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CHARACTERIZATION OF HELA NUCLEAR FACTORS WHICH INTERACT WITH A CONDITIONALLY PROCESSED RAT FIBRONECTIN PRE-MRNA

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It is demonstrated that several HeLa nuclear proteins interact in a magnesium-dependent fashion with a conditionally processed pre-mRNA derived from an alternatively spliced region of the rat fibronectin gene. HnRNP C proteins crosslink under both permissive and non-permissive conditions (high and low magnesium, respectively), whereas hnRNP I/PTB is observed only under the latter. Thus hnRNP I/PTB represents a potential inhibitor of splicing for this intron. A protein that was observed to crosslink under permissive conditions has been identified as splicing factor U2AF65, which thus represents a candidate positive factor in the splicing of this pre-mRNA. We interpret these results with a working model for the conditional selection of the 3' splice site of alternative exon EIIIB.

Constitutive pre-mRNA processing requires the ordered, step-wise assembly of a functional splicing complex, or spliceosome containing several snRNPs, which are involved in the recognition of splice sites and branch sites (1,2,3). However, biochemical fractionation of mammalian nuclear extracts has demonstrated additional requirements for non-snRNP proteins (3). Many eukaryotic genes have been shown to produce multiple mRNAs by alternative RNA splicing (18,19,20), and it is of interest to understand what factors control these events. The finding that the concentration of the mammalian splicing factor SF2/ASF affects the choice of competing 5' splice sites (6,7) suggested that alternative splice site utilization may be controlled by modulation of the relative concentrations of general splicing factors. Recent studies have implicated the polypyrimidine tract region in the control of

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alternative 3' splice site selection and multiple proteins have been described which interact with the polypyrimidine tract/3' splice site region of the pre-mRNA (8-13).

The fibronectin (FN) gene gives rise to multiple mRNAs by alternative splicing (reviewed in 14). As all forms derive from a single pre-mRNA, it follows that cellular factors interact directly with the RNA to dictate splice site selection. Alternative exon EIIIB undergoes a striking developmental regulation, declining from virtually 100% inclusion of EIIIB during early embryogenesis to low levels of inclusion in most adult tissues examined (15-18). When a truncated pre-mRNA containing exon EIIIB and flanking intron sequences was processed *in vitro* by HeLa nuclear extracts under standard splicing conditions (3mM Mg^{++}) the dominant spliced product lacked EIIIB (19). This reaction recapitulated the splicing events observed with transfected HeLa cells. However, increasing Mg^{++} concentration in the reaction from 3 to 5 mM stimulated the processing of the intron upstream of EIIIB, IVS1 (19). Formation of a slowly migrating ATP-dependent complex was similarly conditional. Using ultraviolet light to crosslink nuclear proteins to radiolabelled FN pre-mRNA under permissive and non-permissive conditions, we have identified candidate factors which may modulate EIIIB selection.

MATERIALS AND METHODS

DNA templates and *in vitro* synthesis of pre-mRNAs

The fibronectin pre-mRNA 7iB was derived from the p7iB template DNA (19). Linearization with BamHI resulted in transcripts containing IVS1 (184 nucleotides) flanked by 5' exon III7b (154 nucleotides) and alternative exon EIIIB (131 nucleotides). The adenovirus template pBSAd1 contains sequences derived from the adenovirus 2 major late transcription unit (20). pBSAd1 was linearized with Sau3AI (within exon L2). Transcripts were prepared using T7 (7iB) or T3 (BSAd1) RNA polymerases essentially as described (19).

Preparation of nuclear extracts and fractions, and incubation with pre-mRNAs

Nuclear extract preparation and splicing reactions were performed essentially as described (19); the $MgCl_2$ concentration is indicated for each experiment. Reactions were incubated at 30°C for 30 minutes unless otherwise indicated.

Fractionation of nuclear extract by Poly-U Sepharose chromatography was as described (10). Briefly, 0.5 ml of nuclear extract was adjusted to 500 mM KCl and applied to a 1.0 ml column of polyU Sepharose (Pharmacia). Three sets of fractions were collected: flowthrough material, a 1.0 M KCl step and a 2.0 M guanidine-HCl step. A single peak fraction from each step was identified by protein assay (BioRad), dialyzed as for nuclear extract, and stored at -80°C . Fractionated material was incubated with pre-mRNAs as described for nuclear extract.

UV crosslinking and analysis of labelled proteins

Following incubation of pre-mRNA with protein, reactions were irradiated with 254 nm light for 10 minutes at 0°C, (2500 mW/cm^2). RNAs were digested by addition of 1/10 vol of RNase A (10 mg/ml). Samples were adjusted to 0.1% SDS and 0.1 M DTT, and heated to 100°C. Electrophoresis was through 10% polyacrylamide separating gels. Gels were fixed, dried and exposed to X-ray film.

Immunoprecipitation of crosslinked proteins

Monoclonal antibodies directed against hnRNP proteins C (21) and I (22) were obtained from G. Dreyfuss. After crosslinking, reactions were diluted with cold 150mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.2% NP40 and specific antibody was added. Samples were incubated on ice for one hour, then Protein A-agarose beads (GIBCO-BRL) were added and incubation continued for an additional hour. The washed beads were resuspended in electrophoresis sample buffer and SDS-PAGE and autoradiography were performed.

RESULTS

Different proteins interact with IVS1 under different cation conditions

Exposure of splicing reactions to ultraviolet light has been shown to result in covalent linkage of radiolabelled RNA to bound proteins, which can be visualized by SDS-polyacrylamide gel electrophoresis and autoradiography (9-11,23). FN pre-mRNA 7iB and adenovirus pre-mRNA BSAd1 were incubated at various Mg^{++} concentrations, irradiated and the electrophoretic patterns of crosslinked proteins compared (Figure 1). For both RNAs, the most prominent bands were doublets of ca. 32 and 38 kd. The only change in the proteins labelled by the adenovirus RNA was the appearance of a doublet of 56-58 kilodaltons at the lowest Mg^{++} concentration (1 mM; lane 7). The adenovirus precursor spliced at 2-3 mM Mg^{++} (20, and data not shown), conditions under which IVS1 removal from 7iB was not detected (19). In contrast, the FN pre-mRNA crosslinked to the 56-58 kDa doublet at 1-3 mM Mg^{++}, with decreasing amounts at 4 mM and none at 5-6 mM (lanes 1-6). A diffuse band in the 60-65 kDa range exhibited a similar transition. A band of 62 kDa intensified at the highest Mg^{++} concentrations; this species co-migrated with a band present in all of the adenovirus samples. A transcript consisting solely of vector sequences crosslinked weakly to the 32 kDa proteins and not to the others (not shown), indicating specificity for the pre-mRNA sequences. Thus, transitions in protein crosslinking to the FN pre-mRNA coincided with splicing of IVS1.

Identification of several crosslinked proteins.

Immunological and biochemical criteria were used to establish whether the crosslinked bands corresponded to previously identified pre-mRNA binding proteins. The 38 kDa proteins resemble hnRNP C in approximate molecular size and as well as affinity for uridine-rich tracts of RNA (21,24). The identity of the 38 kDa proteins was confirmed by immunoprecipitation with hnRNP C-specific monoclonal antibody 4F4 (Figure 2, lanes 1 and 2). Monoclonal antibody 7G12, which is hnRNP I-specific (22), immunoprecipitated the 56-58 kDa doublet preferentially at 2 mM (lane 3) versus 6 mM Mg^{++} (lane 4). (A small amount of the heavily labelled hnRNP C bands persisted through the washing steps.) Thus, the p56-58 protein doublet which binds the FN pre-mRNA in a Mg^{++}-dependent

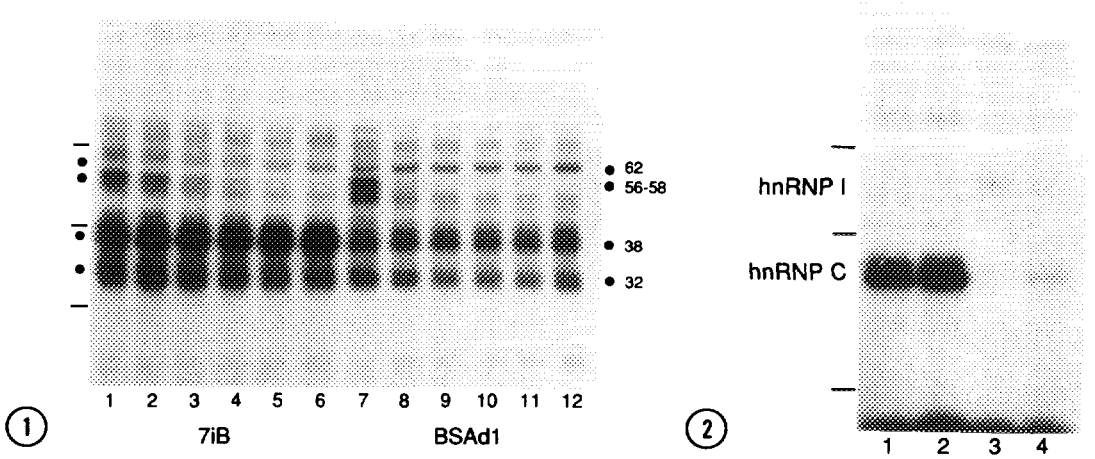

Figure 1. UV crosslinking of adenovirus and fibronectin pre-mRNAs to nuclear factors.

Magnesium concentration in splicing reactions was varied from 1 to 6 mM for FN pre-mRNA 7iB (lanes 1-6) or adenovirus pre-mRNA BSAd1 (lanes 7-12). Crosslinked proteins were analyzed by SDS-PAGE. The positions of molecular size markers are indicated at the left (dashes); from top, apparent M_r= 68, 43 and 27 x 10^3. Proteins referred to in the text are indicated with bullets and labelled according to apparent size relative to markers.

Figure 2. Immunoprecipitation of crosslinked material.

7iB pre-mRNA was incubated with nuclear extract at either 2 (lanes 1 and 3) or 6 mM Mg^{++} (lanes 2 and 4). Crosslinked proteins were precipitated with monoclonal antibody 4F4 (lanes 1 and 2) or 7G12 (lanes 3 and 4). The presence of hnRNP C in lanes 3 and 4 presumably represents incomplete removal of these heavily labelled proteins during washing; the amount of such carryover varied in different experiments (not shown). Note that 7G12 is poorly precipitated with Protein A (12). Markers are indicated as in Figure 1.

fashion is hnRNP I. Sequence comparisons indicated that hnRNP I is identical to the polypyrimidine tract-binding protein, PTB, a putative splicing factor (10,22,25).

To further characterize the differentially crosslinked bands, nuclear extract was fractionated and the fractions crosslinked to 7iB pre-mRNA. Nuclear extract was applied to a poly U Sepharose column and the flow through material (FT) collected; the column was eluted with 1.0 M KCl, then 2.0 M guanidine-HCl. These latter fractions are enriched for PTB and U2 snRNP auxiliary factor, U2AF, respectively (10). Crosslinking of FT material to 7iB RNA demonstrated that this fraction was depleted of p56-58 and p62 (Figure 3, compare lanes 3-4 to lanes 1-2). The 1.0 M KCl fraction (KCl) was highly enriched for p56-58 (lanes 5-6), as expected for hnRNP I/PTB; partially purified p56-58 crosslinked preferentially at low Mg^{++} concentration. In contrast, p62 was present in the 2.0 M guanidine-HCl fraction (GuCl) (lanes 7-8), and crosslinked preferrentially at high Mg^{++} concentration. The GuCl fraction should be enriched for splicing factor U2AF (10,12); we have confirmed its presence in our preparation by Western blotting (data not shown). Several additional lines of

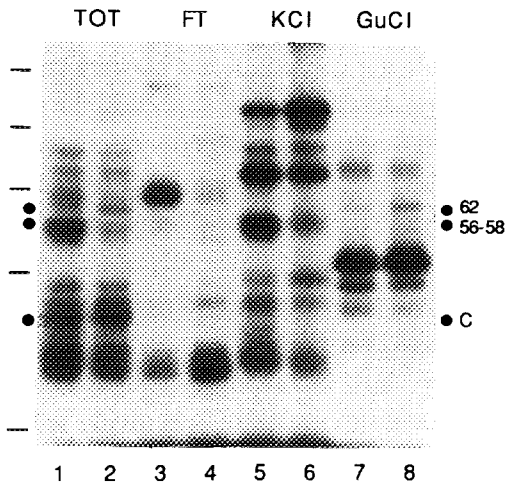

Figure 3. Crosslinking of proteins in extract fractions to 7iB pre-mRNA. Nuclear extract was fractionated by poly-U sepharose chromatography. 10 µl of flow-through (FT), 1.0 M KCl (KCl) or 2.0 M guanidine-HCl (GuCl) fractions, or total nuclear extract (TOT) were crosslinked to 7iB RNA at 2 (odd numbered lanes) or 6 (even lanes) mM $MgCl_2$. Crosslinked proteins were analyzed by SDS-PAGE. HnRNP C and hnRNP I /PTB (p56-58) are indicated, as is p62/U2AF65. The positions of molecular weight markers are indicated at the left (dashes); from the top, apparent M_r= 220, 100, 68, 43 and 27 x 10^3.

evidence indicate that the 62 kDa protein is related to U2AF. p62 is similar in mobility to the U2AF 65 kDa component (15) and the two proteins co-fractionate on DEAE-Sepharose (data not shown). Cytoplasmic S100 extracts are depleted of both U2AF and p62 (15 and data not shown). Based on the above evidence, we conclude that p62 is likely to be identical to splicing factor U2AF65.

DISCUSSION

We have employed a UV crosslinking strategy to identify proteins which bind to conditionally spliced FN pre-mRNA 7iB. Crosslinking of several proteins to 7iB is influenced by Mg^{++} concentration. The functional relevance of the observed protein-RNA interactions is suggested by the similarity in the pattern of proteins which crosslink to 7iB under splicing permissive conditions (high Mg^{++}) and those which crosslink to adenovirus pre-mRNA under all but the lowest cation concentration; the latter may represent a condition non-permissive for splicing, due to chelation of Mg^{++} by ATP.

Proteins which crosslink specifically at low or high Mg^{++} are candidate inhibitors or co-factors, respectively, for IVS1 splicing. hnRNP C has previously been implicated in splicing (26); however, we see these proteins bound under either condition. PTB has been postulated to be an essential splicing factor; our data do not

lend any support for this with regard to IVS1, as we do not see these proteins crosslinked to 7iB under splicing conditions nor do they crosslink to the adenovirus intron under normal splicing conditions (ca. 3 mM Mg^{++}). Our data are more consistent with hnRNP I/PTB acting as a negative regulator of splicing; a similar role has recently been proposed for this protein with regard to an alternatively spliced β-tropomyosin intron (27). p62/U2AF65 crosslinking does correlate with splicing, however, consistent with the known role of this protein early in splicing complex formation. A similar reciprocal relationship between PTB and U2AF crosslinking has been reported recently for an alternative intron of the preprotachykinin gene (28). Thus, this report extends studies of other workers who have examined various constitutively and alternatively spliced pre-mRNAs.

The data we have presented suggest the following model: Mg^{++}-dependent splicing is due to the cation-dependent binding of one or more proteins to IVS1. An obvious candidate is hnRNP I/PTB , which remains Mg^{++}-sensitive when purified away from most other extract components (Figure 3). Both hnRNP I/PTB and U2AF bind to the polypyrimidine tract of the 7iB intron (P.A.N., manuscript in preparation), suggesting that PTB could directly prevent U2AF binding at low Mg^{++}. In keeping with the idea that negative regulation of EIIIB selection is associated with the extended polypyrimidine tract of IVS1, we find that replacement of this sequence by an adenovirus 3' splice site leads to inclusion of the EIIIB exon (29). We are currently testing various aspects of this model, which should lend insight into possible mechanisms for modulating selection of exon EIIIB.

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