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Detection and Characterization of a Factor Which Rescues Spliceosome Assembly from a Heat-Inactivated HeLa Cell Nuclear Extract

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Mild heat treatment of HeLa cell nuclear extracts (NE) selectively inhibits pre-mRNA splicing. Heat-inactivated extracts can be complemented by a small amount of untreated NE. Utilizing this complementation assay and a combination of ion-exchange, affinity, and hydrophobic chromatography, a heat reversal factor (HRF) was purified from NE that is required to rescue pre-mRNA splicing from a heat-inactivated extract. This activity in its most purified form consistently copurified in a fraction containing two 70-kDa proteins and a minor polypeptide of approximately 100 kDa. It was free of the major small nuclear RNAs, sensitive to protease, and required to rescue spliceosome formation from a heat-inactivated nuclear extract. These results suggest that this factor is a protein that may be an important component in pre-mRNA splicing, or alternatively, it may be involved in renaturation of a heat-sensitive splicing factor.

Nuclear pre-mRNA splicing takes place in a complex structure of proteins and RNA called a spliceosome (9, 10, 24). Several small nuclear ribonucleoproteins (snRNPs) consisting of U1, U2, U4, U5, and U6 are required for assembly of this particle. U1 and U2 recognize and bind to sequence elements within the pre-mRNA and are essential for the initial stages of spliceosome assembly (11, 12, 29, 32). The association of U4/U6 and U5 snRNPs with this presplicing complex results in the formation of the spliceosome, within which the reaction intermediates of pre-mRNA splicing can be detected (12, 19).

In addition to the snRNPs, numerous protein factors have been implicated in this important process. Several factors have been identified which influence 5′ splice site selection prior to the cleavage-ligation reactions (7, 13, 31). A number of factors have been purified which are essential for spliceosome assembly (5, 16, 23, 30). In addition, several proteins have been detected which specifically bind the pre-mRNA (6, 8, 25, 26) or are specifically required for the cleavage-ligation reactions (27).

The successful purification and biochemical characterization of non-snRNP factors required for pre-mRNA splicing depends on the development of assays that target single activities. Krainer and Maniatis (14) have demonstrated that mild heat treatment of nuclear extracts (NE) results in the inhibition of pre-mRNA splicing. In *Saccharomyces cerevisiae*, it has also been shown that heat-inactivated extracts could be complemented by fractions from wild-type extracts (28). This report describes a similar approach for the detection, characterization, and purification of a factor that rescues pre-mRNA splicing from a heat-inactivated mammalian NE.

MATERIALS AND METHODS

Cells and NE preparation. Suspension cultures of HeLa cells (5×10^5 cells per ml) were grown in Joklik minimal essential medium supplemented with 5% calf serum. The cells were harvested by centrifugation at 2,000 rpm in an SS-34 Sorvall rotor at 4°C and washed once in phosphate-buffered saline, and the NE was prepared by the method of Dignam et al. (2). The NE obtained by this method was dialyzed against buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 50 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol) for 5 h at 4°C. After dialysis, the resulting extract was clarified by centrifugation for 20 min at 25,000 × *g*, divided into aliquots, and stored at −70°C (2). The protein concentrations of the clarified extracts were determined by the method of Bradford (1) (usually 4 to 8 mg/ml).

Fractionation procedures. NE containing 50 mg of protein equilibrated in buffer D (supplemented with 1.5 mM $MgCl_2$) was layered on a DEAE-Sepharose CL-6B column (10-ml bed volume) (Pharmacia). Bound material was step eluted with four column volumes each of buffer D containing 0.15, 0.25, and 0.5 M KCl, respectively. Fractions of 5 ml were collected at a flow rate of 1 ml/min, and aliquots containing activity (see below) were pooled and dialyzed against buffer D. An aliquot of this pooled material (usually about 2.5 mg of protein) was applied to an ATP-agarose column (1-ml bed volume) (ribose linkage; Pharmacia) equilibrated in buffer D. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The flowthrough (FT) from this column usually contained 60 to 70% of the total protein and was discarded. The bound material (approximately 0.5 mg) was eluted with buffer D containing 0.5 M KCl, adjusted to 1 M KCl, boiled for 5 min, and layered onto a phenyl-Sepharose column (0.5-ml bed volume). This column was washed extensively with buffer D containing 1 M KCl, and the bound material was eluted with buffer D containing 0.01 M KCl. Fractions containing activity were pooled and stored at −70°C.

Synthesis of pre-mRNA transcripts. Substrates for the in vitro splicing reactions were synthesized by transcription on linearized templates. Transcription and purification of pSP64HβΔ6 (22) pre-mRNA were done by standard procedures (15) and yielded precursor with a specific activity of approximately 200,000 cpm/pmol of transcript. T3 transcription of pBSAd-1 was done by similar methodology (6) and

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resulted in a precursor with a specific activity of approximately 40,000 cpm/pmol of transcript.

Splicing reactions. Conditions used for in vitro splicing reactions were identical to those described elsewhere (15). Briefly, 0.1 to 0.3 pmol of precursor (10,000 to 100,000 cpm/μl, depending on dilution) was combined with 50% (vol/vol) NE, 2.6% polyvinyl alcohol, 1 mM ATP, 20 mM creatine phosphate, and 3 mM $MgCl_2$. Standard nonkinetic assays were performed for 2 h at 30°C. Several standard nonkinetic reaction mixtures containing between 0 and 90% (vol/vol) NE (15) were assayed to determine the amount of NE required for efficient in vitro splicing. Pre-mRNA splicing was usually abolished in reaction mixtures containing less than 40% (vol/vol) NE. For kinetic reaction mixtures containing the adenovirus type 1 precursor, the polyvinyl alcohol was omitted and instead 88% (vol/vol) NE was used. Each splicing reaction mixture was then typically fractionated on a 5% polyacrylamide–8 M urea gel in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) for the β-globin precursor or a 10% polyacrylamide–8 M urea gel in 1× TBE for the adenovirus type 1 precursor. Gels were dried and stored overnight at −70°C with film for autoradiography. Conditions for the reactions containing heat-treated NE (HNE) were identical to those above except that the reaction mixtures utilized for complementation contained 2.6% polyvinyl alcohol. HNE were prepared from aliquots of NE. These aliquots (200 μl or occasionally smaller volumes) of NE were thawed on ice for 15 min and then incubated at 46°C for various times. At 5, 10, 15, 20, and 25 min, aliquots were removed and stored on dry ice. After samples were collected at all time points, these heat-treated fractions were stored at −70°C until needed for in vitro splicing reactions. To assay fractionated material, we combined an aliquot (usually 16% of the total reaction volume) of a given column fraction with 50% (vol/vol) HNE in a 12.5- or 50-μl splicing reaction mixture. The aliquots of HNE (50%, vol/vol) contained 125 μg of protein, which we define as 1.0 NE equivalent. To determine the NE equivalents of heat reversal factor (HRF) used for the complementation, the amount of complementing material is divided by 125 μg. Thus, the micrograms of starting NE are divided by 125 μg to determine the total NE equivalents used at the start of the purification. Hence, the ratio of HRF-containing NE equivalents (micrograms of HRF-containing protein/NE equivalents) can be calculated for each column step by normalizing the protein recovered to the starting NE equivalents. The amount (micrograms) of HRF used in the complementation assay (contained in 8 μl of a column fraction) was divided by the previous ratio to determine the NE equivalents used in a specific assay.

Native gel electrophoresis. Fractionation of splicing complexes generated with the adenovirus type 1 precursor was done under conditions previously described by Zillmann et al. (33, 34). In this case, aliquots were removed from splicing reaction mixtures and stored on dry ice. After a typical experiment was completed, the samples were thawed at room temperature, adjusted to ~2 mg of heparin (Sigma) per ml and 8 mM EDTA, and further incubated at 30°C for 10 min. For the kinetic assays, the reactions were stopped prior to incubation on dry ice with the heparin-EDTA solution. During this 10-min period, each sample was mixed three times by pipetting. An aliquot of this mixture was fractionated on a 3.5% polyacrylamide (1:80, ratio of bisacrylamide to acrylamide)–0.5% agarose native gel in 50 mM Tris-glycine (pH 8)–10 mM EDTA. Electrophoresis was performed for approximately 5 h at room temperature, and the resulting gel was stored overnight at −70°C with film for autoradiography. The identification and assignment of the complexes A, A′, and B were determined by a qualitative comparison of the gel pattern with the published results of Zillmann et al. (33, 34), by two-dimensional gel analysis of the RNA contained within the various complexes, and by oligonucleotide-specific degradation of U1, U2, and U4/U6.

Quantitation of radiolabeled material. Gel slices were carefully excised from the gel and their radioactivity was counted in a Beckman scintillation counter or the gels were dried and scanned by an Ambis gel scanner. HRF activity was quantitated by titrating HNE with HRF obtained from NE, DEAE-Sepharose, ATP-agarose, and phenyl-Sepharose chromatography. One unit of HRF activity was arbitrarily determined to be percent complex A/milligram of protein or, in some cases, percent intervening sequence (IVS)-exon/milligram of protein.

Sodium dodecyl sulfate (SDS) protein gel electrophoresis. Proteins were electrophoresed by the method of Laemmli (18). Gels were stained overnight with Coomassie blue R-250 (Sigma) or silver stained by the method of Morrissey (20).

Trypsin digestion of HRF. In general, an 8-μl aliquot of HRF-containing material from ATP-agarose or phenyl-Sepharose chromatography was combined with 0.5 μl (0.25 mg/ml) of trypsin (Boehringer Mannheim) for 30 min at either 30 or 37°C. The reaction was stopped by the addition of 2 μl (1 mg/ml) of trypsin inhibitor, 1 μl (10 mg/ml) of leupeptin, and 1 μl (10 mg/ml) of aprotinin (all from Boehringer Mannheim). It was found that a cocktail of inhibitors had to be used to completely block the activity of trypsin or the digestion of NE and HNE occurred. In the mock reactions, trypsin was combined with the inhibitors before addition of the column fraction. In addition, trypsin inhibitors were added to the NE control reactions to confirm they would not inhibit the normal in vitro splicing reaction.

pCp end labeling of small nuclear RNAs in column fractions. Aliquots of fractionated material were labeled with [^{32}P]pCp by standard methods (3).

RESULTS

Heat sensitivity of HeLa cell NE. It has been demonstrated that mild heat treatment of HeLa cell NE can inhibit efficient splicing of a human β-globin pre-mRNA (14). To study this phenomenon more closely, we preincubated aliquots of HeLa NE at 46°C for various times and then combined them with a human β-globin transcript in a standard splicing reaction. An analysis of the reaction intermediates indicated that splicing was inhibited at all pretreatment times tested (Fig. 1). After 15 min of heat treatment, 3′ cleavage and ligation of the exons was blocked (Fig. 1, lane 4). Pretreatment of the NE for 20 min at 46°C abolished 5′ cleavage as well (Fig. 1, lane 5). The differential sensitivity of the two steps in splicing was obtained with numerous HeLa NE preparations. However, the time at 46°C required to selectively inhibit initially the second step and subsequently both steps of the splicing reaction varied with the extract preparations. Further studies with an adenovirus pre-mRNA transcript indicated that heat inactivation was not intron specific (data not shown); 3′ cleavage and exon ligation of an adenovirus pre-mRNA were abolished after 5 min and 5′ cleavage was blocked after 10 min at 46°C.

Development of complementation assay. The observation that various steps in pre-mRNA splicing could be selectively inhibited by heat treatment was used as a basis for developing an assay to identify factors involved in this process.

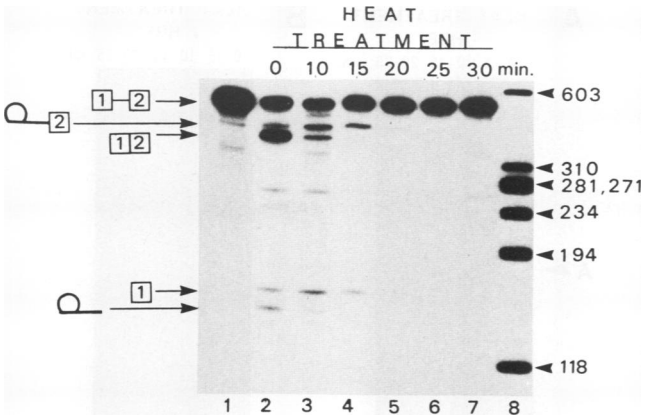

FIG. 1. Heat sensitivity of HeLa NE. The NE was heat treated for the times indicated at the top of the figure, pre-mRNA was added to each aliquot, and reaction mixtures were incubated under standard splicing conditions (see Materials and Methods). The RNA products from each splicing reaction were analyzed by denaturing gel electrophoresis. Lane 1, human β-globin pre-mRNA in the absence of NE; lanes 2 to 7, in vitro splicing reaction mixtures containing NE pretreated with heat for the times indicated at the top of the figure; lane 8, *Msp*I markers (in nucleotides). Intervening sequences ( ) and exons 1 and 2 are indicated on the left.

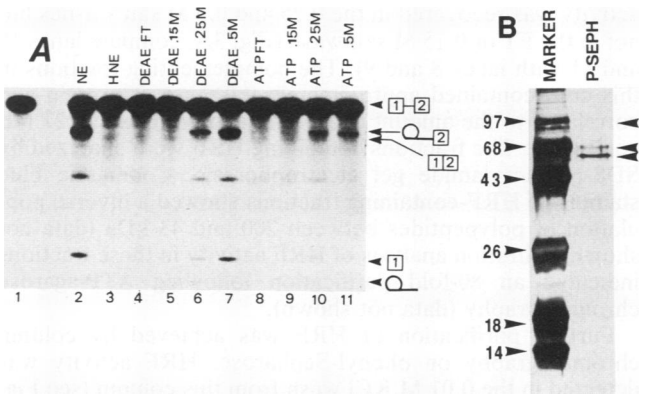

FIG. 3. Purification of HRF. (A) HNE was complemented with aliquots of purified material from DEAE-Sepharose and ATP-agarose chromatography. Lanes: 1, β-globin pre-mRNA; 2, untreated NE; 3, HNE; 4, DEAE unbound FT; 5, DEAE 0.15 M KCl wash; 6, DEAE 0.25 M KCl wash; 7, DEAE 0.5 M KCl wash; 8, ATP affinity unbound FT; 9, ATP affinity 0.15 M KCl wash; 10, ATP affinity 0.25 M KCl wash; 11, ATP affinity 0.5 M KCl wash. Intervening sequences ( ) and exons 1 and 2 are indicated on the right. (B) SDS-polyacrylamide gel electrophoresis and silver staining of HRF purified from phenyl-Sepharose (P-SEPH) chromatography. The molecular weight standards (in thousands) are shown schematically on the left. The protein bands (~1 to 50 ng) that copurify in the fraction containing HRF activity are indicated by the arrowheads. To visualize these bands by silver staining, 500 to 700 μl of a 1-ml column fraction was precipitated with 5% trichloroacetic acid (18).

Specifically, we attempted to restore splicing in HNE by the addition of a small fraction of untreated NE. We began by determining the amount of NE required for efficient in vitro splicing. These experiments showed that a minimum of 50% NE (vol/vol; ~125 μg of NE protein or 1 NE equivalent; see Materials and Methods) was required to obtain efficient splicing. This amount of NE or HNE was then used in standard assays. To prepare HNE, we heated NE under controlled conditions (usually 15 to 20 min) to inactivate 5′ cleavage and IVS-exon formation. If the heat inactivation was specific for a single factor, then 5′ cleavage might be rescued by the addition of a limiting quantity of NE. As shown in Fig. 2, the addition of 16% NE (vol/vol; 0.32 NE equivalent) was observed to rescue the first cleavage ligation reaction from HNE (lane 3). Further titration experiments have shown that as little as 2% NE (vol/vol; ~5 μg of protein; 0.04 NE equivalent) is sufficient to rescue 5′ cleavage from a reaction mixture containing 50% (vol/vol) HNE (data not shown). These results demonstrated that the heat inactivation of pre-mRNA splicing was reversible since it could be rescued by the addition of a limiting amount of NE. The addition of creatine kinase (0.32 mg/ml) also failed to stimulate 5′ cleavage (Fig. 2, lane 7). This result demonstrates that heat inactivation of splicing was not due to the absence of an ATP-regenerating system. The addition of 1.2 mg of bovine serum albumin (BSA) per ml, 0.4 mg of polyethylene glycol per ml, and 0.6 mg of tRNA per ml failed to stimulate 5′ cleavage in HNE (Fig. 2, lanes 4 to 6), thus confirming that the reconstitution of 5′ cleavage was not due to the trivial addition of bulk protein or nucleic acid.

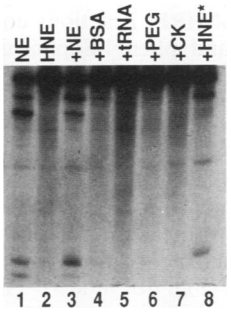

FIG. 2. Complementation of HNE. NE inactivated for pre-mRNA splicing by moderate heat treatment can be complemented by the addition of a limiting amount of untreated NE. Lanes: 1, untreated NE; 2, HNE; 3, complementation of HNE with NE; 4, BSA; 5, tRNA; 6, polyethylene glycol (PEG); 7, creatine kinase (CK); 8, HNE* (HNE deficient for the second cleavage-ligation reaction but still competent for 5′ cleavage and IVS-exon formation).

Purification of HRF. The observation that an activity in NE was able to stimulate pre-mRNA splicing from HNE suggested a potential assay for purifying this factor(s). The strategy was to subject NE to various fractionation procedures and then to rescue pre-mRNA splicing by complementing HNE with column-purified material. The purification of a factor based on this assay (an HRF) is presented in Fig. 3A. Untreated HeLa NE was fractionated over DEAE-Sepharose utilizing a step gradient with buffer D containing KCl (see Materials and Methods). Four protein-containing fractions were collected: an FT, a low-salt wash (Fig. 3A, DEAE .15M), and two high-salt washes (Fig. 3A, DEAE .25M and DEAE .5M). HRF activity was detected in the 0.25 and 0.5 M KCl DEAE washes but not in the FT or 0.15 M KCl wash (Fig. 3A, lanes 6 and 7, respectively). The DEAE aliquots utilized to complement HNE contained ~2 μg of protein and correlated with the amount of HRF in approximately 0.31 NE equivalent. The fractions containing HRF activity were pooled, dialyzed, and then chromatographed over an ATP affinity column. The bound material was eluted as described above for the DEAE-Sepharose column. HRF

activity was recovered in the 0.25 and 0.5 M salt washes but not in the FT or 0.15 M salt wash (Fig. 3A, compare lanes 10 and 11 with lanes 8 and 9). The complementing fractions in this case contained approximately 0.012 μg of protein and correlated to the amount of HRF in approximately 0.27 NE equivalent. The fractions containing HRF were analyzed by SDS-polyacrylamide gel electrophoresis. Coomassie blue staining of HRF-containing fractions showed a diverse population of polypeptides between 200 and 43 kDa (data not shown). Titration analysis of HRF activity in these fractions indicated an 80-fold purification following ATP-agarose chromatography (data not shown).

Further purification of HRF was achieved by column chromatography on phenyl-Sepharose. HRF activity was detected in the 0.01 M KCl wash from this column (see Fig. 7C, lane 5). Silver staining of the HRF-containing fractions allowed detection of two major polypeptides that migrated in the 70-kDa range and a minor band that migrated in the 100-kDa region of the gel (Fig. 3B). The minor 100-kDa polypeptide was barely detectable with silver and subsequently does not show up in Fig. 3B. When a linear gradient (1 to 0 M KCl) was used instead of a step purification procedure, HRF was eluted between 200 and 0 mM KCl (data not shown). Silver staining of the HRF-containing fractions again revealed two polypeptides that migrated in the range of 70 kDa. Additionally, phenyl-Sepharose chromatography of HRF resulted in an overall purification of approximately 950-fold as estimated by titration experiments which measured the extent of 5′ cleavage or complex A formation (see Materials and Methods). HRF purified by this methodology was observed to be unstable (~2 weeks at −70°C and several hours at 4°C). Since the characterization of HRF was difficult due to the rapid decline of HRF activity at this stage of the purification, the functional studies described were completed with HRF purified by ATP-agarose chromatography and then confirmed with HRF samples purified by the phenyl-Sepharose chromatography procedure.

Functional studies utilizing HRF. It has recently been demonstrated by Zillman et al. (33, 34) that the addition of pre-mRNA to an NE results in the formation of three complexes; A, A′, and B. The cleavage-ligation reactions reportedly take place within complex B. Since 5′ cleavage was blocked in HNE, we considered that HRF could function directly in the 5′ cleavage reaction or indirectly by rescuing the formation of one or more of the splicing complexes. For simplicity, we adopted the same gel system and nomenclature reported by Zillman et al. (33, 34).

As a first step in understanding the function of HRF, NE which had been heat treated for 0 to 25 min at 46°C was assayed for spliceosome assembly by native gel electrophoresis. Adenovirus type 1 pre-mRNA was added to each aliquot, and splicing complex assembly was monitored after 60 min at 30°C (Fig. 4A). Assembly of complex A was completely blocked after pretreatment of the extract for 20 min at 46°C (Fig. 4A, lane 5). Analysis of the RNA from the aliquots containing NE pretreated at 46°C for 20 min confirmed that the first cleavage-ligation reaction was also completely blocked (data not shown). These results suggested that mild heat treatment of the NE blocks pre-mRNA splicing by disrupting the assembly of complex A.

To test whether HRF could rescue complex A formation from HNE, an aliquot of HRF (~0.27 NE equivalent) was added to HNE incubated for 0 to 25 min at 46°C (Fig. 4B). Complex A was assembled efficiently in the reaction mixtures supplemented with HRF (Fig. 4B, lanes 5 and 6).

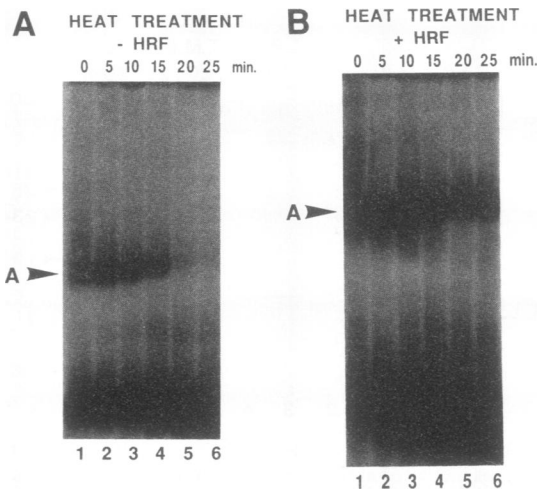

FIG. 4. HNE supplemented with HRF rescues complex A formation. Aliquots of NE were heat treated for the times indicated at the top of the figure, supplemented or not with HRF, and assayed for complex A assembly after 60 min at 30°C by native gel electrophoresis. (A) HNE without supplemental HRF. (B) HNE supplemented with HRF.

Figure 5 shows a time course of complex assembly. NE was initially heat treated for 20 min at 46°C to block complex A formation, an aliquot of HRF (~0.27 NE equivalent) was then added, and native gel electrophoresis was used to monitor complex A formation for 60 min at 30°C. Control reactions were samples containing either untreated NE or HNE without added HRF. Complex A was rapidly assembled in the untreated NE (Fig. 5A). Similarly, complex A was efficiently assembled in the reaction mixture supplemented with HRF (Fig. 5B). In contrast, the control reaction containing HNE (without HRF) failed to assemble complex A in the same 60-min period (Fig. 5C).

Complex A assembly was also stimulated by HRF purified by phenyl-Sepharose chromatography. This result is shown in Fig. 6A, where it is shown that functional HRF was detected in fraction two of the 0.01 M KCl wash from phenyl-Sepharose chromatography (lane 5). These results suggest that HRF rescues HeLa NE from heat inactivation by aiding in the assembly of complex A, which is the first committed step in spliceosome assembly.

Characterization of HRF. Fractions containing HRF from both ATP affinity and phenyl-Sepharose chromatography were sensitive to digestion with trypsin (Fig. 6B). Complex A formation was stimulated when HRF was incubated with the HNE prior to the addition of pre-mRNA (Fig. 6B, lane 3). In contrast, if HRF was first incubated with trypsin prior to incubation with the HNE, assembly of complex A was blocked (Fig. 6B, lane 4). A mock reaction in which trypsin was incubated with protease inhibitors prior to incubation with HRF under splicing conditions showed that trypsin was properly inactivated (Fig. 6B, lane 5). The slight decrease in HRF activity seen in lane 5 was due to the addition of less HRF resulting from the volume constraints of the trypsin reaction (see Materials and Methods). These results show that stimulation of complex A formation was due to the addition of a protein.

Initial characterization of HRF obtained by ATP affinity chromatography revealed that HRF was resistant to digestion with micrococcal nuclease (data not shown). However,

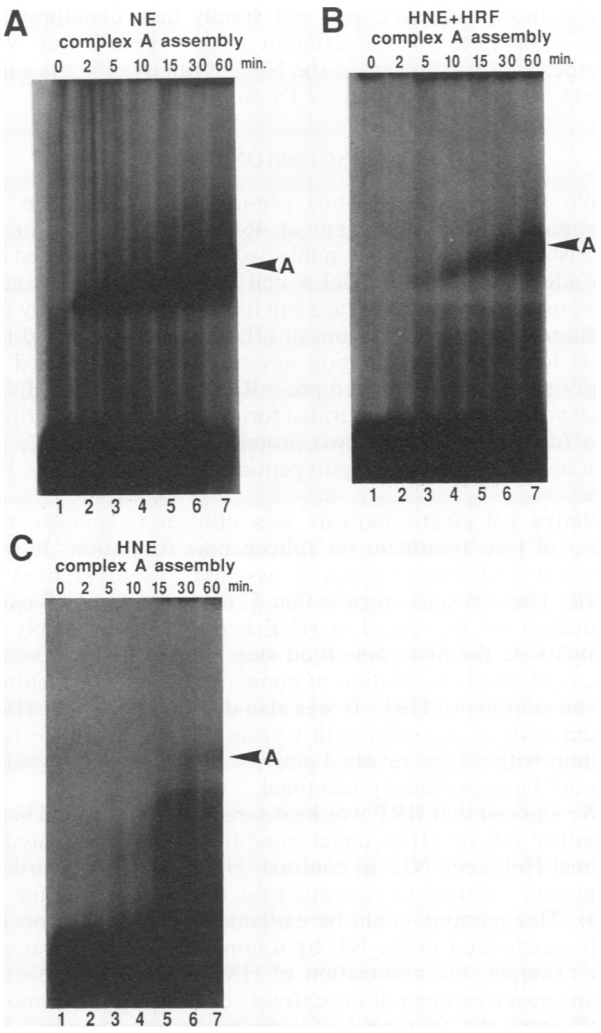

FIG. 5. Time course of complex A assembly in HNE supplemented with HRF. NE was heat treated for 20 min at 46°C to block complex A formation and then complemented with HRF, and complex A assembly was monitored for 60 min at 30°C by native gel electrophoresis. Complex A assembly was monitored at the times indicated at the top of the figure. (A) Time course of complex A formation in untreated NE. (B) Time course of assembly in HNE supplemented with HRF. (C) Time course of assembly in HNE without the addition of HRF.

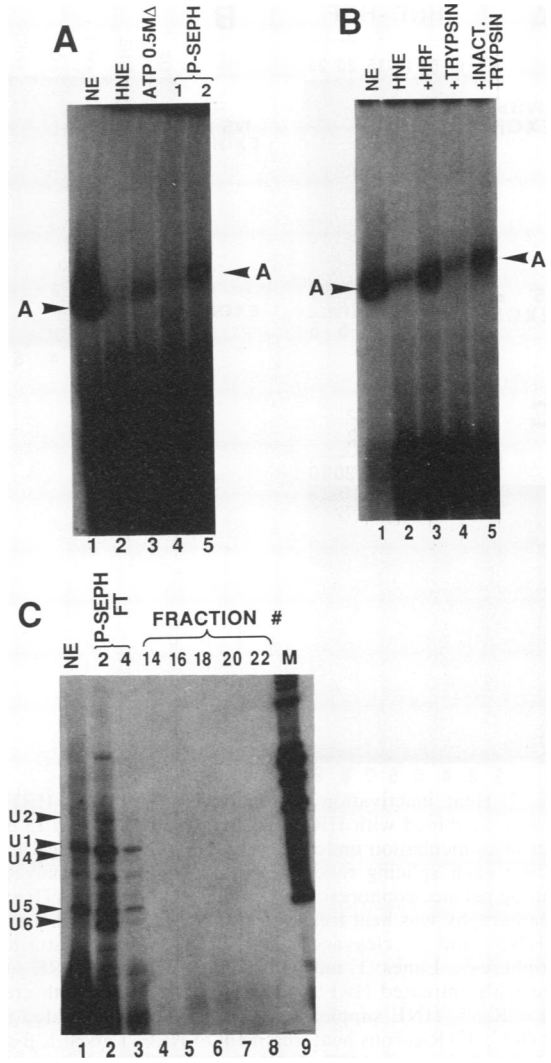

FIG. 6. Characteristics of HRF. (A) Complex A formation in HNE supplemented with HRF purified by phenyl-Sepharose (P-SEPH) chromatography. Lanes: 1, untreated NE; 2, HNE; 3, 0.5 M KCl wash containing HRF from ATP affinity chromatography; 4 and 5, fractions from the 0.01 M KCl wash collected during phenyl-Sepharose chromatography of HRF. Complex A formation was stimulated in HNE supplemented with an aliquot of HRF (<0.21 NE equivalent) containing material from fraction 2. (B) HRF is a protein. Lanes: 1, untreated NE; 2, HNE; 3, untreated HRF; 4, HRF pretreated with trypsin; 5, HRF pretreated with trypsin and trypsin inhibitors. (C) Small nuclear RNA content of phenyl-Sepharose fractions containing HRF. Fractions resulting from phenyl-Sepharose chromatography were deproteinized, labeled with [^{32}P]pCp (see Materials and Methods), and analyzed by denaturing electrophoresis. Lanes: 1, NE; 2 to 4, FT fractions from phenyl-Sepharose chromatography; 5 to 8, 0.20 to 0.00 M KCl wash containing HRF; 9, *Msp*I markers.

the major snRNPs (U1, U2, U4/U6, and U5) were detected in these fractions. For this reason, fractions containing HRF from phenyl-Sepharose chromatography were assayed by labeling snRNPs with [^{32}P]pCp by using RNA ligase (3; see Materials and Methods). The major snRNPs were detected in the FT from this chromatography (Fig. 6C, lanes 2 and 3) but were absent in the fractions containing HRF (lanes 4 to 9). HRF purified by phenyl-Sepharose fractionation was also resistant to micrococcal nuclease digestion. Taken together, these data suggest that HRF is not one of the major snRNPs.

Since HRF had been purified by the criterion of heat sensitivity, experiments were performed in which HRF was incubated under the same conditions which had previously been shown to inactivate an in vitro HeLa cell NE. To simulate the conditions for heat inactivation of HRF in the NE, we combined HRF (16%, vol/vol; ~0.27 NE equivalent) with HNE (50%, vol/vol) to achieve a fully complemented system (i.e., functional 5′ cleavage). This complemented system was then heat treated between 0 and 25 min at 46°C, and 5′ cleavage was monitored by denaturing gel electrophoresis (Fig. 7A). The control reaction resulted in 5′ cleavage (lane 1). In contrast, HRF was inactivated within 10 min at 46°C (lane 3). Similar results were obtained when HRF was combined with NE and heat treated under the same condi-

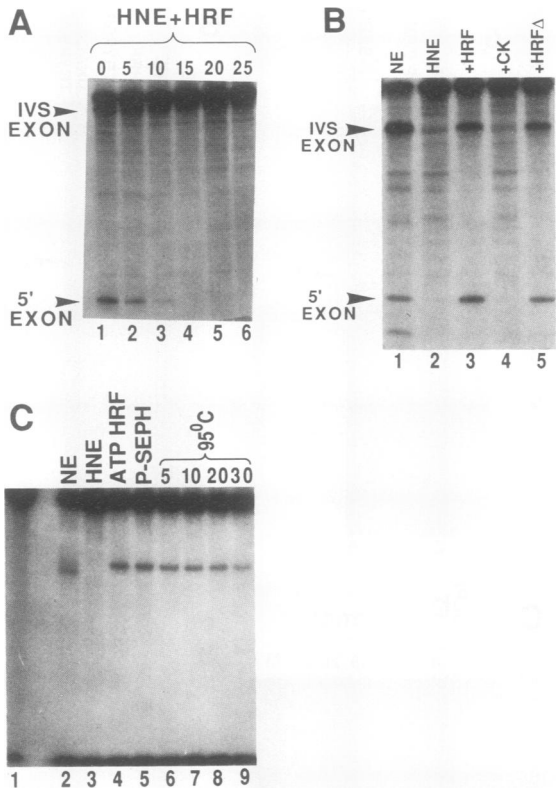

FIG. 7. Heat inactivation of crude versus purified HRF. (A) HNE was combined with HRF and heat treated for 0 to 25 min at 46°C prior to incubation under splicing conditions. The RNA products from each splicing reaction were subsequently analyzed by denaturing gel electrophoresis. (B) HRF resulting from ATP affinity chromatography was heat treated for 25 min at 46°C and combined with HNE, and 5′ cleavage was monitored by denaturing gel electrophoresis. Lanes: 1, untreated NE; 2, HNE; 3, HNE supplemented with untreated HRF; 4, HNE supplemented with creatine kinase (CK); 5, HNE supplemented with HRF heat treated for 25 min at 46°C. (C) Rigorous heat treatment of HRF. Lanes: 1, β-globin pre-mRNA; 2, untreated NE; 3, HNE; 4, HNE supplemented with HRF resulting from ATP affinity chromatography; 5, HRF activity (<0.21 NE equivalent) from phenyl-Sepharose (P-SEPH) chromatography; 6 to 9, HNE supplemented with HRF heat treated at 95°C for the times indicated at the top of the figure (minutes). The 5′ exon in this gel is not shown. HRF activity was based on the presence or absence of the IVS-exon splicing intermediate.

tions; however, NE complemented with HRF was substantially more resistant to heat inactivation at 46°C than the uncomplemented NE control reaction (data not shown). These results suggested that in the presence of NE or HNE, HRF was sensitive to mild heat treatment. However, when HRF was purified by ATP affinity or phenyl-Sepharose chromatography and was heat treated in the absence of NE or HNE, contrasting results were obtained. For example, when HRF-containing fractions from ATP affinity chromatography were heat treated for 25 min at 46°C and then combined with HNE, HRF activity was unaffected (Fig. 7B, lane 5). In addition, if HRF from ATP-agarose or a fresh round of phenyl-Sepharose fractionation was incubated at 95°C for 5 to 30 min prior to the complementation of HNE, HRF was also unaffected (Fig. 7C, lanes 6 to 9). Although unusual for a protein, there is at least one case in which a splicing factor retained some activity after heat treatment at 95°C (7). These results suggested that the mode of heat inactivation in the NE was not simply heat denaturation. HRF appears to be interacting in a *trans*-specific way with another factor(s) present in the NE but removed in the early stages of the purification (see Discussion).

DISCUSSION

We showed that in vitro pre-mRNA splicing can be disrupted by heat treatment at 46°C. This observation is consistent with previously published studies that focused on pre-mRNA splicing in HeLa cell NE (14, 17). We also demonstrated that 5′ cleavage can be restored in HNE by the addition of a limiting amount of NE. This result formed the basis for a complementation assay which was utilized to purify an HRF that rescued pre-mRNA splicing in an HNE. HRF activity in its most purified form consistently copurified in a fraction containing two proteins of approximately 70 kDa along with a minor polypeptide of approximately 100 kDa.

Native gel electrophoresis was utilized to analyze the effect of heat treatment on spliceosome formation. It was demonstrated that complex A assembly was disrupted in HNE. These results suggest that 5′ cleavage and IVS-exon formation are prevented in HNE because the assembly of complex A, the first committed step in spliceosome assembly, is blocked. Formation of complex A could be restored by the addition of HRF. It was also demonstrated that HRF is sensitive to digestion with trypsin. Therefore, HRF is a protein with a heat reversal activity that to our knowledge has not been previously identified.

We showed that HRF was heat sensitive when added back to either NE or HNE under conditions that inactivated a normal HeLa cell NE. In contrast, HRF in its most purified form was resistant to rigorous heat treatment (95°C for 30 min). This paradox could be explained if HRF was specifically inactivated in the NE by a noncovalent modification. For example, the association of HRF with another factor during heat treatment of the extract could effectively remove HRF from the free pool of splicing factors required for complex A formation. Heating HRF in the presence of NE may cause it to unfold and associate with other unfolded or aggregated proteins, preventing HRF from restoring splicing. The subsequent addition of exogenous HRF would rescue complex A formation. An alternative possibility is that HNE is rescued by the addition of a heat shock factor. It has been proposed that heat shock factors bind misfolded or aggregated proteins and catalyze their refolding (4, 21). In this case, a splicing factor required for complex A assembly in an unfolded and aggregated state would be rescued by the addition of one of these factors. Since HSP70 is constitutively expressed in HeLa cells, it is a candidate for this proposed interaction (4, 21). However, preliminary complementation experiments using purified HSP70 homologs (HSC70 and BiP [4]) failed to stimulate complex A formation from HNE (unpublished observations). Additional experiments are now in progress to determine the possible relationship of HRF with HSP70.

It is not clear from our data whether HRF has a precise role in pre-mRNA splicing. Regardless, it is clear that HRF rescues in vitro splicing from a heat-inactivated extract. Although this rescue function may reflect a requisite step of pre-mRNA splicing in vivo, it may also be due to a stress-induced phenomenon (discussed above). Further characterization of the polypeptide responsible for HRF activity should address these questions and also allow comparisons to be made with known splicing factors.

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