced cell death when the cells were incubated at 42 °C for 3 h; rather, cell division was arrested under this condition. It is unlikely that heat shock induced a non-specific inhibitor such as a nuclease, as transcription in the control extract was not altered by the addition of extract from the heat-shocked cells.

None of the past studies has explored the nature of the pol I transcription factors that may be altered by elevated temperatures. We have shown that the DNA-binding activity of E₁BF/Ku was reduced in a time-dependent manner, and complete inhibition was achieved after 3 h of incubation at elevated temperature. This inhibition of DNA-binding activity correlates well with the disappearance of the smaller E₁BF polypeptide as determined by immunoblot analysis. This observation is consistent with the requirement of both small and large E₁BF polypeptides for promoter binding [13]. Hyperthermia may also have caused post-translational modification of E₁BF which resulted in its decreasing affinity for the promoter.

The down-regulation of rDNA transcription under different conditions is brought about by distinct mechanisms (for a review, see ref. [3]). For example, the inhibition of pol I transcription after glucocorticoid treatment of mouse lymphosarcoma cells has been attributed to the reduced activity of a transcription initiation factor called TFIC [40]. TFIC, which consists of three polypeptides of 55, 50 and 42 kDa, appears to be the transcriptionally competent form of pol I. Inactivation of TFIC does not allow formation of the initiation complexes, which can be restored by the addition of exogenous TFIC. A factor, designated TIF-IA, with a molecular mass of 75 kDa, which is tightly associated with pol I, is inactivated in the cells grown to the stationary phase [41]. This factor is analogous to the bacterial σ factor and the pol II transcription factor TFIIF (RAP 30/74) with respect to their requirements in the transcription initiation and interaction with the respective RNA polymerases. In *Acanthamoeba castellanii*, the down-regulation of rDNA transcription in encysting cells is also mediated by the inactivation of the active form of pol I [42],

which is due to a structurally altered 39 kDa subunit of the enzyme. Recent study in our laboratory has demonstrated that E₁BF is converted into a repressor of pol I transcription by a post-translational modification after deprivation of the cells of serum. The modified form of E₁BF can interact with the promoter, but is unable to *trans*-activate the pol I promoter [24]. The present study has shown that heat shock inhibits rDNA transcription which correlates well with the reduction in the amount of E₁BF. The decrease in the level of E₁BF polypeptides results in reduced promoter-binding activity and *trans*-activation. It is evident from these studies that distinct factors are involved in the down-regulation of rDNA transcription by different physiological means.

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