RNA (1998), *4*:1007–1018. Cambridge University Press. Printed in the USA.
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The mammalian homologue of Prp16p is overexpressed in a cell line tolerant to Leflunomide, a new immunoregulatory drug effective against rheumatoid arthritis

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ABSTRACT

Prp2p, Prp16p, Prp22p, and Prp43p are members of the DEAH-box family of ATP-dependent putative RNA helicases required for pre-mRNA splicing in *Saccharomyces cerevisiae*. Recently, mammalian homologues of Prp43p and Prp22p have been described, supporting the idea that splicing in yeast and man is phylogenetically conserved. In this study, we show that a murine cell line resistant to the novel immunoregulatory drug Leflunomide (Arava™) overexpresses a 135-kDa protein that is a putative DEAH-box RNA helicase. We have cloned the human counterpart of this protein and show that it shares pronounced sequence homology with Prp16p. Apart from its N-terminal domain, which is rich in RS, RD, and RE dipeptides, this human homologue of Prp16p (designated hPrp16p) is 41% identical to Prp16p. Significantly, homology is not only observed within the phylogenetically conserved helicase domain, but also in Prp16p-specific sequences. Immunofluorescence microscopy studies demonstrated that hPrp16p co-localizes with snRNPs in subnuclear structures referred to as speckles. Antibodies specific for hPrp16p inhibited pre-mRNA splicing in vitro prior to the second step. Thus, like its yeast counterpart, hPrp16p also appears to be required for the second catalytic step of splicing. Taken together, our data indicate that the human 135-kDa protein identified here is the structural and functional homologue of the yeast putative RNA helicase, Prp16p.

Keywords: DEAH-box; drug tolerance; RNA helicase; spliceosome

INTRODUCTION

The removal of introns from nuclear mRNA precursors (pre-mRNA) is catalyzed by a multisubunit complex termed the spliceosome. The spliceosome is composed of four small ribonucleoprotein particles (snRNPs) U1, U2, U5, and U4/U6, and an undefined number of non-snRNP protein splicing factors, which assemble on the pre-mRNA in an ordered manner. An important consequence of spliceosome assembly is the formation of a dynamic RNA network that involves base pairing between the RNA components of the spliceosome, i.e., small nuclear RNAs (snRNAs) and pre-mRNA. The proper selection and catalytic activation of the pre-mRNA splice sites are accompanied by a series of structural rearrangements within this RNA network (Moore et al., 1993; Madhani & Guthrie, 1994; Guthrie, 1996; Krämer, 1996; Reed, 1996; Will & Lührmann, 1997). It is very likely that spliceosomal proteins mediate these RNA conformational rearrangements. Of particular interest in this respect is a group of spliceosomal proteins that contain DEAD- or DEXH-box RNA helicase domains and that were first shown to be essential for pre-mRNA splicing in the yeast *Saccharomyces cerevisiae* (see Staley & Guthrie, 1998, for a recent review).

To date, eight putative RNA helicases have been identified as spliceosomal components in yeast. These include the two DEAD-box proteins Prp5p (Dalbadie-

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McFarland & Abelson, 1990) and Prp28p (Strauss & Guthrie, 1991), the DEAH-box proteins Prp2p (Chen & Lin, 1990), Prp16p (Schwer & Guthrie, 1991), Prp22p (Company et al., 1991), and Prp43p (Arenas & Abelson, 1997), and the DEXH-box protein Snu246p (also termed Brr2p, Rss1p, Slt22p) (Lauber et al., 1996; Lin & Rossi, 1996; Noble & Guthrie, 1996; Xu et al., 1996). These proteins play an essential role at various stages of the splicing reaction. Prp5p (Dalbadie-McFarland & Abelson, 1990) and Prp2p (King & Beggs, 1990) are putative RNA helicases required before the first transesterification reaction, and both induce an ATP-dependent structural rearrangement of RNA in the pre-catalytic spliceosome (Kim & Lin, 1996; O'Day et al., 1996). Prp28p and probably Snu246p are involved in the first step of the splicing reaction (Strauss & Guthrie, 1991; Lauber et al., 1996), whereas Prp16p is a second-step splicing factor (Schwer & Guthrie, 1991). Prp16p mediates a change in the confirmation of the spliceosome (Schwer & Guthrie, 1992) and also appears to be involved in a proofreading mechanism during splicing (Burgess & Guthrie, 1993). The eventual release of mRNA and the disassembly of spliceosomes are dependent on Prp22p and Prp43p (Company et al., 1991; Arenas & Abelson, 1997).

In contrast to the situation in yeast, our knowledge regarding putative RNA helicases in the mammalian spliceosome is limited and has only recently improved with the discovery of mammalian homologues of Prp22p (Ono et al., 1994; Ohno & Shimura, 1996), Prp28p (Teigelkamp et al., 1997), Prp43p (Gee et al., 1997), and Snu246p (Lauber et al., 1996). These proteins are both structurally and functionally similar to their yeast counterparts, supporting the idea that the mechanism of spliceosomal RNA rearrangements is conserved from yeast to mammals. This raises the interesting question whether homologues of the DEAD-box protein Prp5p or the DEAH-box proteins Prp2p and Prp16p also exist in mammals.

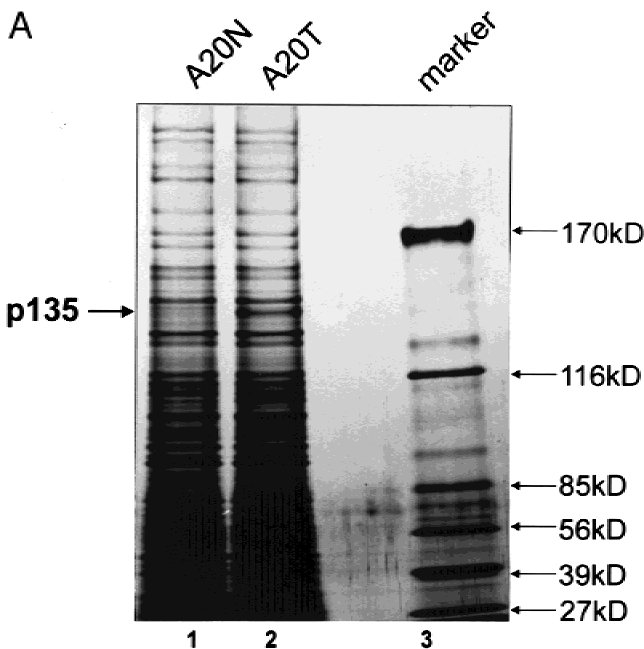
One of the research goals at Hoechst Marion Roussel is to characterize the effects of the new immunoregulatory drug Leflunomide (Arava^{TM}) on proteins in its target cells. Leflunomide has been shown to moderate undesired immune responses, for example, in rheumatoid arthritis and during organ graft rejections (Bartlett et al., 1996; Silva & Morris, 1997). The active metabolite of Leflunomide, A77 1726, inhibits lymphocyte proliferation (Xu et al., 1995; Chong et al., 1996), suppresses immunoglobulin production (Siemasko et al., 1996), and interferes with cell adhesion (Dimitrijevic & Bartlett, 1996). The molecular mechanism that forms the basis of Leflunomide action appears to be inhibition of dihydroorotate dehydrogenase (DHODH), an enzyme of de novo pyrimidine synthesis (Greene et al., 1995; Williamson et al., 1995; Zielinski et al., 1995; Davis et al., 1996; Knecht et al., 1996). Inhibition of DHODH decreases the intracellular concentration of rUMP, which induces a block in cell cycle progression by activating p53 (M. Herrmann, A.C. Frangou, & B. Kirschbaum, in prep.). This antiproliferative effect can be antagonized in vitro by the addition of uridine or cytidine to the cell culture medium, underscoring the significance of this mechanism of action (Cao et al., 1995; Williamson et al., 1995; Zielinski et al., 1995; Silva et al., 1996). However, several effects, such as the inhibition of the switch in immunoglobulin from IgM to IgG (Siemasko et al., 1996) or inhibition of various protein tyrosine kinases (Xu et al., 1995) cannot be completely reversed by the addition of uridine (Silva & Morris, 1997), indicating that Leflunomide may possess additional molecular mechanisms of action.

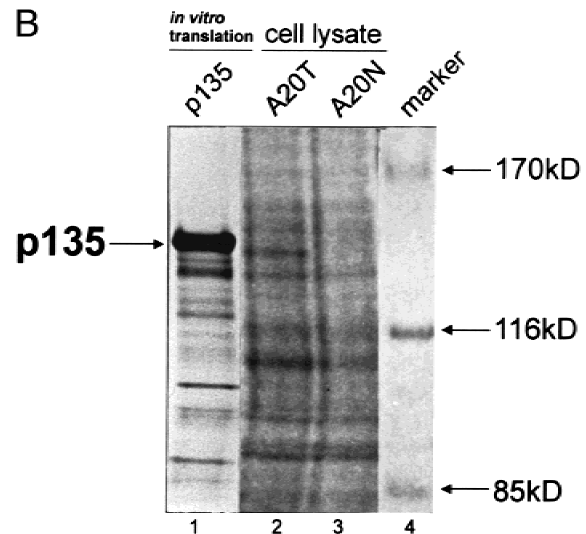
In the course of characterizing Leflunomide, we investigated its effects on protein expression in B cells. A mouse B cell line, A20T, which exhibits a 40-fold higher tolerance to Leflunomide than its parental A20N cell line, was established by culturing for more than one year in the presence of increasing concentrations of A77 1726 (described in patent no. 19545126.0, Hoechst Marion Roussel). We show here that A20T cells overexpress a protein of 135 kDa. We have cloned the human homologue of this protein and shown that it contains the consensus motifs characteristic of the DEAH-box family of ATP-dependent RNA helicases. Interestingly, the human 135-kDa protein (p135) exhibits significant structural similarity with the yeast Prp16p protein (41% sequence identity), suggesting that p135 could be involved in pre-mRNA splicing. This idea was supported by our finding that antibodies specific for p135 inhibit the second step of pre-mRNA splicing in HeLa nuclear extract, an effect also observed in yeast extracts with anti-Prp16p antibodies (Schwer & Guthrie, 1991). Thus, based on their structural and functional similarity, these results indicate that p135 (hereafter termed hPrp16p) is the human homologue of the yeast Prp16p protein.

RESULTS

A 135-kDa protein overexpressed in A20T cells is homologous to the yeast DEAH-box protein, Prp16p

To investigate the molecular mechanism of Leflunomide action, the mouse cell line A20N was exposed to increasing concentrations of this drug over an extended period of time. We were interested in determining whether the pattern of proteins expressed in Leflunomide-tolerant A20T cells differed from that observed in the nontolerant A20N cells. For this purpose, lysates of A20T and A20N were compared by one-dimensional SDS gradient gel electrophoresis. Indeed, at least one protein with an apparent molecular weight of 135 kDa (designated p135), appeared with stronger intensity in A20T compared to A20N cells (Fig. 1A). To further characterize this protein, peptide





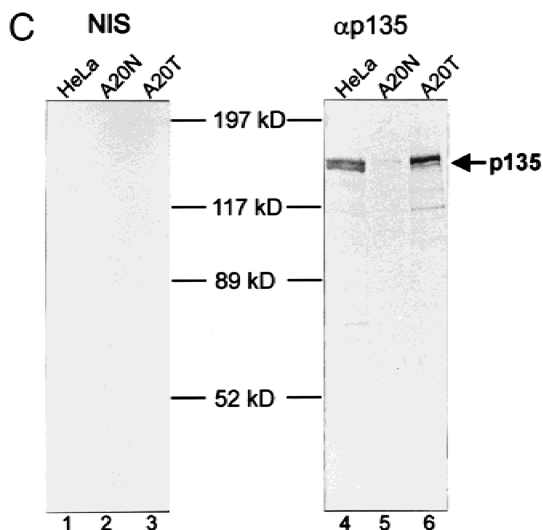


FIGURE 1. Identification of a 135-kDa protein overexpressed in Leflunomide-tolerant mouse cells. **A:** Comparison of protein expression in cells adapted to A77 1726 (A20T) or parental cells (A20N). Five micrograms protein from total cell lysates of A20N (lane 1) or A20T (lane 2), or molecular weight standard (lane 3), were separated by 4–10% gradient SDS-PAGE. The position of the p135 protein is indicated by an arrow on the left. Molecular weights of marker proteins are indicated on the right. Proteins were visualized by silver staining. **B:** Gel migration of endogenous murine p135 protein and recombinant human p135 translated in vitro. Radioactively labeled in vitro translate of human p135 cDNA (lane 1) or total cell lysates of A20T (lane 2) and A20N cells (lane 3) were separated by 7.5% SDS-PAGE. Protein size markers are shown in lane 4, and their molecular weights are indicated on the right. In vitro-translated protein was visualized by autoradiography and proteins analyzed in lanes 2–4 were stained with Coomassie. The position of murine and recombinant human p135 protein is indicated by an arrow on the left. **C:** Anti-p135 antibodies react specifically with p135 on immunoblots. Ten micrograms of HeLa nuclear extract (lanes 1, 4) or lysates of A20N (lanes 2, 5) or A20T cell (lanes 3, 6) were separated by 7.5% SDS-PAGE and transferred to a PVDF-membrane. The blots were probed with nonimmune serum (lanes 1–3) or anti-p135 immune serum (lanes 4–6). The position of p135 is indicated by an arrow to the right. The position of molecular weight markers is indicated between both panels.

fragments were microsequenced and degenerate oligonucleotides deduced from two fragments (KETEPD and TASTVI) were used for PCR amplification of a 612-bp murine cDNA.

A database search with the murine cDNA sequence revealed that it is homologous to a 241-bp human cDNA (clone B185, accession no. T19978), which was subsequently used to screen for the corresponding full-length cDNA (see Materials and Methods). The cDNA of human p135 was found in two overlapping clones that encompassed an open reading frame of 3,684 bp, encoding a 135-kDa protein of 1,227 amino acids (see Fig. 2A). A Kozak consensus sequence consisting of a purine (G) at position -3 and $+4$ (Kozak, 1987) was found in proximity to the first ATG trinucleotide, indicating that translation starts with this codon.

The deduced protein sequences of human and mouse p135 share 94% identity (data not shown), suggesting that they are functional homologues. The identity of the human cDNA clone was further supported by the similar gel migration of the endogenous mouse p135 protein and an in vitro translate of the full-length human p135 cDNA (Fig. 1B). Moreover, antibodies that were raised against a unique sequence of p135 (positions 381–527) specifically recognize a main protein band of 135 kDa in total cell lysates of A20T and A20N, and also in HeLa nuclear extract (see Fig. 1C). Note that p135 was repeatedly recognized as a double band (Fig. 1C), which may be due to posttranslational modification (e.g., phosphorylation, see Discussion). The antibody stained the p135 band more intensely in A20T than in A20N lysates, consistent with the overexpres-

A

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MGDTSEDASI HRLEGTDLDC QVGGLICKSK SAASEQHVFK APAPRPSLLG LDLLASLKRR   60
EREEKDDGED KKKSKVSSYK DWEESKDDQK DAEEEGGDQA GQNIRKDRHY RSARVETPSH  120
PGGVSEEFWE RSRQRERERR EHGVYASSKE EKDWKKEKSR DRDYDRKRDR DERDRSRHSS  180
RSERDGGSER SSRRNEPESP RHRPKDAATP SRSTWEEEDS GYGSSRRSQW ESPSPTPSYR  240
DSERSHRLST RDRDRSVRGK YSDDTPLPTP SYKYNEWADD RRHLGSTPRL SRGRGRREEG  300
EEGISFDTEE ERQQWEDDQR QADRDWYMMD EGYDEFHNPL AYSSEDYVRR REQHLHKQKQ  360
KRISAQRRQI NEDNERWETN RMLTSGVVHR LEVDEDFEED NAAKVHLMVH NLVPPFLDGR  420
IVFTKQPEPV IPVKDATSDL AIIARKGSQT VRKHREQKER KKAQHKHWEL AGTKLGDIMG  480
VKKEEEPDKA VTEDGKVDYR TEQKFADHMK RKSEASSEFA KKKSILEQRQ YLPIFAVQQE  540
LLTIIRDNSI VIVVGETGSG KTTQLTQYLH EDGYTDYGMI GCTQPRRVAA MSVAKRVSEE  600
MGGNLGEEVG YAIRFEDCTS ENTLIKYMTD GILLRESLRE ADLDHYSAII MDEAHERSLN  660
TDVLFGLLRE VVARRSDLKL IVTSATMDAE KFAAFFGNVP IFHIPGRTFP VDILFSKTPQ  720
EDYVEAAVKQ SLQVHLSGAP GDILIFMPGQ EDIEVTSDQI VEHLEELENA PALAVLPIYS  780
QLPSDLQAKI FQKAPDGVRK CIVATNIAET SLTVDGIMFV IDSGYCKLKV FNPRIGMDAL  840
QIYPISQANA NQRSGRAGRT GPGQCFRLYT QSAYKNELLT TTVPEIQRTN LANVVLLLKS  900
LGVQDLLQFH FMDPPPEDNM LNSMYQLWIL GALDNTGGLT STGRLMVEFP LDPALSKMLI  960
VSCDMGCSSE ILLIVSMLSV PAIFYRPKGR EEESDQIREK FAVPESDHLT YLNVYLQWKN 1020
NNYSTIWCND HFIHAKAMRK VREVRAQLKD IMVQQRMSLA SCGTDWDIVR KCICAAYFHQ 1080
AAKLKGIGEY VNIRTGMPCH LHPTSSLFGM GYTPDYIVYH ELVMTTKEYM QCVTAVDGEW 1140
LAELGPMFYS VKQAGKSRQE NRRRAKEEAS AMEEEMALAE EQLRARRQEQ EKRSPLGSVR 1200
STKIYTPGRK EQGEPMTPRR TPARFGL                                    1227
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B

RS-domain **Helicase-domain**

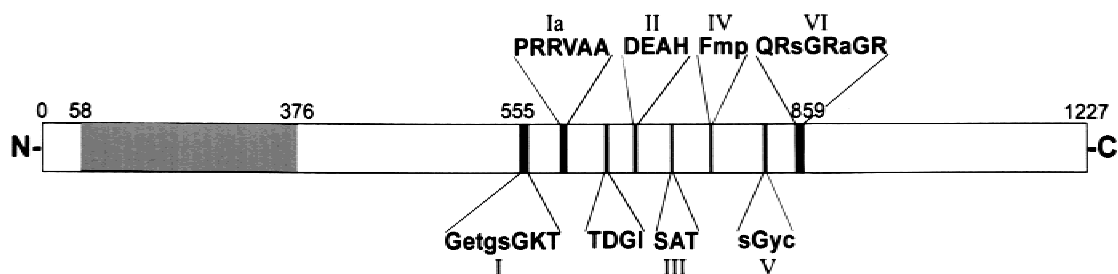

FIGURE 2. Primary structure of p135. **A:** Amino acid sequence of p135. Sequences homologous to the phylogenetically conserved motifs of DEAH-box proteins are indicated by solid black boxes. Arginine–serine (bold-faced letters) and arginine–aspartic acid or arginine–glutamic acid dipeptides, which are prominent in the N-terminal region of the protein, are shaded. A peptide sequence with homology to the nuclear localization signal of SV40 T-antigen is underlined. **B:** Schematic representation of the structural domains of p135. The position of the conserved motifs of the RNA-helicase domain (amino acids 555–859) are boxed and the sequences of these motifs are indicated above or below each box (small letters indicate p135–specific amino acids). The domain rich in RS, RD, or RE dipeptides (amino acids 58–376) is shaded. Relative positions of sequence motifs are according to scale.

sion of p135 in this cell line. Based on these results, we conclude that the cloned full-length cDNA encodes a human homologue of the mouse protein overexpressed in the Leflunomide-tolerant A20T cells.

Sequence analysis revealed that the human p135 protein contains eight consensus motifs that designate it as a member of the DEAH-box family of ATP-dependent RNA helicases and RNA-stimulated ATPases (see Fig. 2A,B) (Gorbalenya et al., 1989; Fuller-Pace & Lane, 1992; Schmid & Linder, 1992). The RNA helicase domain of p135 is located in the central part of the protein, ranging from amino acid position 555 to 859. The most conserved among these motifs are the P-loop (GETGSGKT, positions 555–562), motif II (DEAH, positions 652–655), motif III (SAT, positions 684–686), and motif VI (QRSGRAGR, positions 852–859). In addition to a helicase domain, p135 contains an N-terminal region (amino acids 58–376) with a high content of

arginine–serine (RS), arginine–aspartate (RD), and arginine–glutamate (RE) dipeptides (see shaded boxes in Fig. 2A,B). This region, which, for the sake of simplicity, we have designated an RS domain, is very similar to RS-rich regions found in a number of nuclear splicing factors (Fu, 1995; Neugebauer et al., 1995; Staknis & Reed, 1995). p135 also possesses a short sequence of nine amino acids (Fig. 2A, position 69–77) with strong homology to the nuclear localization signal (NLS) of the SV40 large T-antigen (PPKKKRKVE) (Richardson et al., 1986).

We next performed a sequence homology search to identify homologues of p135. In addition to the aforementioned murine protein, a close relative of human p135 was found in *Caenorhabditis elegans* (clone K03H1.2, accession no. P34498) (Wilson et al., 1994). Strikingly, p135 also exhibits extensive homology with the *S. cerevisiae* DEAH-box proteins Prp2p, Prp16p, Prp22p, and Prp43p, a group of well-characterized proteins required for pre-mRNA splicing (see Introduction).

The p135 protein shares the strongest homology with Prp16p (i.e., 41.1% identity, excluding amino acids 59–376 comprising p135's N-terminal RS domain) (Fig. 3). This homology is not confined to the helicase domain that is generally conserved among DEAH-box helicases (Fig. 3, positions 555–859), but also extends to the N- and C-terminal regions (amino acids 377–555 and 860–1152, respectively). Compared with Prp16p, the similarity between Prp22p, Prp43p, or Prp2p and p135 is less pronounced (not shown). This and additional data described below indicate that p135, henceforth termed hPrp16p, is most likely the human counterpart of the yeast Prp16p protein.

hPrp16p and spliceosomal proteins co-localize in nuclear speckles

Because it shares significant homology with the yeast splicing factor Prp16p, hPrp16p could also be involved in pre-mRNA splicing in humans. To initially test this hypothesis, we investigated whether the subcellular distribution of hPrp16p resembles that of spliceosomal proteins. For this purpose, COS cells were transiently transfected with a cDNA encoding FLAG-tagged hPrp16p protein and subsequently immunostained with fluorescein-labeled αFLAG M5 antibodies (Eastman Kodak Company). As shown in Figure 4A, fluorescence was observed predominantly in the nucleoplasm, whereas the cytoplasm and nucleoli remain essentially unstained. Interestingly, the nucleoplasm was more densely stained in subnuclear structures referred to as speckles (Fig. 4A). Nontransfected cells treated in the same way did not display significant nuclear staining with the αFLAG M5 antibody, indicating that the speckled appearance of the nucleoplasm is due to the specific recognition of hPrp16p by αFLAG antibodies (data not shown).

The localization of the hPrp16p protein in the nucleus is consistent with the fact that it contains a putative NLS (Fig. 2A, positions 69–77) homologous to that of the SV40 large T antigen (Richardson et al., 1986). Moreover, several proteins containing an arginine–serine-rich domain (see Fig. 2A, shaded boxes) have also been found to reside in speckles, suggesting that this RS domain targets such proteins to these nuclear regions (Misteli et al., 1997). Interestingly, most proteins located in speckles have proven to be involved in splicing or transcription (Misteli et al., 1997). To compare the nuclear localization of hPrp16p and a spliceosomal protein, COS cells were also immunostained with a polyclonal antibody raised against the U5 snRNP 116kD protein (U5-116kD) (Fabrizio et al., 1997). As shown in Figure 4B, U5-116kD resides almost exclusively in the nucleus. A digital overlay of both staining patterns revealed that the hPrp16p (Fig. 4A, green) and U5-116kD (Fig. 4B, red) fluorescent signals are, for the most part, superimposable (Fig. 4C, bright yellow). These results thus demonstrate that hPrp16p and U5-116kD are co-localized in the nucleoplasm, in particular, in nuclear speckles.

Finally, we also investigated the steady-state distribution of endogenous hPrp16p in HeLa cells by immunofluorescence microscopy using an antiserum raised against recombinant human Prp16p (i.e., a fragment spanning amino acid positions 381–527, see Materials and Methods). This antiserum reacts predominantly with the Prp16 protein from HeLa cells and exhibited negligible crossreactivity with other nuclear proteins (Fig. 1C). As shown in Figure 4D, predominantly nuclear staining was observed with the anti-Prp16 antiserum. Similar to the situation observed for the transiently expressed FLAG-tagged hPrp16p protein (Fig. 4A), also the endogenous hPrp16p protein was not evenly distributed in the nucleus, but rather showed a number of more intensely stained dots above the less intense general staining of the nucleoplasm and was absent from the nucleoli (Fig. 4D). We conclude that the endogenous hPrp16p exhibits a speckled nuclear distribution similar to other spliceosomal proteins.

hPrp16p is required for the second step of pre-mRNA splicing in vitro

The nucleoplasmic co-localization of hPrp16p with the splicing factor U5-116kD, and its identification as a potential RNA helicase, suggested that it might be involved in pre-mRNA splicing. To test whether hPrp16p is an essential splicing factor, HeLa nuclear extracts were immunodepleted of hPrp16p with anti-hPrp16p antibodies. Extracts that were partially depleted of hPrp16p exhibited a moderate reduction in splicing activity and an accumulation of splicing intermediates (data not shown), suggesting that hPrp16p is required for the second step of splicing. The rela-

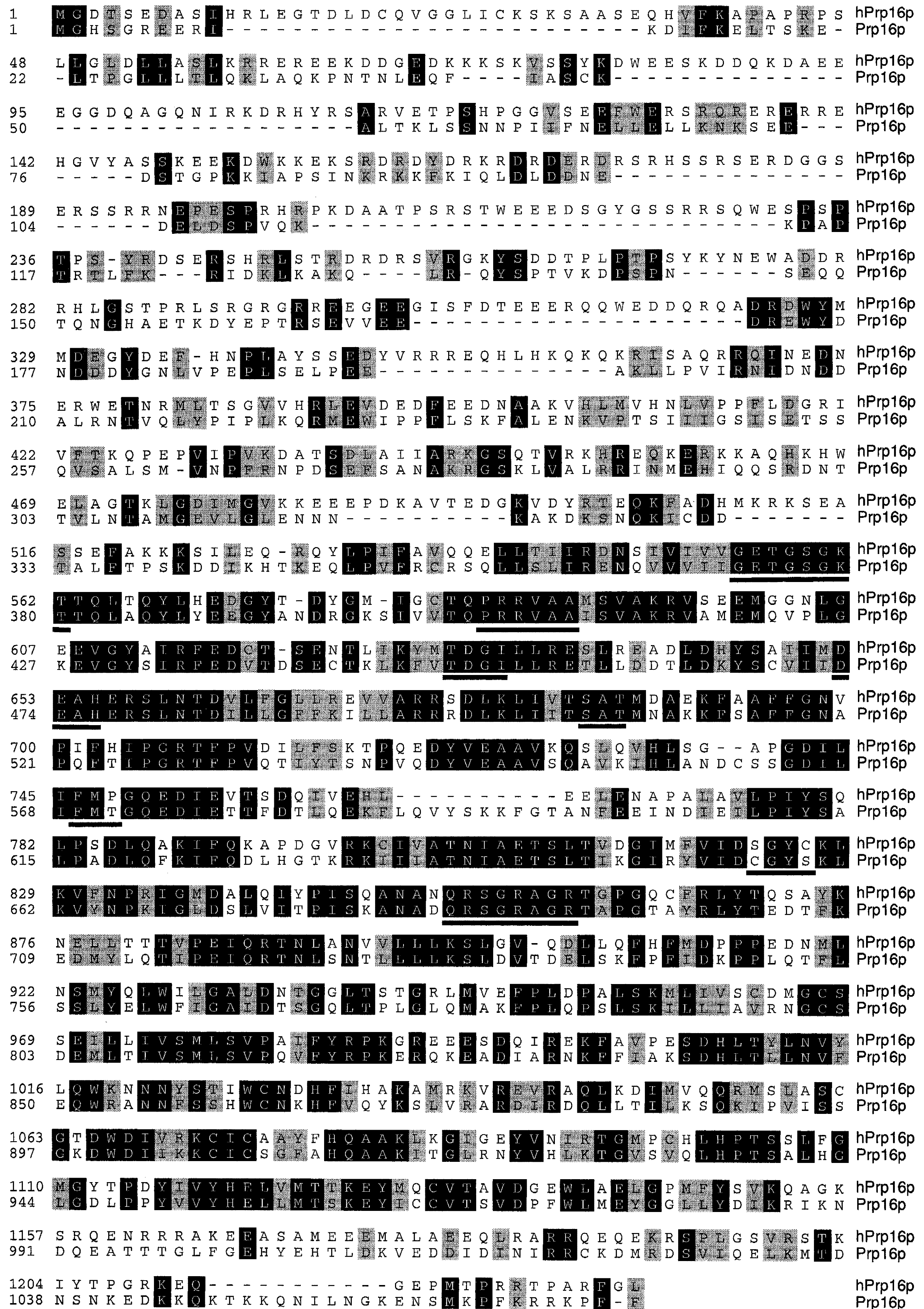

FIGURE 3. (*Legend on facing page.*)

tively high level of residual splicing activity observed with these extracts can be attributed to the fact that ca. 20% of the hPrp16p protein is still present after immunodepletion (as determined by immunoblotting). To provide more convincing evidence that hPrp16p is involved in splicing, immunoinhibition studies were also performed with anti-hPrp16p antibodies. To this end, IgG fractions of the hPrp16p antiserum were purified with protein A–Sepharose and tested directly for inhibition of splicing in nuclear extract. Significantly, the addition of increasing amounts of these antibodies led to the accumulation of first-step intermediates (i.e., the 5′ exon and lariat–exon 2 intermediate), whereas the concentration of second-step products (i.e., intron lariat and mRNA) decreased (Fig. 5, lanes 9–12). The corresponding concentrations of pre-immune serum (Fig. 5, lanes 5–8) or buffer (Fig. 5, lanes 1–4) had no effect on the splicing reaction. Taken together, these results indicate that hPrp16p is required for the second catalytic step of pre-mRNA splicing. Significantly, the structural homologue of hPrp16p in yeast, Prp16p, has also been shown previously to be a second-step splicing factor (Schwer & Guthrie, 1991).

DISCUSSION

We have identified a mammalian DEAH-box, putative RNA helicase that is overexpressed in murine cells with increased tolerance to the immunoregulatory drug Leflunomide. Using comparative cDNA analysis, we have cloned the human cDNA of this 135-kDa protein and provide experimental evidence that it is the human homologue of the splicing factor Prp16p from *S. cerevisiae*. With the exception of an N-terminal RS domain that is only present in hPrp16p, both proteins share significant sequence homology (41% identity, Fig. 3). Other DEAH-box, putative RNA helicases that are also present in the yeast spliceosome, namely Prp2p, Prp22p, and Prp43p, do not exhibit this degree of structural similarity with hPrp16p. Moreover, the mammalian counterparts of Prp22p (HRH1) and Prp43p (mDEAH9) have already been identified (Ono et al., 1994; Ohno & Shimura, 1996; Gee et al., 1997). The sequence homology between the two proteins is not limited to their RNA helicase domains, but also extends to their C termini, and the region between the RS and helicase domain. For example, the region of hPrp16p between amino acids 525 and 1152 exhibits 50% sequence identity with the corresponding region of yeast Prp16p (Fig. 3). In comparison, only 38.5% sequence identity is observed between the corresponding regions of hPrp16p and yeast Prp2p (not shown).

Our notion that p135 is the human homologue of the yeast Prp16p splicing factor is further corroborated by our finding that antibodies specific for p135 inhibit the second step of pre-mRNA splicing in HeLa nuclear extract (Fig. 5); Prp16p has also been shown to be required for the second step of pre-mRNA splicing in yeast (Schwer & Guthrie, 1991). To determine if hPrp16p also resembles yeast Prp16p in its association behavior with spliceosomes, we investigated whether spliceosomal complexes could be co-immunoprecipitated by αhPrp16p antibodies (data not shown). Only a minimal amount of a pre-mRNA lacking a 3′AG dinucleotide, but no wild-type pre-mRNA, could be precipitated from splicing extracts, suggesting that either hPrp16p indeed interacts only transiently with the spliceosome, or that its association is so weak that it does not withstand immunoprecipitation. More detailed immunoprecipitation studies are clearly needed in the future to clarify this point. Our data are consistent with the recent findings of R. Reed and co-workers who independently isolated a cDNA clone encoding the human homologue of yeast Prp16p that is essentially the same protein as p135 described here. They further demonstrated that *PRP16* could be replaced by a gene encoding the C-terminal part of the human homologue in yeast cells in vivo (R. Reed, pers. comm.).

Unlike Prp16p, hPrp16p contains an N-terminal domain that contains SR dipeptides (RS domain). RS domains have also been identified in the spliceosomal DEAH-box, putative RNA helicase HRH1 (the human homologue of Prp22p) and the DEAD-box protein U5-100kD (the human homologue of Prp28p). In both cases, the corresponding yeast proteins do not contain such a domain (Ono et al., 1994; Teigelkamp et al., 1997). Moreover, the biochemical character of the RS domains of the three human putative RNA helicases is similar, all containing a high percentage of alternating mixed charged residues (i.e., RD and RE dipeptides) and only short stretches of interspersed SR dipeptides (see Fig. 2 and Ono et al., 1994; Teigelkamp et al., 1997). What could be the function of the N-terminal RS domain of hPrp16p? RS domains are known to facilitate protein–protein interactions in the spliceosome, in particular, during early spliceosome formation (for review see Fu, 1995; Cáceres & Krainer, 1997). It is therefore attractive to speculate that the RS domain of hPrp16p may interact with other spliceosomal proteins and thus target this putative RNA helicase to its site of action. Of further interest is the fact that RS domain

FIGURE 3. Sequence comparison of p135 with Prp16p from *S. cerevisiae*. p135 (hPrp16p) was aligned with Prp16p from *S. cerevisiae* (accession no. P15938) using the CLUSTAL algorithm. Identical amino acids are boxed in black, whereas conserved residues are shaded gray. Conserved amino acids are grouped as follows: (DE), (HKR), (PILVCMFWY), (NQ), (ST), (AG). The DEAH-box sequence motifs are underlined by black bars.

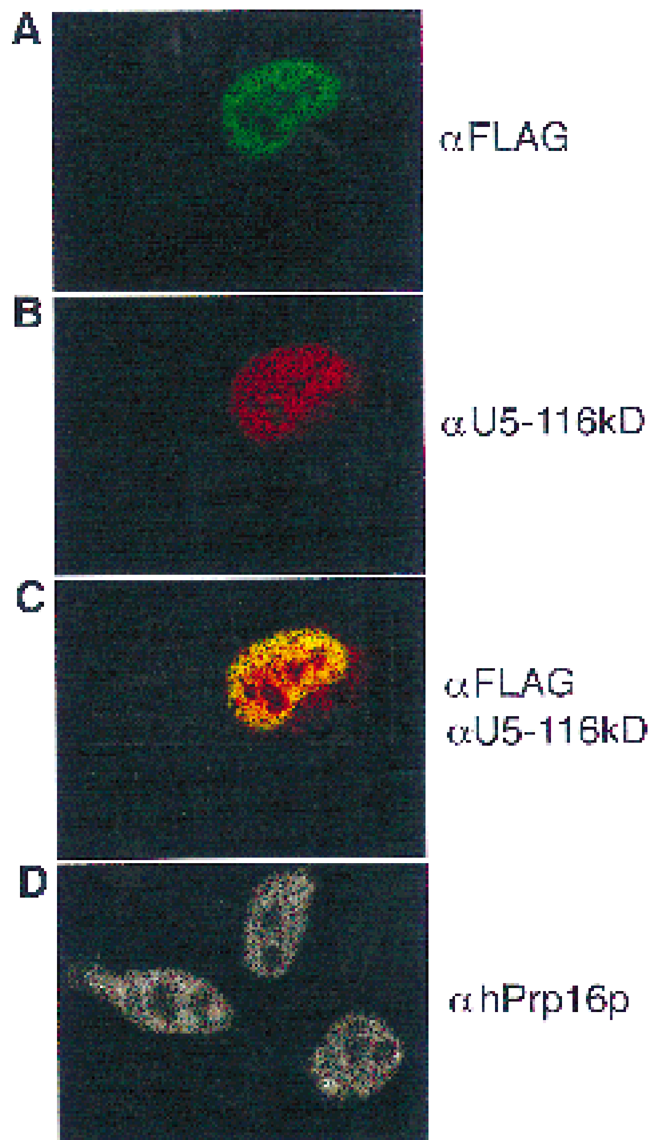

FIGURE 4. Subcellular localization of hPrp16p in transfected COS cells compared to endogenous hPrp16p localization in HeLa cells was investigated by immunofluorescence microscopy. COS cells were transiently transfected with a FLAG-tagged version of hPrp16p and double-stained with monoclonal anti-FLAG-antibodies (**A**) and rabbit antiserum raised against the U5 snRNP-specific 116-kDa protein (**B**). The green and red fluorescence was recorded independently and combined in an overlay image (**C**); yellow color indicates the nuclear regions where hPrp16p and U5-116kD co-localize. Endogenous hPrp16p was visualized by staining nontransfected HeLa cells with a rabbit antiserum raised against recombinant human Prp16p (**D**).

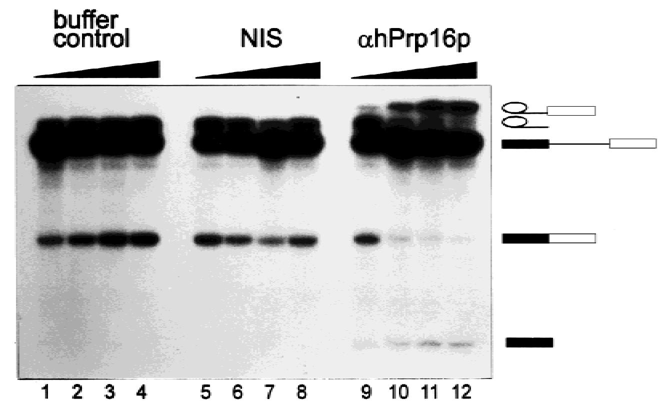

FIGURE 5. Inhibition of the second step of splicing by antibodies raised against hPrp16p. Splicing reactions (12.5 μL) were preincubated for 15 min at 30 °C. Radiolabeled MINX pre-mRNA was added subsequently and the incubation was continued for 1 h in the presence of PAS-purified IgG from rabbit anti-hPrp16p serum (lanes 9–12). The amount of immunoglobulin added was 5, 10, 15, or 20, μg (lanes 9, 10, 11, and 12, respectively). Control reactions contained either the corresponding concentration of immunoglobulin from preimmune serum (lanes 5–8), or equivalent volumes of Tris-buffered glycine (lanes 1–4). The RNA substrate and spliced products were separated by electrophoresis on 14% polyacrylamide/8.5 M urea gels and the RNA was visualized by autoradiography. The positions of the pre-mRNA, splicing products, and splicing intermediates are indicated on the right.

interactions can be modulated by phosphorylation (Mermoud et al., 1992; Xiao & Manley, 1997). The hPrp16p protein also appears to be posttranslationally modified (see Fig. 1C) and our preliminary data indicate that this is due to phosphorylation by an, as yet, unidentified kinase (data not shown). This raises the possibility that the interaction of hPrp16p with other spliceosomal SR proteins could be a regulated process. Finally, consistent with previous observations that RS domains mediate the targeting of certain SR protein splicing factors to subnuclear sites termed speckles (see, e.g., Hedley et al., 1995), hPrp16p immunofluorescence microscopy demonstrated that hPrp16p is also present in such sites (Fig. 4).

Although our antibody inhibition experiments indicate that hPrp16p is implicated in the second step of pre-mRNA splicing (Fig. 5), its exact function remains unclear. In yeast, Prp16p has been implicated in a significant conformational change at the 3′ end of an intron prior to step two of the splicing reaction (Schwer & Guthrie, 1992). Interestingly, a similar conformational change occurs at the 3′ end of the intron in mammalian spliceosomes, raising the possibility that hPrp16p may likewise be involved in this event. Whether hPrp16p exhibits RNA helicase activity in vitro will require the isolation of purified protein, which as yet could not be isolated in a native form. Recent studies in yeast indicate that Prp16p interacts with the 3′ splice site of pre-mRNA in a temporal order together with the second-step splicing factors Prp8p and Slu7p (Umen & Guthrie, 1995a). Alleles of *PRP16*, *SLU7*, and two additional proteins involved in the second step, *PRP17* (Vijayraghavan et al., 1989) and *PRP18* (Vijayraghavan & Abelson, 1990), are synthetically lethal with mutations in *PRP8* (reviewed in Umen & Guthrie, 1995b), raising the possibility that they may also physically interact with each other. Human homologues of Prp8p (Anderson et al., 1989), Prp18p (Horowitz & Krainer, 1997), and Prp17p (R. Reed, pers. comm.) have been de-

scribed recently, and it will be interesting to investigate whether these proteins cooperate with hPrp16p in facilitating step two of the pre-mRNA splicing reaction in the human spliceosome.

Our initial identification of the mammalian homologue of Prp16p as a protein overexpressed in Leflunomide-tolerant A20T cells raises the question whether hPrp16p binds this immunoregulatory drug. Although we have not been able to detect any interaction between hPrp16p and Leflunomide in vitro, we cannot rule out the possibility that the increased levels of this protein play a role in drug tolerance. However, a more likely mechanism for drug tolerance in A20T cells is the overexpression of DHODH (M. Löffler, pers. comm.), an enzyme known to be inhibited by Leflunomide (Knecht et al., 1996). The human genes encoding DHODH and hPrp16p are both located in close proximity on chromosome 16q22 (accession nos. D86977, M94065). Assuming this is also the case in mouse, the overexpression of both these proteins could be due to the amplification of this region of the chromosome in A20T cells. However, it remains to be seen whether DHODH and the murine Prp16p homologue contribute to Leflunomide tolerance in a synergistic manner.

MATERIALS AND METHODS

Cell lines and analysis of overexpressed proteins

The Leflunomide-resistant (IC50 100 μM) cell line, A20T, was generated by gradually exposing the mouse B lymphocyte cell line A20N to increasing concentrations of A77 1726, the active metabolite of Leflunomide. To compare its protein expression pattern with that of the nontolerant cell line A20N ($IC_{50} = 2$ μM) (described in patent #19545126.0, Hoechst Marion Roussel, Germany), suspension cultures of A20T or A20N were grown (Iscove medium, Biochrom, Berlin, Germany) at the logarithmic phase in the presence or absence of 100 μM A77 1726, respectively. Cells were harvested by centrifugation, and the cell pellet (10^7 cells) was incubated at 95 °C for 10 min in Laemmli solution (Laemmli, 1970). The protein concentration was determined according to Popov et al. (1975). Five micrograms of each protein preparation were analyzed by SDS-PAGE on gels containing a 4–10% polyacrylamide gradient (Laemmli, 1970) and proteins were visualized by silver-staining (Heukeshoven & Dernick, 1988). The overexpressed 135-kDa protein (p135) was excised from such gels and microsequenced.

Cloning of the full-length p135 cDNA

Peptide sequences obtained from the mouse p135 protein (KETEPD, TASTVI; sequencing performed by TopLab mbH, Munich, Germany) were used to design synthetic oligonucleotide primers (primers 1 and 2, see below) for PCR amplification of a 612-bp murine cDNA (B. Kirschbaum, pers. comm.). A 241-bp human cDNA (clone B185, accession no. T19978), highly homologous to the murine sequence, was subsequently isolated from a human cDNA library (peripheral T-cells, 5′-STRETCH PLUS cDNA, Clontech) by PCR with primers 3 and 4. The B185 cDNA fragment was nonradioactively labeled (DIG-High Prime, Boehringer Mannheim), and used to screen a human skeletal muscle 5′-STRETCH PLUS cDNA Library (Clontech). Two clones, which overlap in a region of 530 bp and encode the open reading frame of the human p135 protein, were identified by sequencing (performed by Medigene, Munich, Germany). A full-length clone was obtained by PCR amplification of the N-terminal part (primers 5 and 6), and inserting the resulting fragment into the *Hin*d III and *Pvu* I sites of the clone containing the C-terminal part of p135. The sequence of the full-length cDNA was verified (Sequenase™ Version 2.0, Amersham). The p135 cDNA was translated in vitro (TNT™ System, Promega) and the electrophoretic mobility of recombinant p135 protein was compared to that of the endogenous protein from A20N and A20T cell lysates. Proteins were separated by 7.5% SDS-PAGE and visualized by Coomassie-staining or autoradiography.

Antibody preparation

Antibodies were raised against an N-terminal fragment (amino acid position 381–527) expressed as a glutathione-S-transferase fusion protein. The expression vector was generated by cloning a 455-bp *Nde* I–*Xba* I fragment (amplified by PCR using primers 7 and 8) into pGEX B (a variant form of pGEX-2T, Pharmacia, kindly provided by M. Meisterernst). Overexpression of the GST-fusion protein in *Escherichia coli* XL-1 blue was induced by incubation with isopropyl-β-D-thiogalactoside (1 mM) for 4 h at 28 °C. Cells were disrupted by sonification. The fusion protein was bound to glutathione Sepharose 4B (Pharmacia) and washed with PBS. The p135 fragment was released from the matrix-bound GST-peptide by cleaving with thrombin protease (Pharmacia). This p135 fragment was used subsequently for immunization of a rabbit (Harlow & Lane, 1988). For the immunological detection of proteins, nuclear extracts and cell lysates were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes (Millipore). The blots were incubated with nonimmune serum or anti-p135 serum and immunostained as described by Lehmeier et al. (1990).

For inhibition of in vitro splicing, IgGs were isolated from pre-immune or immune serum using protein A–Sepharose (Pharmacia). In particular, 3 mL of rabbit serum were diluted with 9 mL of PBS, pH 8.0, containing 0.05% (v/v) Nonidet P40, and incubated with 1 mL of protein A–Sepharose for 2 h at 4 °C. The Sepharose was washed with 10 mL of PBS/0.05% (v/v) Nonidet P40, and the IgG fraction was eluted with 5 mL of 100 mM glycine, pH 2.7. Ten 500-μL fractions were collected and immediately neutralized with Tris/HCl. The protein concentration of the eluates was determined photometrically.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed with COS cells transiently transfected with a FLAG-tagged version of p135, or with untransfected HeLa cells, respectively. In order to insert a FLAG epitope (Prickett et al., 1989) at the N-terminus of p135, a 1,075-bp DNA fragment was amplified

by PCR with primers 9 and 10. The upstream primer (9) was designed to introduce a new *Hin*d III restriction site and a new start codon with an optimal translation initiation site adjacent to the FLAG tag. The mutated N-terminus was cloned into the *Hin*d III and *Pvu* I sites of the original pcDNAI-vector (Clontech) containing the p135 cDNA. Introduction of the FLAG tag was verified by DNA sequencing (Sequenase™ version 2.0 DNA Sequencing Kit, Amersham).

This p135 construct was transfected into COS cells via DEAE-dextran (Lopata et al., 1984). Transfected cells were grown for 24 h in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) containing 10% FCS (Sigma Cell Culture), trypsinized, and seeded onto sterile coverslips, incubating for an additional 24 h. Nontransfected HeLa cells were grown on glass coverslips to ~70% confluency. HeLa and transfected COS cells were then processed for immunofluorescence at room temperature.

Coverslips were initially washed three times with PBS. The cells were then fixed with 2% (w/v) paraformaldehyde (Merck), rinsed three times with PBS, and permeabilized with PBS/0.2% (v/v) Triton X-100 (Sigma). After washing one time with PBS, blocking and antibody hybridization were performed with PBS containing 1% (w/v) bovine serum albumin (BSA, Sigma). Primary antibodies used were αU5-116kD rabbit antiserum, diluted 1:1,000 (Fabrizio et al., 1997), M5 α-FLAG murine monoclonal antibodies at 10 μg/mL (Eastman Kodak Company), and αhPrp16p rabbit antiserum, diluted 1:50. α-Mouse FITC-conjugated and α-rabbit Cy3-conjugated secondary antibodies (Amersham) were diluted 1:200 and 1:500, respectively. Primary and secondary antibody incubations were performed for 1 h, followed by three washes with PBS. After the final wash, coverslips were air dried and mounted with Fluoprep. Cells were visualized with a fluorescence microscope (Zeiss) at excitation wavelengths of 543 nm (Cy3) and 488 nm (FITC). The images were printed with an HP DeskJet 870Cxi using Adobe Photoshop Software.

In vitro splicing reactions

HeLa nuclear extracts were prepared as described by Dignam et al. (1983). [^{32}P]-labeled pre-mRNAs used for in vitro splicing reactions were derived from pMINX (Zillmann et al., 1988), or from wild-type or mutant (ΔAG) forms of the adenovirus 2 major late transcript (AdML) (Gozani et al., 1994; kindly provided by R. Reed). RNAs with a specific activity of 4×10^6 c.p.m./pmol were synthesized by run-off transcription and capped with G(5′)ppp(5′)G (Pharmacia). In vitro splicing was performed essentially as described by Krainer et al. (1984). Reactions (12.5 μL in total) contained 30% (v/v) nuclear extract, 2×10^4 c.p.m. of [^{32}P]-labeled pre-mRNA, 3 mM $MgCl_2$, 2 mM ATP, 40 mM KCl, and 10 mM creatine phosphate. For inhibition assays, nuclear extracts were preincubated for 15 min at 30 °C with protein A–Sepharose-purified IgG fractions or Tris-glycine buffer (80 mM Tris/HCl, 0.1 M glycine, pH 7.0) before addition of the RNA substrate (MINX). After addition of pre-mRNA, incubation was performed for 1 h at 30 °C and reactions were stopped by the addition of 200 μL PK buffer [0.1 M Tris/HCl, pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% (w/v) SDS]. Samples were extracted with 1 volume of phenol/chloroform/isoamylalcohol (PCA) (25:24:1), and the RNA was precipitated from the aqueous phase with 2.5 vol ethanol. RNA was analyzed on 14% polyacrylamide/8.5 M urea gels.

Oligonucleotide primers

1. 5′-AAGGAGACGGAGCCGGACAAA-3′
2. 5′-CGATGTAACCATGAGCTTCAG-3′
3. 5′-TGTGATCTGCAAACATCTGCACTGGCC-3′
4. 5′-GCCGGTGATTGCCAGTGAAGGATGCCA-3′
5. 5′-GGGGTGGAAGCTTCGGCGCCC-3′
6. 5′-GCCTCCTCACGTAGTCCTCGG-3′.
7. 5′-ACAAACCATATGCTCACCAGTGGG-3′
8. 5′-TGCCTCTGCTCTAGAATGGACTTC-3′
9. 5′-CCCAAGCTTGCCACCATGGACTACAAGGACGACGATGACAAGGGGGACACCAGTGAGGATGCCTC G-3′
10. 5′-CCGCCTCCTCACGTAGTCCTCGGA-3′

Underlined sequences indicate the following restriction sites: 5, *Hin*d III; 7, *Nde* I; 8, *Xba* I; and 9, *Hin*d III.

ACKNOWLEDGMENTS

We are grateful to Robin Reed for providing the AdMLΔAG plasmid and for communicating results prior to publication. We also thank Irene Öchsner and Gabi Lambert for technical support, Cindy Will for critical reading of the manuscript, and Udo Cronshagen for help in immunofluorescence microscopy.

Received April 6, 1998; returned for revision April 17, 1998; revised manuscript received June 1, 1998

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