An Intronic Polypyrimidine-rich Element Downstream of the Donor Site Modulates Cystic Fibrosis Transmembrane Conductance Regulator Exon 9 Alternative Splicing*

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Two intronic elements, a polymorphic TGmTn locus at the end of intron 8 and an intronic splicing silencer in intron 9, regulate aberrant splicing of human cystic fibrosis transmembrane conductance regulator (CFTR) exon 9. Previous studies (Pagani, F., Buratti, E., Stuani, C., Romano, M., Zuccato, E., Niksic, M., Giglio, L., Faraguna, D., and Baralle, F. E. (2000) *J. Biol. Chem.* 275, 21041–21047 and Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M., and Baralle, F. E. (2001) *Embo J.* 20, 1774–1784) have demonstrated that *trans*-acting factors that bind to these sequences, TDP43 and Ser/Arg-rich proteins, respectively, mediate splicing inhibition. Here, we report the identification of two polypyrimidine-binding proteins, TIA-1 and polypyrimidine tract-binding protein (PTB), as novel players in the regulation of CFTR exon 9 splicing. In hybrid minigene experiments, TIA-1 induced exon inclusion, whereas PTB induced exon skipping. TIA-1 bound specifically to a polypyrimidine-rich controlling element (PCE) located between the weak 5′-splice site (ss) and the intronic splicing silencer. Mutants of the PCE polypyrimidine motifs did not bind TIA-1 and, in a splicing assay, did not respond to TIA-1 splicing enhancement. PTB antagonized *in vitro* TIA-1 binding to the PCE, but its splicing inhibition was independent of its binding to the PCE. Recruitment of U1 small nuclear RNA to the weak 5′-ss by complementarity also induced exon 9 inclusion, consistent with the facilitating role of TIA-1 in weak 5′-ss recognition by U1 small nuclear ribonucleoprotein. Interestingly, in the presence of a high number of TG repeats and a low number of T repeats in the TGmTn locus, TIA-1 activated a cryptic exonic 3′-ss. This effect was independent of both TIA-1 binding to the PCE and U1 small nuclear RNA recruitment to the 5′-ss. Moreover, it was abolished by deletion of either the TG or T sequence. These data indicate that, in CFTR exon 9, TIA-1 binding to the PCE recruits U1 small nuclear ribonucleoprotein to the weak 5′-ss and induces exon inclusion. The TIA-1-mediated alternative usage of the 3′-splice sites, which depends on the composition of the unusual TGmTn element, represents a new mechanism of splicing regulation by TIA-1.

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Cystic fibrosis (CF)[1] is the most common autosomal recessive disorder in Caucasians, and it is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1). CFTR mutations can also be associated with non-classical forms of CF in which the disease shows a tissue-specific variability such as congenital bilateral absence of vas deference and idiopathic pancreatitis (2). In some cases, the phenotypic variability has been associated with a variable proportion of aberrant CFTR exon 9 skipping, which produces a nonfunctional protein (3–7) and can be modulated by splicing factors. Extensive studies on CFTR exon 9 alternative splicing have provided a paradigmatic example of the complexity of its regulation and, in this case, the possibility of aberrant exon skipping that leads to pathological consequences.

Several *cis*-acting elements and *trans*-acting factors have been identified to modulate CFTR exon 9 alternative splicing. The *cis*-acting elements described so far are the (TG)m(T)n polymorphic locus at 3′-end of IVS8 (3), the juxtaposed enhancer and silencer exonic elements (8), and the intronic splicing silencer (ISS). Several *in vitro* and *in vivo* studies have shown that a high number of TG repeats and a low number of T tracts in the TGmTn locus induce exon skipping. In intron 9, the ISS element is located 75 bp downstream of the weak 5′-splice site (9). In our previous studies, we identified TDP43 and members of the Ser/Arg-rich (SR) protein family as inhibitors of CFTR exon 9 alternative splicing via their interaction with the TG tract and the ISS, respectively (9, 10); interestingly, the expression level of these proteins is higher in the mostly affected CF tissues (10). No enhancing splicing factor has been identified so far to activate CFTR exon 9 inclusion. Its role could be important to counteract splicing inhibition and to modulate the tissue-specific phenotypic expression in non-classical CF forms.

TIA-1 and polypyrimidine tract-binding protein (PTB) are two splicing factors involved in the regulation of alternative splicing. Both recognize intronic polypyrimidine-rich sequences, mainly located in introns, and promote exon inclusion or skipping, respectively. TIA-1 binding to polypyrimidine-rich sequences downstream of the 5′-splice site (ss) is involved in the recognition of exons that contain weak 5′-splice sites. Sequence analysis of TIA-1, initially identified as an apoptotic protein (11), revealed that it contains three RNA recognition motifs, and its amino acid sequence shows close homology to the *Saccharomyces cerevisiae* Nam8p protein, a U1 small nu-

clear ribonucleoprotein (snRNP) complex component (12). *In vitro* selection/amplification assays have shown a preference for TIA-1 binding to RNAs containing short stretches of uridines, although a core binding sequence has not been recognized (13). Acting on weak 5′-splice sites followed by U-rich stretches, TIA-1 modulates the alternative splicing of *Drosophila msl-2* and human *fas* receptor pre-mRNAs (14). The TIA-1 effect may be critical for U1 snRNP recruitment and stabilization to these weak 5′-splice sites. Interaction between TIA-1 and U1-C, one of the three U1-specific polypeptides (15), stabilizes the association of both proteins and consequently of U1 snRNP with the adjacent 5′-ss followed by the U-rich stretch. In one case, TIA-1 *in vivo* depletion resulted in a different choice of a wild type normal 3′-ss partner, but the mechanism involved is not known (14).

U-rich polypyrimidine tracts can also interact with PTB. PTB is a well characterized splicing factor involved in repression of the cell-specific splicing pattern in inappropriate cell types (16). Multiple intronic PTB-binding sites distributed on the entire length of pre-mRNAs have been found to mediate splicing repression in several alternatively spliced mRNAs (17–22). The ability of PTB to multimerize led to the suggestion that it establishes a "zone of silencing" within a pre-mRNA, preventing splice site usage and that, in this manner, it mediates exon silencing (16, 23).

In this study, we show that the region between the weak 5′-ss of CFTR exon 9 and the ISS has an important regulatory role in alternative splicing. This polypyrimidine-rich controlling element (PCE) contains a peculiar arrangement of three polypyrimidine-rich (PY) motifs. TIA-1 binding to the PCE increased exon 9 inclusion, possibly stabilizing U1 snRNP interaction with pre-mRNA. In contrast, PTB showed an opposite effect and caused an increase in exon 9 skipping.

In addition, we also report that TIA-1 induced the selection of a cryptic exonic 3′-ss independently of its facilitating role in 5′-ss recognition mediated by U1 snRNP. Taken together, these data increase the number of factors involved in the complex regulation of CFTR exon 9 alternative splicing and for the first time show the involvement of a splicing factor with enhancing activity in CFTR exon 9 recognition.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The wild-type constructs carrying variable combinations of (TG)m(T)n repeats at the polymorphic locus and hCF-Δint2 have been described previously (9). The mutations introduced in the PY motifs were obtained by PCR with different oligonucleotide sets using templates with different mutations inserted. The resulting products were digested with BamHI/KpnI and cloned into the CFTR intron 9 sequence contained in the NdeI insert of the modified pBluescript plasmid (pBSCFNde) described previously (24). The mutagenized NdeI fragment was then subcloned in the NdeI-digested TG11T5 minigene. The BamHI/KpnI cassettes were created by PCR-mediated site-directed mutagenesis using different primers for each cassette: M1, CF9junAdir (5′-ctggatccactggagcaggcaaggtagttcatttg-3′); M2, CFA/3-2bsdir (5′-ctggatccactggagcaggcaaggtagttcttttgttcatc-3′); M1,2, the same primer as M1, but on the M2 template; M2A, CF9/2bsmut (5′-ctggatccactggagcaggcaaggtagttcttttgtaaatc-3′); and M1,2A, CF9/1,2bsmut (5′-ctggatccactggagcaggcaaggtagtgaatttgtaaatc-3′). For all amplifications, the reverse primer used was UNI REV (5′-ggaaacagctatgaccatg-3′). For M3, a two-step overlap extension method was used. For the first amplification, the two sets of primers used were as follows: hCFex9/in9dir (5′-ctggatccactggagcaggcaaggtagttcttttg-3′) and ΔYrev (5′-caaactgcaggacaccaaattaagttcttaat-3′); and ΔYdir (5′-gtgtcctgcagtttgtagtgctggaaggtat-5′) and UNI REV. For the second amplification, the hCFex9/in9dir and UNI REV set was used. For M1,2,3, CF9/1,2bsmut (5′-ctggatccactggagcaggcaaggtagtgaatttgtaaatc-3′) and UNI REV on the ΔM3 template were used. A derivative of pHU1 (25) was used to prepare EX9U1-5′-ss. This variant was created as described (25) using oligonucleotides EX9-U1-5′-ssDIR (5′-gatctcagaactacctggcagggggagataccat-3′) and EX9U1-5′-ssREV (5′-gatcatggtatctccctgccaggtagttctga-3′).

Analysis of Hybrid Minigene Expression—Transient transfection of

Hep3B cells, RNA extraction, reverse transcription (RT)-PCR, and quantitation of PCR products were done as described previously (9). The α2 and B2 primers have been described previously (9). The sequence of the cry2 primer is (5′-ggaggaacagcttgcctgctcc-3′). The pHI plasmid was kindly provided by Dr. M. G. Romanelli. The pTIA-1 expression plasmid was kindly provided by Dr. R. Breathnach. If not specified otherwise, 1 μg of pHI or pTIA-1 plasmid was used in cotransfection experiments.

UV Cross-linking Assay—The T7SW Eco competitor was prepared by cutting and religating the pBSCFNde construct with EcoRI and KpnI enzymes. The 3′-h3′int competitor was prepared by cutting and religating the pBSCFNde construct with SacI and BamHI enzymes. The pBIND competitor was prepared by cloning the EcoRI/BamHI fragment of exon 9 into pBSSK. The competitors in intron 9 that span the first 77 nucleotides were prepared directly by cloning the PCR product (direct primer, hCFex9/in9dir; and reverse primer, IN9Hind/Kpn (5′-ggaagcttccaaaagcttccagcac-3′)) digested with BamHI/HindIII into pBSSK. The pEND competitor was prepared digesting the ΔM3 PCR product with PstI/KpnI and cloning the insert into pBSSK. Each competitor was linearized and used for *in vitro* transcription and UV cross-linking assays as described previously (9).

Immunoprecipitation Experiments—Polyclonal antiserum against PTB was obtained by immunizing a New Zealand White 3-month-old rabbit according to standard protocols. PTB immunoprecipitation experiments were done as described previously (10). TIA-1 immunoprecipitation experiments were performed as described (26) using a commercially available anti-TIA-1 polyclonal antibody (Santa Cruz Biotechnology).

Pull-down Assay—The unlabeled RNA probes were prepared as described previously (9) and placed in a 400-μl reaction mixture containing 0.1 M NaOAc (pH 5.0) and 5 mM sodium metaperiodate (Sigma). Reaction mixtures were incubated for 1 h in the dark at room temperature. The RNA was ethanol-precipitated and resuspended in 100 μl of 0.1 M NaOAc (pH 5.0). Then, 300 μl of a 50% adipic acid dihydrazide-agarose bead slurry (Sigma) were washed four times with 10 ml of 0.1 M NaOAc (pH 5.0) and pelleted after each wash at 3000 rpm for 3 min. After the final wash, 300 μl of 0.1 M NaOAc (pH 5.0) were added to the beads. The slurry was then mixed with the periodate-treated RNA and incubated for 12 h at 4 °C on a rotator. The beads with the bound RNA were pelleted and washed three times with 2 ml of 2 M NaCl and three times with 3 ml of buffer A (20 mM HEPES-KOH (pH 7), 6.5% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol). They were incubated with 0.6 mg of HeLa cell nuclear extract for 20 min at 30 °C in a final volume of 650 μl, pelleted by centrifugation at 1000 rpm for 3 min, and washed five times with 5 ml of buffer A containing 4 mM MgCl$_2$. After the final centrifugation, 60 μl of SDS-PAGE sample buffer were added to the beads and heated for 5 min at 90 °C before loading onto a 10% SDS-polyacrylamide gel.

RESULTS

PY Motifs near the CFTR Exon 9 5′-ss Are Intronic Splicing Enhancers—The intronic region between the 5′-ss of CFTR exon 9 and the previously reported ISS contains three distinct pyrimidine-rich elements, PY1, PY2, and PY3 (Fig. 1*B*). PY1 and PY2 are separated only by a purine residue and together span 13 nucleotides close to the exon/intron junction. The PY3 element is located 30 nucleotides downstream of PY2 and is composed of 15 contiguous pyrimidine residues (Fig. 1*B*). We prepared CFTR exon 9 hybrid minigenes containing mutations in the pyrimidine-rich elements as shown in Fig. 1*B*. Hybrid minigenes were transfected in Hep3B cells, and the pattern of splicing was analyzed by RT-PCR amplification using specific primers. Fig. 1*C* shows the effects of single and multiple mutations on the efficiency of splicing. The control wild-type hCFTG11T5 construct (*lane 1*) resulted in 65 ± 5% exon 9 inclusion, as previously reported (9). Using the M1 and M3 constructs, with mutations in PY1 and PY3, the level of exon 9 inclusion was ~60%, *i.e.* not significantly different from TG11T5 (Fig. 1*C*, *lanes 2* and *7*). Disruption of the PY2 motif in M2 (TCTT motif in PY2 changed to TC<u>A</u>T) and M2A (TCTT motif changed to <u>AAA</u>T) produced 35 ± 3 and 37 ± 4% exon inclusion, respectively (Fig. 1*C*, *lanes 3* and *5*). Significantly lower levels of exon 9 inclusion were observed in the double mutants M1,2 and M1,2A, corresponding to 22 ± 4 and 23 ± 2%, respectively (Fig. 1*C*, *lanes 4* and *6*), indicating that mu-

A

TG$_m$T$_n$AACA<u>G</u>

ex 9 +

ex 9 cryp

ex 9 −

ISS

PCE

B

	PY1 PY2 PY3

CFwt   GTAGTTCTTTTGTTCTTCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M1   GTAGTTC**A**TTTGTTCTTCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M2   GTAGTTCTTTTGTTC**A**TCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M2 A   GTAGTTCTTTTGT**AAA**TCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M1,2   GTAGTTC**A**TTTGTTC**A**TCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M1,2 A GTAGT**GAA**TTTGT**AAA**TCACTATTAAGAACTTAATTTGGTGTCCATGTCTCTTTTTTTTTTCTAGTTTGTAG

M3   GTAGTTCTTTTGTTCTTCACTATTAAGAACTTAATTTGGTGTCC--TGCAGTTTGTAG

M1,2,3 GTAGT**GAA**TTTGT**AAA**TCACTATTAAGAACTTAATTTGGTGTCC--TGCAGTTTGTAG

C

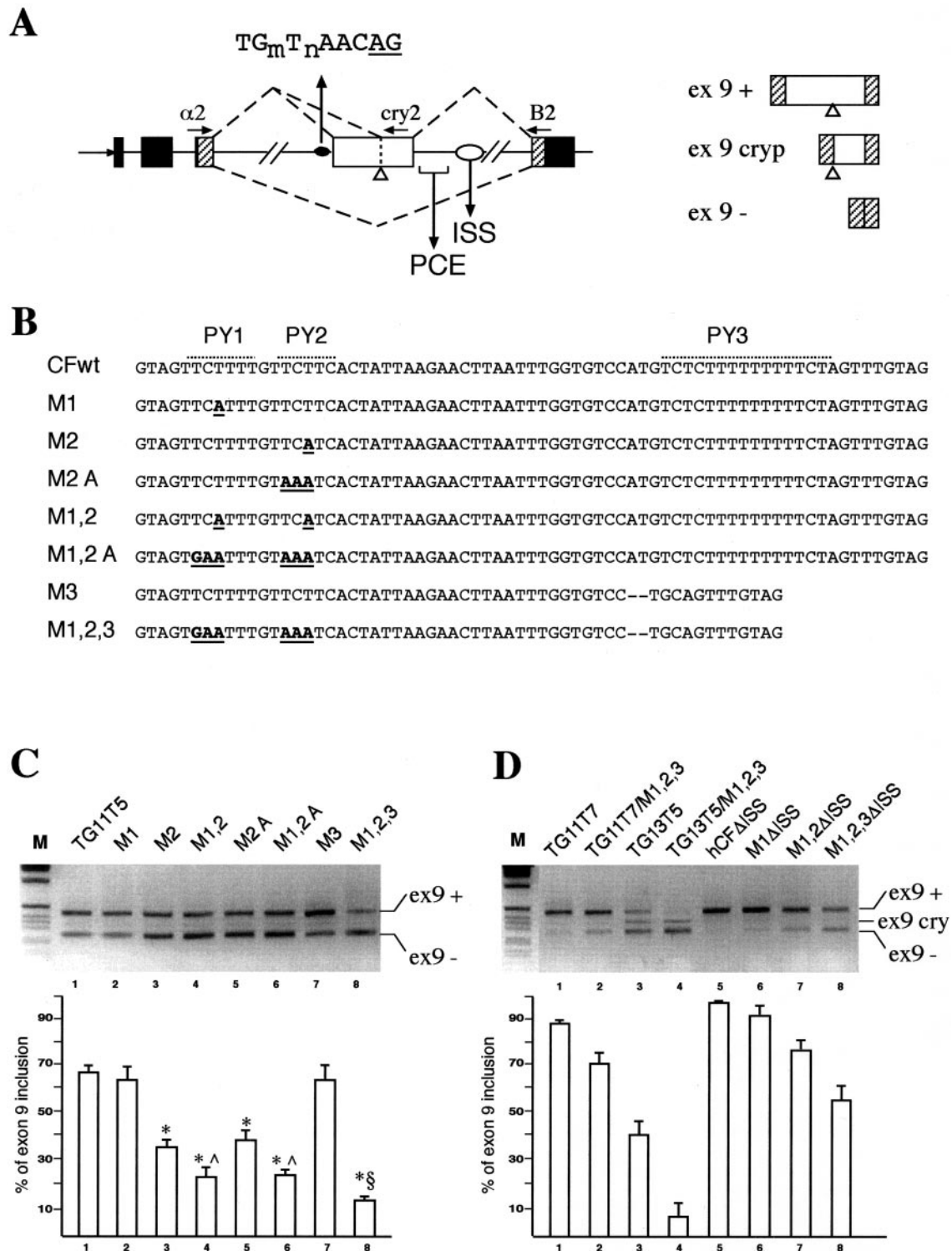ex9 +
ex9 −

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ex9 +
ex9 cry
ex9 −

FIG. 1. **Mutational analysis of the PCE in CFTR intron 9.** *A*, schematic representation of the CFTR exon 9 (*ex 9*) hybrid minigene construct used in transfection experiments showing the positions of the intronic regulatory elements: the polymorphic TGmTn locus, the PCE, and the ISS. *Black boxes* correspond to the α-globin exons, *hatched boxes* to the fibronectin EDB exons, and the *white box* to CFTR exon 9. The *triangle* in exon 9 indicates the position of the cryptic (*cryp*) 3′-ss. The *underlined* AG nucleotides correspond to the normal 3′-ss. The *arrows* indicate the primers used in RT-PCR assays. The three spliced forms originating from alternative pre-mRNA processing of the minigene are shown on the right. *B*, base pair composition of intron 9 wild-type (CFwt) and mutant PCE sequences. The three polypyrimidine-rich sequences, PY1, PY2, and PY3 (*dotted lines*), and the mutations introduced (*boldface* and *underlined*) are indicated. *C*, RT-PCR assays of transient transfection with the α2 and B2 primers using the indicated minigene constructs. The *upper* and *lower bands* correspond to exon 9 inclusion (*ex9 +*) and exclusion (*ex9 −*), respectively. The intermediate band corresponding to usage of the cryptic 3′-ss was present in low amounts (<5%) and is not included in the histogram. *D*, RT-PCR assays of transfection with the α2 and B2 primers using the PCE mutants and variants of the polymorphic TGmTn locus or without the ISS. Significant usage of the cryptic 3′-ss was observed only in the TG13T5 minigenes and is better analyzed in Fig. 4. The histogram shows the percentage of exon 9 inclusion *versus* exon 9 exclusion detected by 6% polyacrylamide gel analysis of radioactive PCR products and quantitated using a Cyclone. Data are expressed as percentage ± S.D. of exon inclusion and are the means of at least three independent experiments done in duplicate. *Lanes M*, markers (1-kb ladder); *, *p* < 0.01 *versus lane 1*; ^, *p* < 0.05 *versus lane 3*; §, *p* < 0.05 *versus lane 4*.
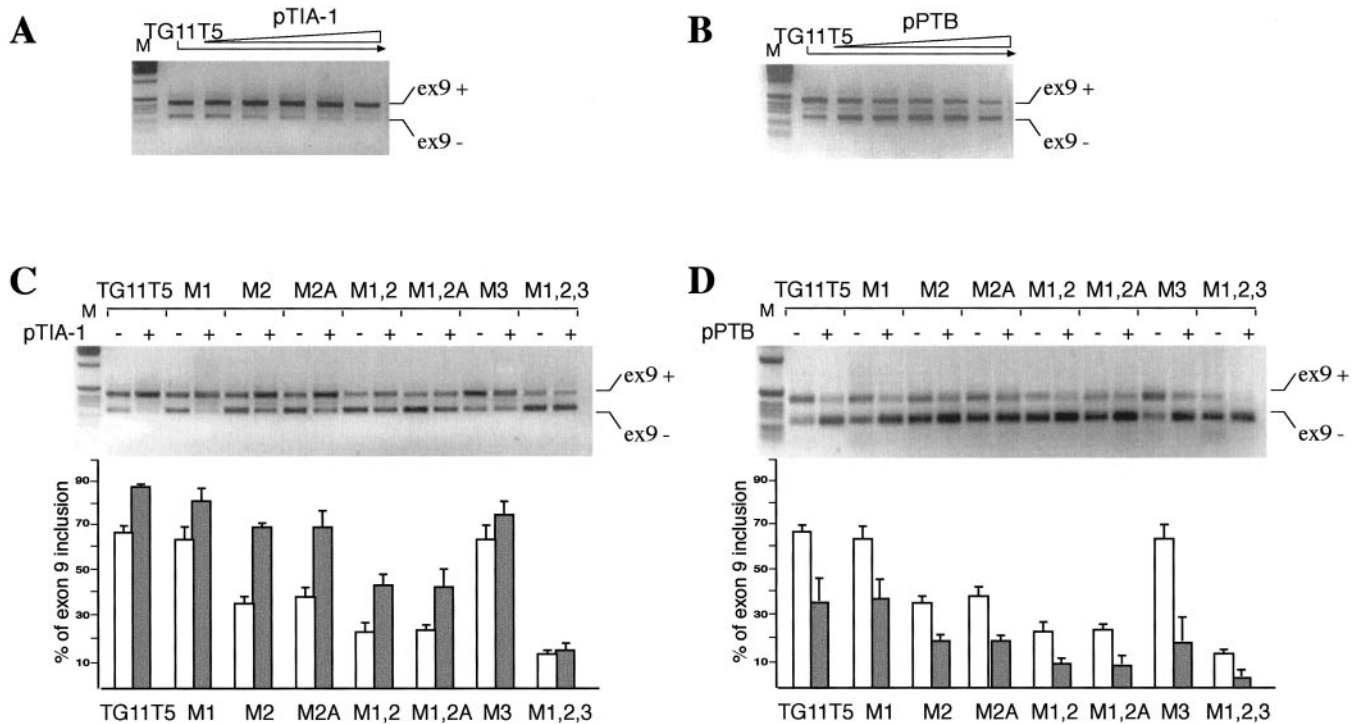
FIG. 2. **Effect of TIA-1 on CFTR exon 9 alternative splicing.** *A* and *B*, dose-response experiments of exon 9 splicing. The TG11T5 minigene was cotransfected along with increasing amounts of pTIA-1 or PTB expression vector, respectively. The amounts of plasmid used for each cotransfection were 100 ng, 200 ng, 500 ng, 1 μg, and 2 μg (*second* through *sixth lanes*, respectively). *C* and *D*, effects of TIA-1 and PTB, respectively, on CFTR exon 9 minigenes containing PCE mutants. The indicated minigenes were cotransfected with 1 μg of pTIA-1 expression vector (+) or empty vector (−). RNA splicing variants were detected with the α2 and B2 primers. Amplified fragments were loaded onto 1.5% agarose gels. The histograms show the percentage of exon 9 inclusion *versus* exon 9 exclusion detected by 6% polyacrylamide gel analysis of radioactive PCR products and quantitated using a Cyclone. Data are expressed as percentage ± S.D. of exon inclusion and are the means of two independent experiments done in duplicate. *Lanes M*, markers (1-kb ladder).

tations in PY1 contribute to splicing regulation when associated with mutations in PY2 (Fig. 1*C*, compare *lanes 3* and *4*). The disruption of all three PY motifs (M1,2,3) resulted in the lowest level of exon 9 inclusion (13 ± 2%) (Fig. 1*C*, *lane 8*), which was also significantly different compared with the M1,2 and M1,2A mutants, suggesting a combined effect of the three PY motifs. Our previous work also showed that an exon 9 cryptic band originated from the recognition of a cryptic 3′-ss in exon 9 (10). Using the same construct (hcFTG11T5), the percentage of this cryptic band was very low and did not change significantly upon comparison of the wild-type and mutant constructs. These results indicate that the intronic sequence between the 5′-ss and the ISS, which we have named PCE for polypyrimidine-rich controlling element, constitutes a new intronic splicing regulatory element involved in the modulation of CFTR exon 9 alternative splicing.

Functional Relationship between the (TG)m(T)n Locus, ISS, and PCE—Previous studies have shown that exon 9 inclusion is modulated by the polymorphic (TG)m(T)n locus near the 3′-ss and by the ISS in intron 9 (3, 9). An increase in the length of the TG tract or a reduction of the U-rich stretch induces exon 9 skipping. In addition, a high number of TG repeats (>13) and a low number of T repeats (<5) induces a significant activation of a cryptic 3′-ss in exon 9. We have now evaluated the enhancing effect of the PCE in relation to the ISS and to the composition of the polymorphic (TG)m(T)n locus. Mutations in the three PY motifs in the PCE were evaluated in the context of the TG11T7 and TG13T5 variants of intron 8 or in combination with deletion of the ISS. The levels of exon 9 inclusion in the wild-type TG11T7 and TG13T5 minigenes were 90 and 40%, respectively (Fig. 1*D*, *lanes 1* and *3*). In comparison with these constructs, the TG11T7/M1,2,3 and TG13T5/M1,2,3 minigenes resulted in reduced exon 9 inclusion (80 and 7%, respectively)

(Fig. 1*D*, *lanes 2* and *4*). The TG13T5 minigenes showed, as expected, usage of the cryptic 3′-ss, but the percentage of inclusion was not significantly affected in the PCE mutants (see additional experiments in Fig. 5). Deletion of the ISS from the wild-type minigene (hCFΔISS) resulted in 95% exon inclusion (Fig. 1*D*, *lane 5*) because of the absence of the ISS inhibitory element, as previously reported (9). In comparison with hCFΔISS, mutations in all of the PY motifs (M1,2,3ΔISS) reduced the level of exon inclusion to 55% (Fig. 1*D*, *lane 8*). Mutation in PY1 (M1ΔISS) or in both PY1 and PY2 (M1,2ΔISS) produced 90 and 75% exon inclusion, respectively (Fig. 1*D*, *lanes 6* and *7*). These results indicate that the enhancing effect of the PCE is modulated by differences in the (TG)m(T)n locus and by the ISS element. Thus, the PCE represents a new intronic element that, along with the ISS in intron 9 and the (TG)m(T)n element in intron 8, modulates CFTR exon 9 alternative splicing. This new intronic element could affect the efficiency of recognition of the upstream weak 5′-ss through binding of a specific splicing factor to the PY motifs.

Role of the Polypyrimidine-binding Proteins TIA-1 and PTB in CFTR Exon 9 Alternative Splicing—To study the influence of TIA-1 and PTB on CFTR exon 9 alternative splicing, we performed transient cotransfection experiments. Hep3B cells were transfected with the TG11T5 minigene alone or with increasing amounts of TIA-1 or PTB expression vector. TIA-1 expression induced a dose-dependent increase in exon 9 inclusion (Fig. 2*A*). In fact, in comparison with the basal level of exon 9 inclusion obtained using the TG11T5 minigene (65%), TIA-1 overexpression increased inclusion to ~90% at the highest vector concentrations (Fig. 2*A*). In contrast, cotransfection with increasing amounts of the PTB expression vector (pHI) resulted in dose-dependent inhibition of exon 9 splicing. It is interesting to note that TIA-1 is the only splicing factor iden-
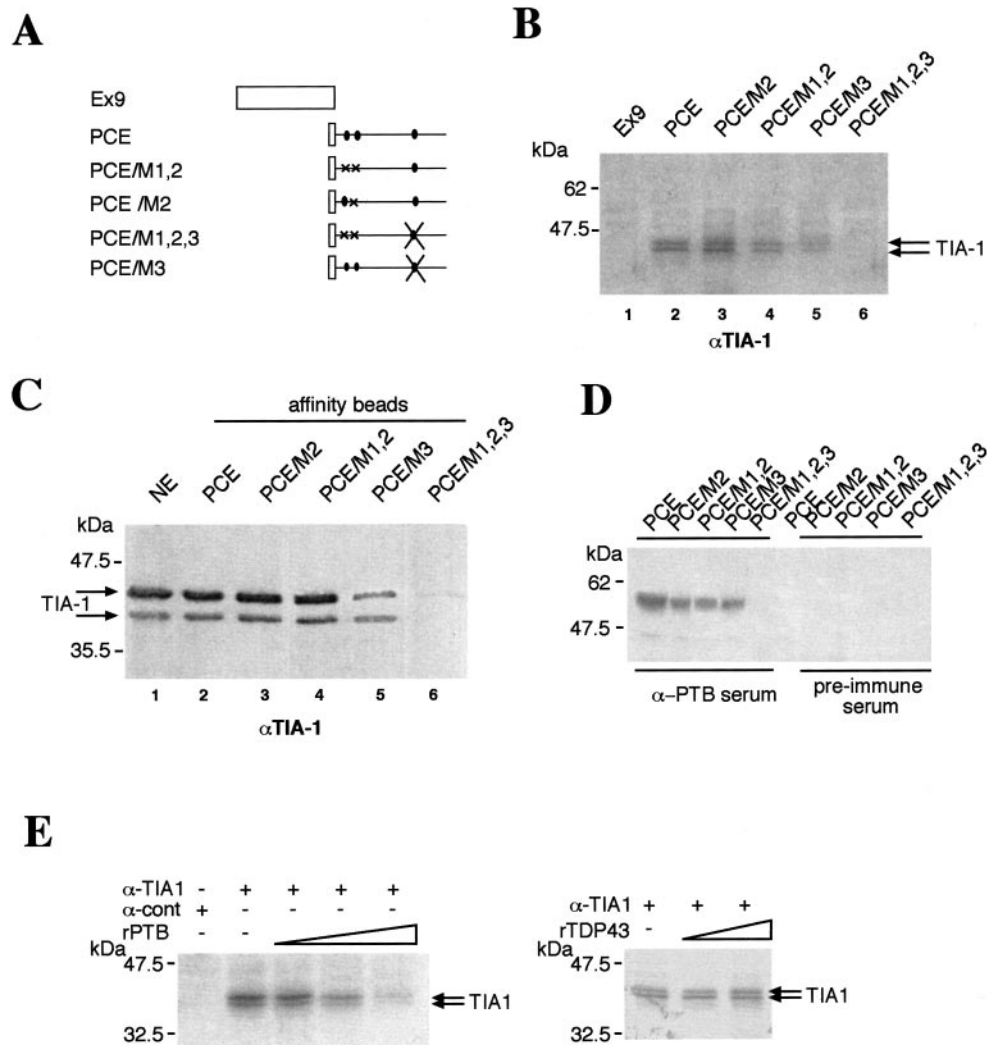
FIG. 3. **TIA-1 binding to the PCE.** *A*, schematic representation of the RNA probes used in *in vitro* protein binding assays. PY elements (*black ovals*) and their mutations (×) are indicated. *Ex9*, exon 9. *B*, immunoprecipitation experiment using anti-TIA-1 polyclonal antibody with the labeled RNA probes previously UV-cross-linked with 30 μg of HeLa nuclear extract. A control unrelated antibody did not show any immunoprecipitated material (not shown). *C*, pull-down assay using adipic acid dihydrazide beads derivatized with the RNA probes (indicated on the top of the gel) following incubation with HeLa nuclear extract (*NE*). The samples were loaded onto a 10% SDS-polyacrylamide gel, Western-blotted, and analyzed with anti-TIA-1 polyclonal antibody. ECL was done after incubation with secondary antibody. *D*, immunoprecipitation experiment using anti-PTB polyclonal antibody with the labeled RNA probes UV-cross-linked with 30 μg of HeLa nuclear extract. As a negative control, immunoprecipitation was carried out using preimmune serum. Only the PCE/M1,2,3 probe was not able to bind PTB. *E*, immunoprecipitation assays performed on PCE RNA probes UV-cross-linked with 30 μg of HeLa nuclear extract in the presence of increasing amounts of recombinant PTB (*rPTB*) (*left panel*) or recombinant TDP43 (*rTDP43*) (10) as a control (*right panel*). The *arrows* on the right indicate the TIA-1 doublet.

tified so far that stimulates CFTR exon 9 inclusion. In fact, heterogeneous nuclear ribonucleoprotein A1, TDP43, PTB, and several other SR proteins induce CFTR exon 9 skipping (9, 10).

To evaluate the role of the multiple PY motifs, we studied the effect of TIA-1 and PTB overexpression using several mutant PCE minigenes (Fig. 2*C*). The enhancing effect of TIA-1 was observed in the case of the M1, M2, and M2A mutants and the double mutants M1,2 and M1,2A, whereas a lower response was observed with the M3 mutant (Fig. 2*C*). In contrast, TIA-1 overexpression did not induce exon 9 inclusion with the M1,2,3 minigene, in which all of the PY motifs of the PCE had been mutated (Fig. 2*C*). This indicates that the effect of TIA-1 on CFTR exon 9 splicing requires mainly the contribution of PY3. However, the complete absence of any response to TIA-1 over-expression in the M1,2,3 minigene suggests the possible additional contribution of the other polypyrimidine-rich sequences of PCE. These results also establish that TIA-1 increases CFTR exon 9 inclusion in a PCE-dependent manner. In contrast, overexpression of PTB reduced exon 9 inclusion with all of the

minigenes (Fig. 2*D*). The percentage of exon 9 inclusion was ~33–35% with the wild-type, M1, and M3 minigenes and was almost 10–12% with the other mutants. Interestingly, the M1,2,3 minigene was sensitive to PTB overexpression, and the level of exon 9 inclusion was reduced to ~4% (Fig. 2*D*). These results are in agreement with the general splicing inhibitory role of PTB, mediated by binding to multiple exonic and intronic sequences (18, 19, 27).

TIA-1 and PTB Binding to the PCE—To investigate the ability of TIA-1 and PTB to bind to the PCE in intron 9, different *in vitro* transcribed, [³²P]UTP-labeled RNA probes (Fig. 3*A*) were incubated with HeLa nuclear extracts, followed by UV cross-linking. The samples were then immunoprecipitated with specific anti-TIA-1 or anti-PTB polyclonal antibodies. The RNAs used correspond to the last 10 bases of exon 9 and extend down to the ISS element in intron 9.

The anti-TIA-1 antibody immunoprecipitated a double band at 40–44 kDa when the wild-type PCE RNA was used (Fig. 3*B*, *lane 2*). This doublet corresponds to the previously reported

isoforms of this splicing factor (28, 29). The TIA-1 bands were completely absent with the PCE/M1,2,3 RNA, in which all three PY motifs were disrupted. A reduced amount of immunoprecipitate was found with the PCE/M1,2 and PCE/M3 RNAs (Fig. 3*C*, *lanes 4* and *5*). Instead, the CFTR exon 9 did not show any TIA-1 binding (Fig. 3*B*, *lane 1*). To validate the results obtained in the immunoprecipitation experiments, we performed RNA pull-down assays. As expected, a TIA-1 doublet was specifically pulled down by the wild-type PCE RNA, but not by the PCE/M1,2,3 probe (Fig. 3*B*, *lanes 2* and *6*). PCE/M3 showed reduced TIA-1 binding, whereas other RNAs did not show any difference in comparison with the wild-type probe. The reduced TIA-1 binding to M3 is in agreement with the low splicing enhancing effect of TIA-1 overexpression with the M3 minigene (Fig. 2*C*). The PCE/M1,2 RNA probe gave bands of different intensity in pull-down and immunoprecipitation experiments (compare lanes for PCE/M1,2 in Fig. 3, *B* and *C*). This discrepancy could be due to smaller amounts of ^{32}P-labeled RNA protected after RNase digestion in the case of UV cross-linking. In fact, the M1,2 RNA contains fewer uridines at the TIA-1-binding site compared with the PCE RNA. This result suggests that PY1 does not contribute significantly to TIA-1 binding.

The anti-PTB antibody immunoprecipitated a characteristic doublet at ~60 kDa when the wild-type PCE RNA was used (Fig. 3*D*). In contrast, there was no immunoprecipitated material with the PCE/M1,2,3 probe. The PTB doublet was also evident with the PCE/M1,2 and PCE/M3 RNAs (Fig. 3*D*), indicating that PTB binding is mediated by all of the PY motifs in the PCE. The facts that both TIA-1 and PTB bound the PCE and that these splicing factors had antagonistic activity in our hybrid minigene experiments suggest a possible competition between them. To test this hypothesis, we performed immunoprecipitation experiments using radiolabeled PCE RNA. This RNA was incubated with a combination of nuclear extract and increasing amounts of recombinant PTB, subjected to UV cross-linking, and then immunoprecipitated with anti-TIA-1 polyclonal antibody. Fig. 3*E* (*left panel*) shows that an increase in the amount of recombinant PTB resulted in a proportional reduction of TIA-1 binding. In a control experiment, the unrelated recombinant RNA-binding protein TDP43 (10) did not affect PTB binding (Fig. 3*E*, *right panel*). Taken together, these results indicate that CFTR intron 9 sequences close to the exon 9/intron 9 junction bind both TIA-1 and PTB and that the three PY elements in the PCE mediate this binding. In addition, the antagonistic activity of PTB in relation to TIA-1 binding to the PCE might be one of the factors involved in the splicing inhibition mediated by PTB.

TIA-1 Activates a Cryptic 3′-ss in the Presence of a Suboptimal Acceptor Site—Previous studies have shown the activation of a cryptic 3′-ss in the presence of a high number of TG repeats and/or a low number of T repeats at the polymorphic locus of CFTR exon 9 (9, 10). To analyze whether TIA-1 affects cryptic 3′-ss selection, we performed cotransfection experiments with minigenes containing different TGmTn repeats (Fig. 4). With TG11T7 and TG11T5, overexpression of TIA-1 resulted in an increase in the exon 9+ form, but had no effect on the usage of the cryptic 3′-ss. In fact, the percentage of the spliced form with the cryptic site was <5% with respect to the other spliced variants and was not significantly affected by TIA-1. In contrast, in TG13T5 and TG13T3, TIA-1 induced an increase in not only the exon 9+ form, but also the cryptic site variant (Fig. 4). In fact, this splicing factor increased the percentage of the cryptic site from 9 to 27% with TG13T5 and from 20 to 35% with TG13T3 (Fig. 4). Thus, TIA-1 has a double effect on CFTR exon 9 alternative splicing: it increases exon inclusion and, in
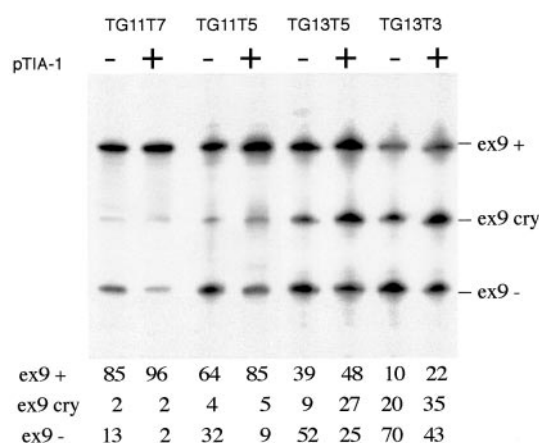

	TG11T7		TG11T5		TG13T5		TG13T3	
pTIA-1	−	+	−	+	−	+	−	+
ex9 +	85	96	64	85	39	48	10	22
ex9 cry	2	2	4	5	9	27	20	35
ex9 −	13	2	32	9	52	25	70	43

FIG. 4. **Effect of TIA-1 on the activation of the cryptic exonic 3′-ss in relation to the composition of TGmTn.** The TG11T7, TG11T5, TG13T5, and TG13T3 minigenes were transfected with 1 μg of control empty vector (−) or pTIA-1 expression vector (+) and amplified with the α2 and B2 primers, and the resulting radioactive splicing variants were analyzed on 6% polyacrylamide gel. The *numbers* below each lane are the percentages of the three different forms detected. Data are the means of two independent experiments done in duplicate. *ex9 +*, exon 9 inclusion; *ex9 cry*, exon 9 cryptic 3′-ss inclusion; *ex9 −*, exclusion.

the presence of a suboptimal acceptor site caused by an unfavorable composition of the TGmTn locus, activates a cryptic 3′-ss. The selection of an alternative 3′-ss mediated by TIA-1 has been observed previously (14). We also analyzed the effect of PTB on the cryptic 3′-ss, and no changes in the selection of this alternative 3′-ss were observed (data not shown).

Selection of the Cryptic 3′-ss by TIA-1 Occurs Independently of Its Binding to the PCE and of Recruitment of U1 Small Nuclear RNA (snRNA) to the 5′-ss—TIA-1 binding to intronic elements facilitates the recruitment of U1 snRNP to a nearby weak 5′-ss (14, 26). The CFTR exon 9 donor site differs from the consensus site mainly because of a substitution of the conserved guanidine with a uridine at position +5 (Fig. 5*A*) (30). Interestingly, the same substitution is present in the 5′-ss of the *msl-2* gene, whose alternative splicing is regulated by TIA-1 binding to U-rich stretches downstream of the 5′-ss (Fig. 5*A*) (14). To study the functional relationship between TIA-1 binding to the PCE and U1 snRNP recruitment, we performed complementation experiments using a U1 snRNA variant (Fig. 5*A*). This variant (U1-T5) is complementary to the weak CFTR intron 9 5′-ss. TG13T5 minigenes with or without a mutant PCE were cotransfected with TIA-1, U1-T5, or both splicing factors, and the resulting cDNAs were analyzed for the percentage of inclusion of the entire exon (Fig. 5*B*). As shown in Fig. 5*B*, cotransfection of U1-T5 increased the percentage of exon 9 inclusion with both minigenes. In contrast, TIA-1 induced exon inclusion only with TG13T5. Interestingly, for each minigene, the levels of exon 9 inclusion were not affected when U1-T5 and TIA-1 were cotransfected alone or in combination (Fig. 5*B*). This functional observation is consistent with the role of TIA-1 binding to the PCE as a splicing enhancement mediator through recruitment of U1 snRNP to the weak 5′-ss.

In a second set of experiments, we evaluated whether the U1 snRNP recruitment mediated by TIA-1 binding to the PCE might be also involved in the alternative selection of the 3′-splice sites. In this case, the cDNAs were amplified with primers that can detect selectively only cryptic and normal 3′-ss usage (Fig. 5*C*). Unexpectedly, with both the normal TG13T5 and PCE-deleted TG13T5/M1,2,3 minigenes, U1-T5 increased the percentage of exon 9 inclusion, but not that of cryptic 3′-ss inclusion. This indicates that 3′-ss alternative selection does
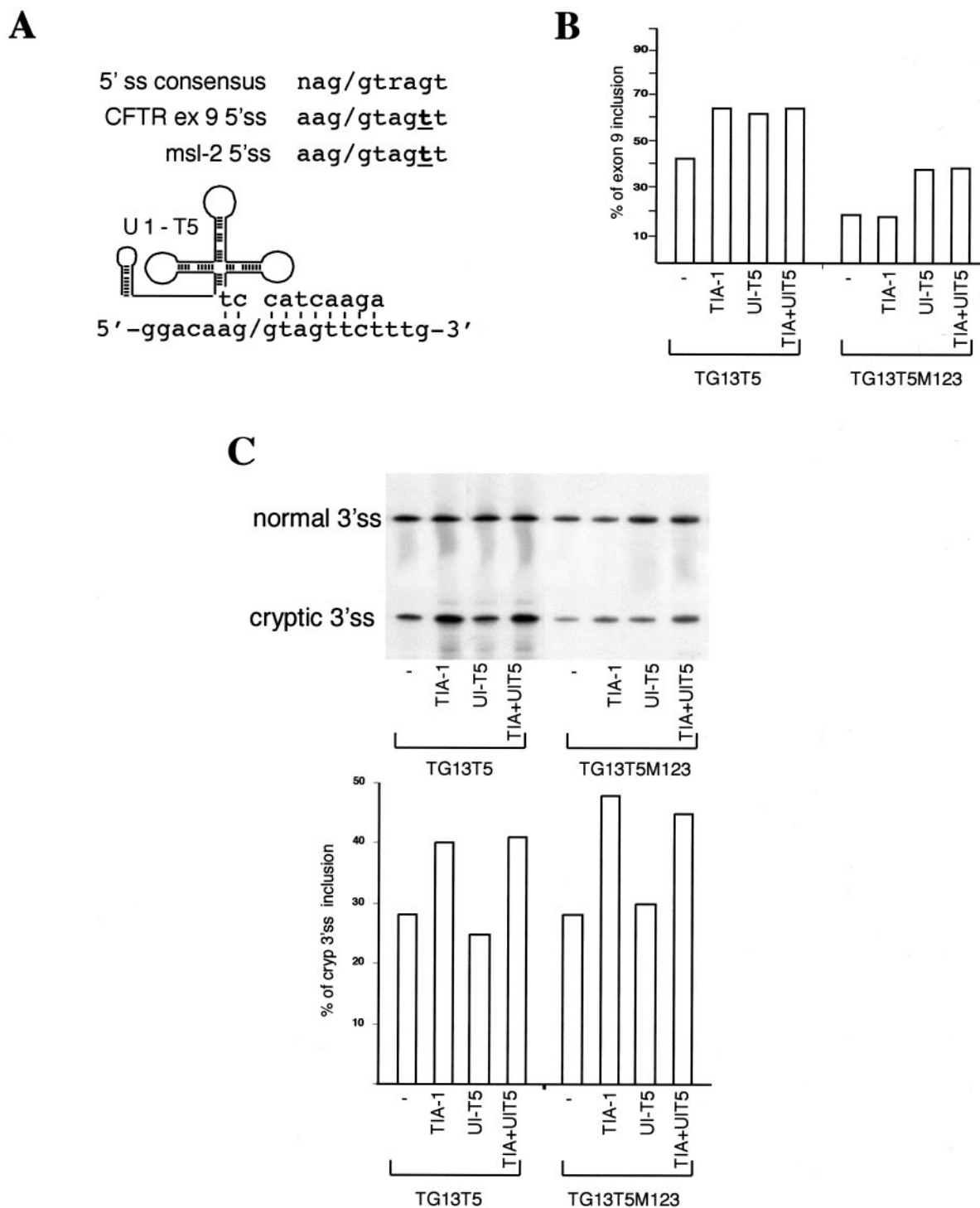
A

5' ss consensus	nag/gtragt
CFTR ex 9 5'ss	aag/gtag**t**t
msl-2 5'ss	aag/gtag**t**t

U 1 - T5

tc catcaagaa

5'-ggacaag/gtagttctttg-3'

B

C

normal 3'ss

cryptic 3'ss

FIG. 5. **Functional relationship between TIA-1 and U1 snRNA overexpression.** *A*, shown is a comparison of the CFTR exon 9 (*ex 9*), *msl-2*, and consensus 5'-splice sites. The schematic representation shows the restored base pair homology between the weak 5'-ss of CFTR exon 9 and U1-T5 snRNA. *B*, the TG13T5 and TG13T5/M1,2,3 minigenes were cotransfected with 1 μg of control empty vector (−) or with 1 μg of pTIA-1 expression vector (*TIA-1*) in the absence or presence of U1-T5 snRNA (500 ng). The histogram shows the percentage of exon 9 inclusion *versus* exon 9 exclusion detected by 6% polyacrylamide gel analysis of radioactive PCR products amplified with the α2 and B2 primers and quantitated using a Cyclone. Data are the means of two independent experiments done in duplicate. *C*, the same transfections as described for *B* were amplified with the α2 and cry2 primers. Shown are the results from autoradiography of radioactive PCR products on a 6% polyacrylamide gel. The histogram shows the percentage cryptic (*cryp*) 3'-splice sites. Data are expressed are the means of two independent experiments done in duplicate.

not depend on the recruitment of U1 snRNP to the weak 5'-ss. In contrast, TIA-1 produced an increase in cryptic 3'-ss usage in the two minigenes. This evidence indicates that TIA-1 binding to the PCE induces CFTR exon 9 inclusion, but not the selection of the cryptic 3'-ss. This observation establishes that the recruitment of U1 snRNA to the weak 5'-ss mediated by TIA-1 activates exon 9 inclusion, but not the alternative selec-

tion of the 3'-splice sites. In control experiments, normal U1 snRNA had no effect on the splicing efficiency (data not shown).

The Polymorphic TGmTn Tract Mediates the Activation of the Cryptic 3'-ss Induced by TIA-1—The experiments shown in Fig. 4 indicate that TIA-1 preferentially induced cryptic 3'-ss selection in the TG13T5 and TG13T3 minigenes. In addition, RNA binding experiments using the entire exon 9 did not

FIG. 6. **Role of the TG and T repeats in the TIA-1-mediated alternative selection of the 3′-splice sites.** The ΔTG and ΔT minigenes were cotransfected in Hep3B cells with 1 μg of pTIA-1 expression vector (*TIA-1*) or empty vector (−). *A*, autoradiography of normal and cryptic 3′-splice sites derived from RT-PCR amplification with the α2 and cry2 primers. *B*, histogram showing the percentage exon 9 inclusion derived from the analysis of exon 9+ and exon 9− after amplification with the α2 and B2 primers.
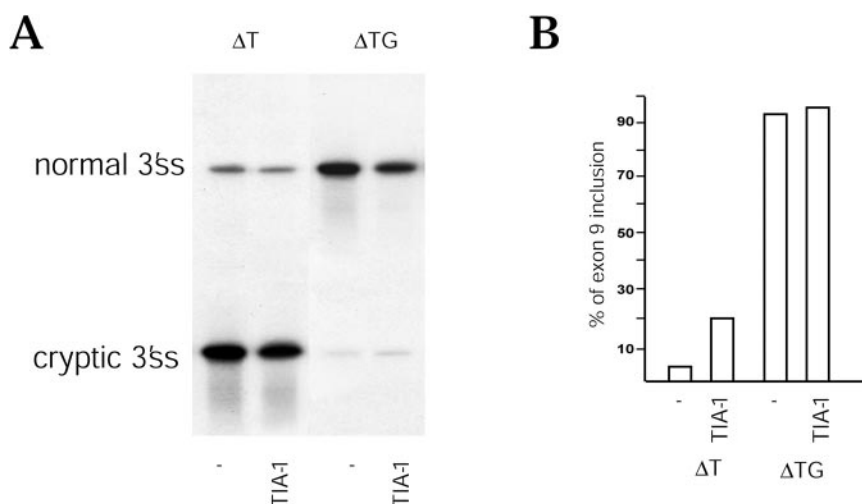
detect any TIA-1 binding (Fig. 3*B*). These observations suggest that the mechanism involved in the selection of the cryptic 3′-ss is not due to exon 9 or intron 9 sequences, but to the conformation of TGmTn in the normal acceptor site. To clarify the mechanism involved in the alternative selection of the 3′-splice sites mediated by TIA-1, we performed cotransfection experiments with minigenes in which the entire TG or T tract had been deleted (ΔGT and ΔT, respectively). With both minigenes, analysis of the splicing assays showed that TIA-1 did not induce preferential usage of the cryptic 3′-ss (Fig. 6*A*). As expected, TIA-1 increased the percentage of the exon 9+ form with the ΔT minigene, which contains the wild-type PCE (Fig. 6*B*). The TIA-1 enhancing effect could not be observed with the ΔGT minigene because exon 9 was included in the mature mRNA even without cotransfection with the splicing factor (Fig. 6*B*). This result indicates that the unusual presence of the TG and T tracts in the normal acceptor site is necessary in the alternative selection of the 3′-splice sites mediated by TIA-1.

DISCUSSION

In this study, we have evaluated the role of the PCE near the weak 5′-ss of CFTR exon 9 and the effect of two polypyrimidine-binding splicing factors, TIA-1 and PTB. This study was prompted by the fact that TIA-1 is the only splicing factor identified with a positive effect on CFTR exon 9 splicing. The effect of TIA-1 on CFTR exon 9 alternative splicing is complex. TIA-1 binding to the PCE recruits U1 snRNP to the weak 5′-ss and causes exon inclusion. In addition, TIA-1 induces alternative usage of a cryptic 3′-ss by interfering with the unusual sequences of the normal acceptor site. This alternative choice of 3′-splice sites, based on the composition of the polymorphic TGmTn sequences present in the normal 3′-ss, constitutes a new splicing regulatory function of TIA-1. On the other hand, PTB may antagonize TIA-1 binding to the PCE; and in this manner, it might negatively regulate the efficiency of CFTR exon 9 alternative splicing.

Complementation experiments with modified U1 snRNA showed that recognition of the weak 5′-ss represents an additional level of regulation of aberrant CFTR exon 9 splicing (Fig. 5*A*). Recruitment of U1 snRNP to the exon 9 5′-ss can occur not only by base complementarity with a modified U1 snRNA, but also through binding of TIA-1 to the downstream PCE. In these last years, several studies have shown that recruitment of U1 snRNP to a class of weak 5′-splice sites is promoted by binding of TIA-1 to U-rich sequences immediately downstream of the 5′-splice sites (14, 26, 31). Interaction of TIA-1 with the U1-C protein has been reported to mediate U1 snRNP recruitment to the 5′-ss (15). We have shown here that, when modified U1

snRNA was cotransfected, TIA-1 did not induce further splicing enhancement (Fig. 5*B*). This suggests, as previously reported for the yeast homolog Nam8p (32), that TIA-1 is required for efficient recognition of the weak 5′-ss, but is not essential in the presence of the consensus 5′-ss and/or when, as described here, U1 snRNP is recruited by complementarity.

The majority of the previous studies analyzed the effect of TIA-1 on alternative splicing using multiple substitution mutants that present complete disruption of U-rich stretches near the 5′-splice sites (14, 26). Likewise, we observed that the CFTR mutant with the three polypyrimidine sequences disrupted did not bind to TIA-1, showed the highest level of exon 9 skipping in minigene experiments, and did not respond to TIA-1 overexpression. Each of the PY elements contributes in a different manner to splicing regulation. PY1, which is very close to the 5′-ss, has a minor role, as its absence did not significantly affect TIA-1 stimulation of exon inclusion (Fig. 2*C*) or TIA-1 binding (Fig. 3*C*). In contrast, PY2 was the most active element under basal conditions (Fig. 1*C*), whereas PY3 appeared to be the most important element, mediating TIA-1 stimulation of exon inclusion (Fig. 2*C*) and binding (Fig. 3*C*). However, as PY2 alone did not contribute significantly to TIA-1-mediated regulation (Fig. 2*C*), its effect under basal conditions could be related to RNA secondary structures or binding to other splicing factors. Conformational changes induced by PY2 mutants might affect accessibility of the upstream 5′-ss to U1 snRNP, as previously reported for other gene systems (33). More detailed site-directed mutagenesis experiments and RNA secondary structure data are necessary to better clarify the role of PY2 in CFTR exon 9 splicing. The effect of TIA-1 on 5′-ss usage has been related to polypyrimidine sequences located very close to that site. However, activation of donor site usage can occur when U1 snRNP binds farther downstream of the 5′-ss (34). It is possible that, in CFTR intron 9, the TIA-1-mediated recruitment of U1 snRNP to the distal PY sequences may also contribute to 5′-ss selection.

Binding of TIA-1 to the U-rich stretch near the 5′-ss and the subsequent recruitment of U1 snRNP have been found to be affected by antagonistic splicing factors. In *msl-2*, the *Drosophila* regulator Sex-lethal (SLX) interferes with the binding of TIA-1 near the 5′-ss and, in this manner, prevents the U1 snRNP-mediated recognition of the 5′-ss (35). In *in vitro* experiments with CFTR exon 9, PTB competed with TIA-1 for binding to the PCE (Fig. 3*E*). This result suggests that one of the mechanisms by which PTB induces CFTR exon 9 skipping might be through antagonizing the binding of TIA-1 to the PCE. However, as the minigene with mutations in the PCE

responded to PTB overexpression, additional mechanisms must be necessarily involved. Altogether, the mechanism by which PTB regulates alternative splicing is still unclear (reviewed in Ref. 16). One of the proposed models suggests that PTB-mediated exon silencing depends on the ability of PTB to multimerize across the exon, masking in such a way the exon definition (16). The binding of PTB at only one of the intron sides may result in exon silencing due to its ability to multimerize (16). Consistent with this hypothesis, the CFTR exon 9 splicing inhibition induced by PTB could be due to its binding to multiple sites, *i.e.* not only to the PCE, but also to other intronic or exonic sequences (Fig. 3*D*). Indeed, we found that this splicing factor bound to CFTR exon 9 (data not shown). Additional studies are necessary to analyze the combined role of these two antagonistic polypyrimidine-binding splicing factors in our system.

The effect of TIA-1 on the selection of the cryptic 3′-ss of CFTR exon 9 was largely unexpected (Fig. 4). In the case of *fas* alternative splicing, binding of TIA-1 to the 5′-ss region of intron 5 induces exon 6 inclusion at the expense of transcripts in which exon 6 is skipped, suggesting that TIA-1 modulates the choice between 3′-splice sites (35). In the context of our CFTR exon 9 minigene, the 5′-ss region of the upstream exon does not contain polypyrimidine-rich sequences; therefore, TIA-1 cannot bind to this region and, as a result, selects the cryptic 3′-ss. Our results also exclude that TIA-1 binding to the PCE and U1 snRNP recruitment to the 5′-ss of exon 9 are involved in the alternative selection of the 3′-ss. In fact, these two interactions, separately or together, activated exon 9 inclusion, but not the cryptic 3′-ss inclusion (Fig. 5). In contrast, evidence indicates that the polymorphic TGmTn locus at the 3′-end of CFTR intron 8 is involved. We have shown previously that high numbers of TG repeats and/or low numbers of T repeats activate the cryptic 3′-ss and that UG repeats bind the inhibitory splicing factor TDP43 (9, 10). We found here that the same TGmTn composition is necessary for the TIA-1-mediated activation of the cryptic 3′-ss (Fig. 5). An intriguing possibility is that TIA-1 could interact directly with TDP43 bound to the UG sequences. Alternatively, it might interfere with the binding of general splicing factors that recognize the 3′-ss, such as U2AF or the U2 snRNP complex. This will result in masking of the normal 3′-ss and preferential selection of the downstream cryptic acceptor site. In minigenes in which either the TG or T repeat is deleted, the lack of activation of the cryptic 3′-ss by TIA-1 (Fig. 6) is consistent with a complex mechanism in which both TDP43 and basic splicing complexes are involved. Indeed, we detected TIA-1 binding to the last 250 nucleotides of intron 8 (data not shown). Additional experiments are necessary to clarify the sequence specificity of this binding and its effect on the multiple interactions occurring at the CFTR intron 8 3′-ss.

TIA-1 is the only splicing factor known so far to enhance CFTR exon 9 recognition. SR proteins, which commonly have an enhancing effect, instead induce CFTR exon 9 skipping by interacting with the ISS in intron 9 (9). PTB (as shown in this work) and heterogeneous nuclear ribonucleoprotein A1 and TDP43 (9, 10) induce exon 9 skipping. In non-classical forms of CF, the phenotypic variability has been associated with a variable proportion of aberrant CFTR exon 9 skipping (3–7). The positive effect of TIA-1 on CFTR exon 9 inclusion will result in

a protecting role of this splicing factor regarding the development of the disease. The enhancing role of TIA-1 represents an additional factor that regulates the disease-associated aberrant alternative splicing of exon 9.

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**An Intronic Polypyrimidine-rich Element Downstream of the Donor Site Modulates
Cystic Fibrosis Transmembrane Conductance Regulator Exon 9 Alternative Splicing**

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