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Inactivation of Splicing Factors in HeLa Cells Subjected to Heat Shock*

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The nuclear extracts from HeLa cells subjected to heat shock at 43 or 46 °C for 2 h were unable to splice pre-mRNA *in vitro*. Analysis of snRNPs in the extracts revealed that the U4·U5·U6 small nuclear ribonucleoprotein particle (snRNP) complex was disrupted at both temperatures while U1 and U2 snRNPs remained unaffected at 43 °C but were disrupted to certain extent during heat shock at 46 °C. During splicing reaction, the extract from cells heat shocked at 43 °C formed intermediate splicing complexes α and β but was unable to form a functional spliceosome, complex γ. Addition of fractions from a normal nuclear extract restored splicing activity only in the extract from cells subjected to heat shock at 43 °C. Using this complementation assay, we have partially purified the factor(s) inactivated at this temperature. The purified factor(s) was essentially devoid of snRNAs and snRNPs and resistant to micrococcal nuclease, indicating that the factor(s) inactivated by *in vivo* heat shock at 43 °C is a protein.

We have also subjected the nuclear extracts from normal HeLa cells to *in vitro* heat treatment at 43 or 46 °C. The results indicate that during *in vitro* heat treatment of the extracts the damage to splicing machinery is more extensive than that during *in vivo* heat shock. These experiments also suggest that the factor(s) inactivated by heat shock at 43 °C is different from previously identified thermolabile splicing factors.

The response of cells to a number of environmental stresses results in the increased intracellular level of proteins termed heat shock proteins (hsps).[1] This response is elicited by heat as well as by a number of chemicals, such as ethanol, sodium arsenite, and heavy metals, and could be seen in all organisms examined, from *Escherichia coli* to man (1–3). Heat shock genes can also be activated either by denatured (4) or modified proteins (5). In addition to heat shock proteins, other proteins can be induced during stress response. These include ubiquitin (6, 7) metalloproteinases (8), and heme oxygenase (9). Other reported effects of heat shock include alteration in antigenic reactivity of hnRNPs (Ref. 10 and references cited therein) and snRPNs (11).

Induction of heat shock proteins is always accompanied by a significant decrease in the synthesis of other proteins. The mechanism of suppression is not well understood. Inhibition of translation seems to be at least partially responsible, possibly via phosphorylation of initiation factors (12). Earlier studies of heat shock in *Drosophila* suggested inhibition of transcription of mRNA coding for non-heat shock proteins (reviewed in Ref. 13). In contrast, recent studies have shown that heat shock of HeLa cells does not affect the rate of mRNA transcription but nevertheless lowers the concentration of non-hsp mRNA in the cytoplasm (14). The latter finding indicates that post-transcriptional events, such as inhibition of pre-mRNA splicing or nuclear transport may be responsible for this result. Since the majority of heat shock genes do not contain introns, inhibition of pre-mRNA splicing would provide a potential mechanism of discrimination between heat shock-inducible and non-heat shock transcripts. Indeed, it has been shown that *in vivo* splicing of pre-mRNA was inhibited during heat shock of *Drosophila* (15, 16) or mammalian cells (7, 17, 18).

It seems likely that inhibition of splicing during heat shock is caused by inactivation of certain components of the splicing machinery. If this is the case, one should be able to study the mechanism of inactivation and identify the factors damaged by heat shock using an *in vitro* splicing system. We report here that nuclear extracts from HeLa cells subjected to heat shock are not able to splice mRNA precursors. The splicing activity can be rescued by chromatographically purified fractions of nuclear extracts from untreated cells suggesting that a specific thermolabile splicing factor is inactivated by heat shock. Our results indicate that the inactivated factor is different from other known splicing factors.

EXPERIMENTAL PROCEDURES

Cell culture—HeLa cells were grown in suspension in Joklik's modified minimal Eagle's medium containing kanamycin and gentamycin, supplemented with 10% calf serum (Hyclone Labs). For heat shock experiments the cells were pelleted (1000 × *g*, 10 min), resuspended in medium prewarmed to 43 °C (half of the original volume), and incubated at this temperature for 2 h. Immediately thereafter the cells were pelleted, washed with ice-cold phosphate-buffered saline, and nuclear extracts were prepared (see below). For recovery experiments heat shocked cells were pelleted, resuspended in a medium prewarmed to 37 °C, and nuclear extracts were prepared at specified times (see Fig. 1*C*). Cell survival was assayed by trypan blue exclusion.

Preparation of the Nuclear and Cytoplasmic (S100) Extracts—Splicing nuclear extract was prepared essentially according to Dignam *et al.* (19) and Krainer *et al.* (20) except that nuclei were subjected to an additional wash with buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol; see Ref. 19). The cytoplasmic S-100 fraction was prepared and concentrated by precip-

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[1] The abbreviations used are: hsp, heat shock protein; snRNP, small nuclear ribonucleoprotein particle; hnRNP, heterogeneous nuclear ribonucleoprotein particle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

itation with ammonium sulfate as described by Hernandez and Keller (21).

Fractionation of the Nuclear Extract—Nuclear extract from 1.2×10^{10} cells (approximately 10 ml) obtained as described above was dialyzed for 5 h against buffer D (19) supplemented with 12.5 mM $MgCl_2$ so that the final composition of the dialysis buffer was: 20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.5 mM dithiothreitol, and 12.5 mM $MgCl_2$. After dialysis the extract was centrifuged for 30 min at 16,000 rpm in Sorvall SS34 rotor. The resultant pellet, referred to as the "magnesium pellet," was resuspended in 10 ml of buffer D and loaded onto a fast protein liquid chromatography Mono Q (HR 5/5) column preequilibrated with buffer D containing 10% glycerol. The column was washed with 5 bed volumes of the same buffer and eluted, in 1-ml fractions, with the same buffer containing 0.5 M KCl. Activity peak was pooled, dialyzed against buffer D, and reloaded onto a Mono Q column. The column was washed with buffer D containing 10% glycerol and eluted with a linear gradient of 0.1–0.5 M KCl in the same buffer. 3-ml fractions were dialyzed against buffer D and concentrated approximately 5-fold by treating the dialysis bags with dry Sephadex G-200. Concentrated fractions were aliquoted and stored at −70 °C. Protein concentration in the fractions was assayed with a Bio-Rad reagent following manufacturers protocol.

Analysis of snRNPs in Nuclear Extracts—1-μl aliquots of nuclear extracts, containing approximately 3 μg of protein, were electrophoresed on 4.5% nondenaturing polyacrylamide gels as described (22). The separated material was electroblotted onto nylon membranes (GeneScreen) and hybridized to ^{32}P-labeled RNA probes for U1, U2, U4, U5, and U6 snRNAs. The probes were obtained by *in vitro* transcription of plasmids carrying anti-U-RNA sequences fused to SP6 promoter. The plasmids were a kind gift of Drs. U. Bond and J. Steitz (Yale University).

Analysis of Spliceosome Formation—Aliquots (1 μl) of standard splicing reaction were separated on nondenaturing polyacrylamide gel as described (22) except that the concentration of polyacrylamide was lowered to 3.8%. For analysis of spliced products and intermediates in the spliceosome complexes, appropriate bands were excised from the gel, eluted, and the RNA analyzed on sequencing polyacrylamide gels (23).

Analysis of RNA from Nuclear Extracts and Column Fractions—Aliquots of the nuclear extract or column fractions were extracted with phenol and precipitated with ethanol. The RNA was electrophoresed on a 12.5% polyacrylamide, 7 M urea sequencing gel (20 × 40 × 0.04 cm), and the gel was stained with silver (Gelcode, Pierce Chemical Co.). As little as 3 μl of the extract is sufficient to visualize the RNA by this procedure.

Transcription of pSP64HBΔ6 Template and Splicing—These reactions were carried out according to published procedures (24–26). In all experiments, a ^{32}P-labeled human β-globin transcript was obtained by SP6 RNA polymerase transcription of the plasmid pSP64HBΔ6 linearized within the second exon at the *Bam*HI restriction site. In the standard splicing assay, a 25-μl reaction mixture contained 15 μl of extract. In the complementation assays, tested fractions (usually 3–5 μl), normalized to equal amount of protein, were added to the standard reaction mixture containing 10–12 μl of the nuclear extract. All the splicing reactions were carried out at 30 °C for 90 min, unless indicated otherwise. The RNA from the reaction was isolated by phenol extraction and ethanol precipitation and separated by electrophoresis on an 8% polyacrylamide sequencing gel.

Digestion of snRNPs with Micrococcal Nuclease—The extract or purified fractions were treated with 600 units/ml of micrococcal nuclease (Boehringer Mannheim) in the presence of 1 mM Ca^{2+} as described elsewhere (25). After removal of calcium with 4 mM EGTA, nuclease-treated material was used in complementation assays as described above.

RESULTS

Inactivation of Splicing Activity in Nuclear Extracts from Heat-shocked Cells—To examine the effect of heat shock on pre-mRNA splicing, we have subjected HeLa cells to heat shock under various conditions and prepared nuclear extracts from these cells immediately thereafter. The extracts were tested in the *in vitro* splicing assay with human β-globin pre-mRNA as substrate. The RNA was analyzed on an 8% polyacrylamide sequencing gel. The intermediates and products of splicing were identified on the basis of their mobility on the gel and previous characterization (26, 27).

The effects of the 2-h heat shock of HeLa cells at 40, 43, or 46 °C on the splicing activity of the extracts is shown in Fig. 1*A*. Extract obtained after 40 °C treatment retained full splicing activity (*lane 2*), as compared with control extract (*lane 1*), whereas heat shock of cells at 43 or 46 °C completely abolished the splicing activity (*lanes 4* and *6*, respectively). If the activation of splicing is caused by the damage of specific splicing factors and not pleiotropic heat denaturation of a large number of proteins, one should be able to rescue the splicing activity by complementation of the inactive extract with fractions of splicing extracts from untreated cells. As can be seen from the figure, the nuclear extract from cells heat shocked at 43 °C was reactivated by a fraction of the extract from untreated cells (*lane 5*), whereas the extract from cells heat-shocked at 46 °C was not (*lane 7*). The fraction alone is inactive in splicing (*lane 8*). This fraction, termed the magnesium pellet (see "Experimental Procedures" and below), is enriched in snRNPs and contains approximately 10% of extract proteins. Thus, it appears that heat shock at 43 °C caused less extensive damage to splicing machinery than heat shock at 46 °C. Additional support for this conclusion is provided by the following findings. First, heat shock of HeLa cells for at least 1 h at 43 °C is necessary for inactivation of splicing (Fig. 1*B*, *lane 3*), but even after 4 h at this temperature, the nuclear extracts from treated cells could be rescued by the magnesium pellet (Fig. 1*B*, *lane 10*). Essentially the same results were obtained with fractions of much higher purity after column chromatography (see below, Fig. 6*A*). Second, splicing activity was recovered within 2 h after the cells were returned to normal growing conditions at 37 °C (Fig. 1*C*), and no apparent cell death was detected by trypan blue exclusion (not shown).

To ascertain that the lack of splicing activity in the nuclear extract from heat-shocked cells was due to inactivation of the factor(s) rather than its excessive leakage from the nuclei to the cytoplasm, we have supplemented the extract with the cytoplasmic, S-100, fraction from normal or heat shocked cells. The S-100 fraction from untreated cells rescued the splicing activity in this assay, as could be expected since a number of splicing factors leaks out to the cytoplasm during preparation of the extract (25). In contrast, the S-100 from cells heat-shocked at 43 °C did not reactivate the extract suggesting that the factor(s) required for activity is inactive in both cytoplasmic and nuclear fractions (not shown).

Inactivation of Splicing in Heat-treated Nuclear Extracts—To compare loss of splicing activity during *in vivo* heat shock of cells *versus in vitro* heat treatment of the nuclear extracts, we have incubated the extracts from normal cells at 43 and 46 °C for 2 h and supplemented them with the magnesium pellet fraction. Similarly to extracts from heat-shocked cells, heat-treated extracts lost their splicing activity (Fig. 2, *lanes 2* and *3*). The splicing activity in the extract heated at 43 °C was poorly restored after supplementation with the magnesium pellet fraction (Fig. 2, *lane 5*), as compared with reactivation of the extract from heat shocked cells (Fig. 2, *lane 7*). The activity in the extract heated at 46 °C was not restored at all (Fig. 2, *lane 6*). One interpretation of these results is that *in vitro* heating of the extract inactivates different splicing factors than *in vivo* heat shock. Alternatively, the damage to splicing machinery may be more extensive and include the factor(s) inactivated by *in vivo* heat shock as well as additional splicing factors.

It has been shown previously that incubation of nuclear extracts from normal cells at elevated temperatures inactivates splicing and that these extracts can be reactivated when

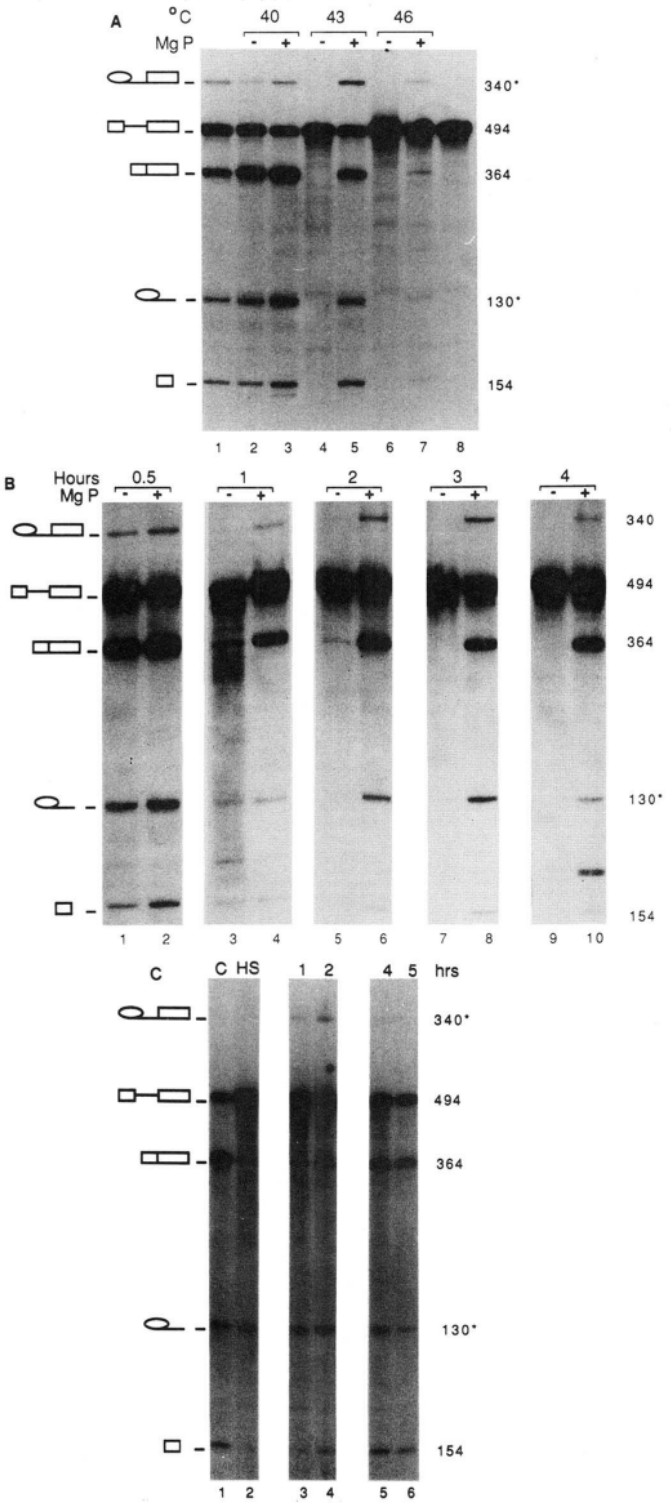

FIG. 1. *A*, effect of heat shock temperature on inactivation and reconstitution of splicing activity. Nuclear extracts from HeLa cells subjected to a 2-h heat shock at 40 (*lanes 2* and *3*), 43 (*lanes 4* and *5*) and 46 °C (*lanes 6* and *7*) were assayed for splicing activity with human β-globin pre-mRNA as substrate as described under "Experimental Procedures." In *lanes 3, 5,* and *7* extracts were supplemented with the magnesium pellet fraction (*MgP*). *Lane 1,* control extract from untreated cells. *Lane 8,* magnesium pellet fraction. The RNA was analyzed on an 8% polyacrylamide sequencing gel. The structures of the input, intermediates, and products of splicing are shown schematically on the *left,* their length in nucleotides on the *right. Asterisk* denotes aberrant mobility of the lariat containing products. *B*, time course of inactivation and reconstitution of splicing activity in extracts from cells subjected to heat shock at 43 °C. Nuclear extracts from HeLa cells heat-shocked at 43 °C for 30 min (*lanes 1* and *2*), 1 h (*lanes 3* and *4*), 2 h (*lanes 5* and *6*) 3 h (*lanes 7* and *8*), and 4 h (*lanes 9* and *10*) were assayed for splicing activity as described above. In *lanes 2, 4, 6, 8,* and *10* the extracts were supplemented with a magnesium pellet fraction. *C*, recovery of splicing activity after heat shock. HeLa cells heat-shocked at 43 °C were transferred to 37 °C, allowed to recover for 1, 2, 4, and 5 h, and the nuclear extracts were prepared immediately thereafter (*lanes 3, 4, 5,* and *6,* respectively). Splicing activity was assayed as above. *Lane 1,* control (*C*) extract from untreated cells. *Lane 2,* extract from cells after heat shock (*HS*) without recovery at 37 °C.

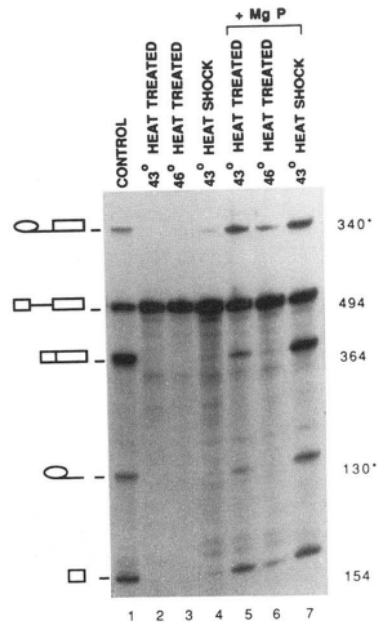

FIG. 2. **Inactivation and reconstitution of splicing activity in extracts heated *in vitro*.** Extracts from untreated cells were heated for 2 h at 43 and 46 °C and assayed for splicing activity alone (*lanes 2* and *3*, respectively) or after reconstitution with the magnesium pellet fraction (*lanes 5* and *6*, respectively). *Lane 1,* control extract from untreated cells; *lane 4,* extract from cells heat shocked at 43 °C; *lane 7,* same as *lane 4,* reconstituted with magnesium pellet fraction (*MgP*).

supplemented with either S-100 (25) or certain fractions of nuclear extract from HeLa cells (28). To examine whether the factor(s) inactivated by *in vivo* heat shock at 43 °C are similar or different from previously identified thermolabile factors we have carried a complementation experiment. When an inactive extract, from cells heat shocked at 43 °C for 2 h, was mixed with an equal amount of a nuclear extract inactivated by heating for 10 min at 45 °C (conditions used by Krainer and Maniatis, Ref. 25), the splicing activity of the extracts was restored (not shown). We conclude that different splicing factors were inactivated by these treatments.

Characterization of the Damage to Splicing Machinery by Heat Shock and Heat Treatment—Since snRNPs are involved in pre-mRNA splicing (29, 30), it seemed possible that these particles are damaged during heat shock. To analyze the changes in the structure and properties of the snRNPs, we have analyzed the nuclear extracts on nondenaturing gels, in which snRNPs are presumably in a native state. Furthermore, the complex of U4, U5, and U6 snRNAs, shown to pre-exist in active splicing extracts (31), can also be detected in these gels. The separated material was blotted onto a nylon membrane and hybridized with RNA probes for snRNAs. Fig. 3 shows that the bands hybridizing to U1 and U2 RNA probes had the same mobility and intensity in control extracts and in those from cells heat shocked at 43 °C, indicating that the structures of U1 and U2 snRNPs were not affected by this

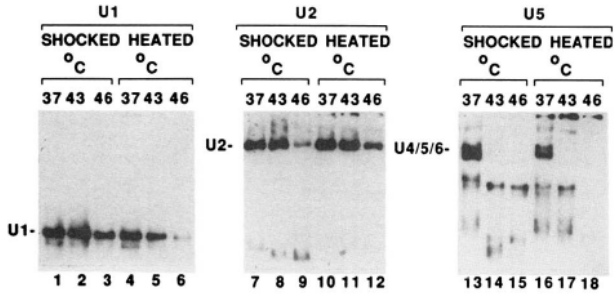

FIG. 3. **Analysis of snRNPs in extracts inactivated by *in vivo* heat shock and *in vitro* heat treatment.** Extracts were electrophoresed on nondenaturing polyacrylamide gels, and the separated material was blotted onto nylon membrane and hybridized to ^{32}P-labeled RNA probes for U-snRNAs (see "Experimental Procedures"). The probes used are indicated at the *top* of the panels. Extracts from cells subjected to *in vivo* heat shock (denoted "*shocked*" in the figure) are shown in *lanes 1–3, 7–9,* and *13–15. In vitro* heat-inactivated extracts from untreated cells (denoted "*heated*") are shown in *lanes 4–6, 10–12,* and *16–18.* The temperatures of the treatments are indicated at the *top* of the panels, the positions of U1, U2, and U4·U5·U6 complexes are indicated on the *left.*

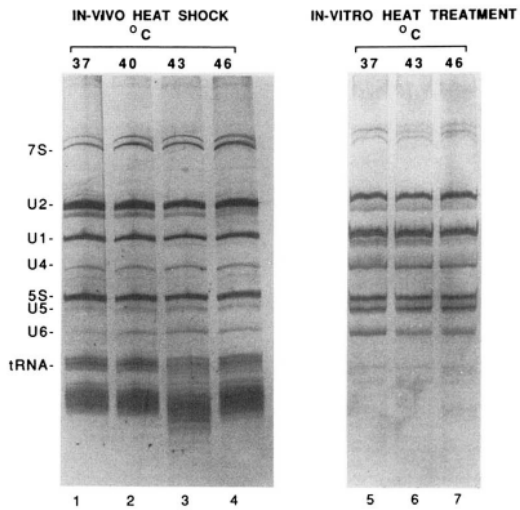

FIG. 4. **Analysis of snRNAs in extracts inactivated by *in vivo* heat shock and *in vitro* heat treatment.** All extracts were adjusted with buffer D to the same protein concentration and the aliquots extracted with phenol and electrophoresed on 12.5% polyacrylamide sequencing gel. RNA from 3 μl of each extract was loaded per lane. The temperatures of *in vivo* heat shock or *in vitro* heat treatment are shown at the *top.* Positions of snRNAs are indicated on the *left.*

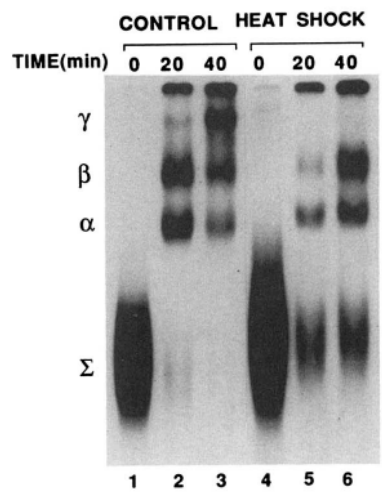

FIG. 5. **Spliceosome formation by extracts from cells heat-shocked at 43 °C.** 1-μl aliquots from standard splicing reactions were electrophoresed on 3.8% nondenaturing polyacrylamide gels (see "Experimental Procedures"), and the gels were autoradiographed. *Lanes 1–3,* control extract from untreated cells; *lanes 4–6,* extract from cells subjected to heat shock at 43 °C. Aliquots were withdrawn from the splicing reaction at 0 (*lanes 1* and *4*), 20 (*lanes 2* and *5*), and 40 min (*lanes 3* and *6*). Positions of α, β, and γ complexes are shown on the *left.* Σ denotes a nonspecific RNA-protein complex.

treatment. (Fig. 3, *lanes 2* and *8*, respectively). In contrast, slowly migrating bands hybridizing to U4, U5, and U6 probes, representing the U4·U5·U6 snRNP complex (31), disappeared after heat shock, whereas lower migrating bands remained visible (Fig. 3, *lane 14*, shows the results of hybridization to U5 probe. Hybridizations with probes for U4 and U6 snRNAs are not shown, since they give essentially the same results). In the extract from cells subjected to heat shock at 46 °C, the U4·U5·U6 complex also disintegrated (Fig. 3, *lane 15*), and, in addition, the level of U1 and U2 snRNPs was decreased (Fig. 3, *lane 3* and *9*, respectively).

We have also analyzed the snRNPs in the heat-inactivated nuclear extracts from normal cells. As in extracts from heat-shocked cells, U1 and U2 snRNPs were essentially unchanged in extracts heat-inactivated at 43 °C (Fig. 3, *lanes 5* and *11*), but their level was decreased at 46 °C (Fig. 3, *lanes 6* and *12*), and the U4·U5·U6 snRNP complex disintegrated at both temperatures (Fig. 3, *lanes 17* and *18*, respectively).

To determine if disintegration of U4·U5·U6 snRNP complex is accompanied by degradation of appropriate snRNAs, we have analyzed them on polyacrylamide sequencing gels stained with silver (Fig. 4). The snRNAs were identified on the basis of their gel mobility and by comparison with blots of similar gels hybridized with RNA probes for snRNAs. The results show that the pattern of stained snRNAs is very similar under all treatment conditions and is not different from that for control extract. Since neither heat shock nor heat inactivation of the extracts led to degradation of snRNAs, one can conclude that the snRNAs are protected by snRNP proteins from nucleases present in the extract and that at least partial integrity of the snRNPs is retained after these treatments. This is further supported by the fact that all species of snRNAs are present in the anti-Sm immunoprecipitates of nuclear extracts from heat-shocked cells labeled *in vivo* with ^{32}P (results not shown).

If heat shock at 43 °C causes only a limited damage to splicing factors, it seemed possible that the formation of splicing complexes, the spliceosomes (32–34), would be impaired only to a limited extent. We have carried out the analysis of spliceosome formation on nondenaturing polyacrylamide gels which resolve three splicing complexes, α, β, and γ (22). We found that during splicing reaction with nuclear extracts from cells heat-shocked at 43 °C, only intermediate splicing complexes α and β were formed, but the functional spliceosome, complex γ, was not (Fig. 5, *lanes 5* and *6*). The mobility of the α and β complexes formed with extract from heat-shocked cells is identical to that of the complexes in the normal extract (Fig. 5, *lanes 2* and *3*). This suggests that splicing factors required for the formation of complexes α and β remain functional and are not damaged by heat shock at 43 °C.

Purification of the Splicing Factor Inactivated by Heat Shock—To characterize the factor inactivated by heat shock at 43 °C, we have subjected a nuclear extract from cells grown under standard conditions to a purification protocol which included the following steps: dialysis of the extract against buffer D containing 12.5 mM $MgCl_2$ and high speed centrifugation of the resulting precipitate, chromatography of the

pelleted material on fast protein liquid chromatography Mono Q column eluted stepwise with 0.5 M KCl, and subsequent chromatography of the eluted material on the same column developed with a salt gradient (0.1–0.5 M KCl). The details of purification are described under "Experimental Procedures." The purified fractions were assayed for their ability to rescue splicing activity of the nuclear extract from HeLa cells subjected to *in vivo* heat shock for 2 h at 43 °C (Fig. 6*A*). In parallel, the snRNA content of the chromatographic fractions was assayed by analyzing their phenol-extracted aliquots on silver-stained polyacrylamide gels (Fig. 6*B*). The rescuing activity precipitated into the magnesium pellet after dialysis against magnesium containing buffer and centrifugation (Fig. 6*A*, *lane 2*). This step removed approximately 90% of extract

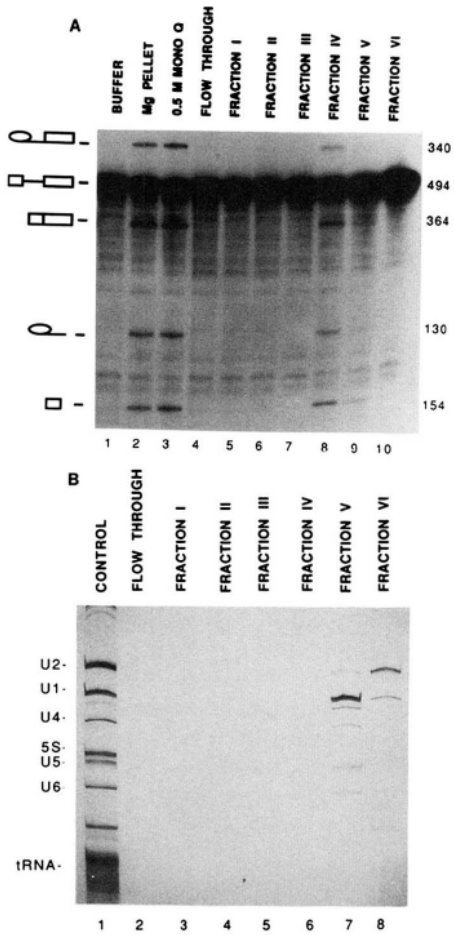

FIG. 6. *A*, fractionation of factor(s) inactivated by heat shock. Extract from normal untreated cells was fractionated according to purification protocol described under "Experimental Procedures" and used to reconstitute splicing activity in extract from cells heat shocked at 43 °C. 10–12 μl of extract from heat-shocked cells was supplemented with 3–5 μl of purified fractions and assayed for splicing activity. Concentration of all added fractions was adjusted to equal amount of protein. Products and intermediates of splicing reaction were assayed on 8% polyacrylamide sequencing gel as in Fig. 1. Fractions used for supplementation were: *lane 1*, buffer as control; *lane 2*, magnesium pellet; *lane 3*, material eluted during stepwise elution of the Mono Q column at 0.5 M KCl. *Lanes 4–10*, material eluted during gradient elution of Mono Q column, fractions assayed are indicated at the *top* of the figure. *B*, analysis of snRNA content in purified fractions. Fractions from the last step of purification (gradient elution of Mono Q column) were phenol-extracted, and the RNA was analyzed on 12.8% polyacrylamide sequencing gel stained with silver. *Lane 1*, RNA from 3 μl of normal control extract. *Lanes 2–8*, 3–5 μl of fractions from Mono Q column as indicated at the *top* of the figure.

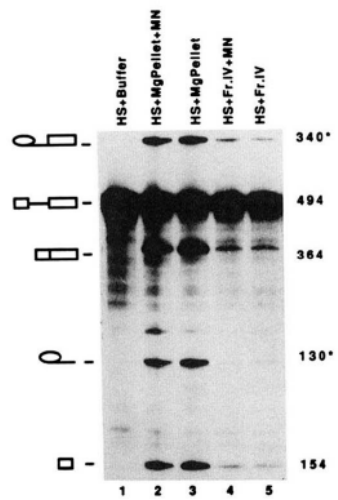

FIG. 7. **Micrococcal nuclease treatment of purified fractions.** Magnesium pellet and fraction IV from Mono Q column were treated with micrococcal nuclease (600 units/ml) as described under "Experimental Procedures." Treated fractions were used to rescue nuclear extract from cells heat-shocked at 43 °C. Fractions used for supplementation were: *lane 1*, buffer as control; *lane 2*, magnesium pellet-treated micrococcal nuclease; *lane 3*, untreated magnesium pellet; *lane 4*, fraction IV treated with micrococcal nuclease; *lane 5*, untreated fraction IV.

proteins which remained in the supernatant. Most of the snRNPs were also precipitated during this procedure (not shown). The material from the magnesium pellet was further purified by chromatography on fast protein liquid chromatography Mono Q column. During stepwise elution the rescuing activity came off the column at 0.5 M KCl (Fig. 6*A*, *lane 3*). In the subsequent chromatographic step, during elution of Mono Q column with a salt gradient from 0.1 to 0.5 M KCl, the activity was eluted at approximately 0.3 M KCl. Attempts of further purification were unsuccessful as the rescuing activity was inactivated during chromatography on ATP-Sepharose, single-stranded DNA-Sepharose, and phenyl-Sepharose.

In contrast to initial steps of purification, the purest active fraction (fraction IV, Fig. 6*A*, *lane 8*) was essentially devoid of snRNAs (Fig. 6*B*, *lane 6*) and separated from the bulk of snRNPs, which were eluted at higher salt concentrations. This result suggested that the rescuing activity is a protein. Additional experiments to support this observation were carried out. The rescuing activity in fraction IV from Mono Q column and in the magnesium pellet was resistant to treatment with micrococcal nuclease at a concentration of 600 units/ml (Fig. 7). This concentration of the nuclease is at least six times higher than that needed to inactivate splicing in nuclear extracts. Moreover, an RNA free fraction of the nuclear extract or magnesium pellet obtained by chromatography on DE-53 anion exchange column as described by Pikielny *et al.* (35) was also able to rescue the activity of the nuclear extract from cells heat-shocked at 43 °C. Finally, the addition of fractions containing snRNPs (fractions V and VI from Mono Q column, Fig. 6*A*, *lanes 7* and *8*) to fraction IV did not increase its ability to reactivate splicing (data not shown). Thus, by these criteria, the factor inactivated by heat shock at 43 °C appears to be a protein and not an snRNP. Since, as we have shown above, heat shock also affects the structure of the U4·U5·U6 snRNP complex and prevents formation of the splicing complex γ, it appears that the inactivated factor is essential for the integrity of both of these complexes.

DISCUSSION

It has been reported in a series of recent papers (7, 14–18) that mRNA metabolism is affected by heat shock or other stress conditions. In most cases the rate of transcription was not inhibited but the level of newly synthesized mRNA in the cytoplasm decreased with a concomitant accumulation of pre-mRNA in the nucleus, suggesting a defect in either splicing or transport of the mRNA to the cytoplasm. In fact, *in vivo* splicing of hsp pre-mRNAs containing introns was inhibited during heat shock of *Drosophila* Shneider cells (15, 16), mouse cells (17), and HeLa cells (18). Inhibition of splicing of other pre-mRNAs such as ubiquitin (7) was also observed. We have found that nuclear extracts prepared from HeLa cells subjected to heat shock exhibit a significant decrease of splicing activity and that this decrease is due to inactivation of specific splicing factor(s). Thus it appears that an important defect in mRNA metabolism during heat shock is due to inactivation of a component of the splicing apparatus.

Several lines of evidence show that the damage to splicing due to heat shock at 43 °C is limited and specific. Splicing activity could be reconstituted by purified fractions only in extracts from cells heat-shocked at 43 °C. U1 and U2 snRNPs were not affected by the treatment and, in spite of the disintegration of the U4·U5·U6 snRNP complex, U4, U5, and U6 snRNAs remained intact in the extract. The latter finding, as well as immunoprecipitation experiments, suggest that these snRNAs are still protected by snRNP specific proteins. The remaining U4, U5, and U6 snRNP complexes are functional to the extent that the extract from cells heat shocked at 43 °C could form apparently normal splicing complexes α and β. It has been shown that these complexes contain other splicing factors (36, 37) in addition to snRNPs (31). Thus, all these factors retain the capability to form the α and β complexes after heat shock at 43 °C, underscoring a limited nature of the damage under these conditions. One can also conclude from these results that the factor inactivated by heat shock at 43 °C is different from those required for formation of the α and β complexes. Another interpretation of the data shown in Fig. 5 is that the factor is required for formation of all three complexes but, due to its partial inactivation during heat shock, formation of the complexes is slower, so that the complex γ is not yet detectable on the gel.

Purification of the factor and reactivation of splicing in extracts from heat-shocked cells with fractions free of RNA strongly suggest that the activity damaged by heat shock at 43 °C is a protein. Although final proof will require purification of the factor to homogeneity, a most likely interpretation of the experiments presented above is that the factor inactivated by heat shock of HeLa cells at 43 °C is a protein that interacts with an snRNP complex composed of U4, U5, and U6 snRNPs. This protein is apparently essential to maintain the integrity of the complex as well as for the formation of the functional spliceosome. Further support for this observation is provided by the fact that addition of the fraction purified through Mono Q column, to the extract from cells heat-shocked at 43 °C was able to restore to some extent the formation of the complex γ during splicing reaction (not shown).

The damage of the splicing factors during heat shock at 46 °C or during heating of the normal extracts at 43 or 46 °C is more extensive. This is seen in the fact that in addition to disintegration of U4·U5·U6 snRNP complex, these treatments lead to decreased levels of U1 and U2 snRNP and an inability of these extracts to be reconstituted by the magnesium pellet fraction. One likely possibility for heat-treated extracts is that during 2-h incubation at elevated temperatures proteolytic and/or nucleolytic degradation of a number of splicing factors contributes to inactivation of splicing.

It has been shown previously that incubation of the extract at 45 °C for 10 min (Ref. 25) or at 37 °C for 40 min (Ref. 38) inhibits only a second step of the splicing reaction, *i.e.* cleavage at the 3′ splice site end exon ligation, without affecting the first step, cleavage at the 5′ splice site and lariat formation. Furthermore, the latter conditions allowed for the formation of the complex γ. This is in contrast to inactivation during heat shock at 43 °C where complex γ formation and therefore the first step of splicing are inhibited. Thus it appears that heating of the extracts at milder conditions leads to inactivation of a factor different from that inactivated by heat shock of the cells at 43 °C. Since none of the factors in question (25, 28, 38) are purified to homogeneity, these results should be interpreted with caution.

Heat shock response is elicited by a variety of chemicals in addition to heat (1–3, 39, 40). We have tested whether such treatments would also inactivate splicing. Surprisingly, we found that nuclear extracts from HeLa cells treated with sodium arsenite and sulfates of zinc, cadmium, and copper retained full splicing activity. These salts were used in concentrations which induced synthesis of heat shock proteins, as assayed by dot blot analysis using a cDNA probe for hsp 70 or by incorporation of [^{3}H]leucine into heat shock proteins (not shown). We interpret these results to mean that the factor inactivated by heat shock is thermolabile but not susceptible to other stress conditions. Since accumulation of nuclear RNA coded by heat shock genes containing introns was observed *in vivo* after sodium arsenite treatment of HeLa cells (18), it appears that *in vivo* inhibition of splicing and/or transport of mature mRNA to the cytoplasm can also be achieved by a mechanism different than the inactivation of a thermolabile splicing factor.

While this work was in progress Ursula Bond reported the effects of heat shock on *in vitro* splicing of adenovirus pre-mRNA (18). This author also noted the disintegration of U4·U5·U6 snRNP complex, and, in contrast to our results, damage to U2 snRNP and aberrant formation of splicing complexes were also observed. This discrepancy is probably due to the difference in temperature during heat shock, since in that work nuclear extracts from cells heat-shocked at 45 °C were studied. As pointed out previously at this temperature a more extensive damage to the components of splicing machinery could be expected. In support of our observations, Bond also noted that heat shock appears to damage a factor different from a thermolabile factor identified by Krainer and Maniatis (25).

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