TIA-1 and TIAR Activate Splicing of Alternative Exons with Weak 5′ Splice Sites followed by a U-rich Stretch on Their Own Pre-mRNAs*

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TIA-1 has recently been shown to activate splicing of specific pre-mRNAs transcribed from transiently transfected minigenes, and of some 5′ splice sites in vitro, but has not been shown to activate splicing of any endogenous pre-mRNA. We show here that overexpression of TIA-1 or the related protein TIAR has little effect on splicing of several endogenous pre-mRNAs containing alternative exons, but markedly activates splicing of some normally rarely used alternative exons on the TIA-1 and TIAR pre-mRNAs. These exons have weak 5′ splice sites followed by U-rich stretches. When the U-rich stretch following the 5′ splice site of a TIA-1 alternative exon was deleted, TIAR overexpression induced use of a cryptic 5′ splice site also followed by a U-rich stretch in place of the original splice site. Using in vitro splicing assays, we have shown that TIA-1 is directly involved in activating the 5′ splice sites of the TIAR alternative exons. Activation requires a downstream U-rich stretch of at least 10 residues. Our results confirm that TIA-1 activates 5′ splice sites followed by U-rich sequences and show that TIAR exerts a similar activity. They suggest that both proteins may autoregulate their expression at the level of splicing.

Many eucaryotic genes are transcribed to yield pre-mRNAs containing exon and intron sequences. The process of splicing then eliminates the introns and joins adjacent exons together (1). This requires precise recognition of exons and introns on the pre-mRNA, but it is not yet clear how this is achieved. Splice sites marking exon-intron junctions have specific sequence characteristics necessary for recruiting some spliceosome components: U1 snRNP at the 5′ splice site (5′ss)1 and U2AF and U2 snRNP close to the 3′ splice site (3′ss), for example (for reviews, see Refs. 1–3). However, additional proteins acting through exon or intron sequences distinct from the splice sites are often required for correct splice site use, particularly, but not only, in alternative splicing (for reviews, see Refs. 4–7). For example, splicing of the FGFR-2 gene alternative exon K-SAM (or IIIb) is repressed by an exon splicing silencer and by upstream intron sequences (8), but activated by three sequences in the downstream intron (9–14). One of these sequences, IAS1, is U-rich and lies immediately downstream from the exon’s 5′ss, suggesting that the protein that binds to it could interact directly with U1 snRNP bound to the adjacent 5′ss.

Nam8p is one of several yeast U1 snRNP proteins that have no counterparts in mammalian U1 snRNP (15). Nam8p binds to nonconserved sequences downstream from 5′ss in yeast commitment complexes (16, 17). This interaction can be important for use of some 5′ss and is most effective if the downstream sequences are U-rich (16). These observations led to the suggestion that a mammalian protein functionally equivalent to Nam8p might activate weak 5′ss followed by U-rich sequences, such as the 5′ss of the K-SAM exon. The closest mammalian relatives of Nam8p are a pair of related proteins, TIA-1 (18) and TIAR (19), which may be involved in apoptosis (20, 21). TIA-1 and TIAR have been shown to participate in translational control (22–25). However, these proteins are mainly nuclear, consistent with an additional role in splicing.

We (11) and others (26) have recently shown that TIA-1 is a splicing regulator. Splicing of the Drosophila melanogaster msl-2 pre-mRNA using a particular 5′ss followed by a U-rich sequence requires TIA-1 in vitro, to promote binding of U1 snRNP (26). In transient transfection assays, increasing TIA-1 levels in cells stimulates use of the FGFR-2 K-SAM exon 5′ss, and this depends on the U-rich IAS1 motif (11), while decreasing TIA-1 levels change the 3′ss chosen for splicing to the 5′ss of the fas gene exon 5, which is followed by a U-rich sequence (26). These experiments linked TIA-1 to splicing. This was an important finding, as until recently, only one other non-SR protein capable of positively influencing alternative splicing, the CUG-binding protein (27), was known.

Relatively little is known about the recently discovered splicing regulators like TIA-1. One important question remaining to be answered is: can TIA-1 activate splicing of pre-mRNA transcribed from a normal, endogenous, chromosome-carried gene? This is not a trivial question, for a number of reasons. It is becoming increasingly clear that splicing and transcription may not be completely independent processes. Splicing factors may be delivered to splice sites during transcription (28), and promoter swapping experiments (29) have shown that splicing of an exon can depend on the promoter used for its transcription. The C-terminal domain of RNA polymerase II could even help exon definition (30). This transcriptional link could clearly be important for splicing regulation, but might not be taken adequately into account in in vitro splicing tests nor in tran-

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1 The abbreviations used are: ss, splice site(s); RT-PCR, reverse transcription-polymerase chain reaction; RRM, RNA recognition motif.
sient transfection assays using only portions of genes under control of heterologous promoters. Characterizing the effect of TIA-1 on splicing of endogenous pre-mRNAs is thus important.

Another point to be cleared up concerns the sequence motifs through which TIA-1 can act. The mammalian K-SAM exon and fas exon 5 5'ss identified as possible targets for TIA-1 are followed by U-rich sequences. However, these particular splice sites were chosen to be tested for the effect of TIA-1 precisely because they were followed by U-rich sequences. This choice was motivated by the similarity of TIA-1 to Nam8p and the identification of sequences containing runs of U as good TIA-1 binding sites by the SELEX approach (31). It is quite possible, however, that TIA-1 will activate splicing through other sequence motifs. A sequence-independent approach to searching for 5'ss activable by TIA-1 was needed to investigate this possibility.

We set out to address the points described above by testing the effect of increasing the levels of TIA-1 on the splicing of a number of endogenous pre-mRNAs. As there was no data available on the possible involvement in splicing of the TIA-1-related protein TIAR, we tested TIA-1 and TIAR in parallel. While neither protein has any significant effect on splicing of several endogenous pre-mRNAs tested, both proteins exert a similar effect on splicing of TIA-1 and TIAR pre-mRNAs, activating specifically the splicing of novel TIA-1 and TIAR gene alternative exons. All the exons have 5'ss which are followed by U-rich sequences. Our results thus validate previous in vitro and transient transfection work on TIA-1, as well as identifying TIAR as a similar splicing regulator. In as far as splicing of the novel exons leads to mRNAs with premature termination codons, our results also suggest that the TIA-1 and TIAR genes are under negative feedback regulation.

EXPERIMENTAL PROCEDURES

Transfections and RT-PCR—Calcium phosphate transfection of 293-EBNA cells (Invitrogen) was as described previously (11, 12). For selection of transfected cells, a Capture-Tec kit (Invitrogen) was used according to manufacturer’s instructions. Briefly, cells were cotransfected with pHook-1 (10 μg) and an expression vector (10 μg). The expression vector pCI-neo was from Promega. The mouse TIA-1 expression vector pTIA-1 and the hnRNP C1 expression vector phnRNP C1 were as described previously (11). 48 h later, cells expressing the cell surface-displayed, single-chain antibody encoded by pHook-1 were selected using hapten-coated magnetic beads. RNA was harvested from them and analyzed by RT-PCR using gene-specific primers as described previously (12).

CD44 primers were 5’-ctctacggaaggttctcagtc-3’ and 5’-tcagtcgtaatgcc-3’. FGFR-2 primers 1–7 were 5’-tcactctgctgggtg-3’ and 5’-aacctga-gttggcag-3’. FGFR-2 primers 7–8 were 5’-atctcgagttggcag-3’ and 5’-gcaatgtaggtaag-3’. cd44 exons were 5’-tcagacagccagcgc-3’ and 5’-cctggttcctgatgtttcgc-3’. caspase-2 primers were 5’-tgagagctgtgtgtgtagtcc-3’ and 5’-gcggccgtctcgctattcgcg-3’. CD44 primers were 5’-ggagacgtggaagcctgtttatttac-3’ and 5’-gctggagcttctctctcttctctctctctctc-3’. CD44 primers were 5’-gagccagcgcggttggcgc-3’ and 5’-ggtgctggtgtggtgtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtg
TIA-1 Overexpression Does Not Alter Splicing of Several Endogenous Pre-mRNAs—We wanted to determine whether TIA-1 overexpression has an effect on the splicing of endogenous pre-mRNAs. To this end, we cotransfected 293-EBNA cells with a TIA-1 expression vector (pTIA-1) and pHook-1, an expression vector for a cell surface-displayed, single-chain antibody. pTIA-1-transfected cells have an ~10-fold overexpression of TIA-1 (data not shown) and can be recovered using hapten-coated magnetic beads. For control experiments, we replaced pTIA-1 either by the expression vector phnRNP C, which codes for hnRNP C1, another protein binding to U-rich sequences, or by the empty expression vector pCI-neo. RNA from recovered cells was analyzed by RT-PCR, using primers from several different genes whose pre-mRNAs are known to undergo alternative splicing.

The FGFR-2 gene contains several alternative exons, some of which are shown in Fig. 1. The K-SAM (S) and BEK (B) exons are mutually exclusive exons. 293-EBNA cells normally splice the BEK exon (11) between exons 7 and 8. We have shown previously (11) that in 293-EBNA cells TIA-1 overexpression, but not hnRNP C overexpression, activated splicing of the FGFR-2 gene K-SAM exon in pre-mRNA from a transiently transfected minigene. This led to splicing of the K-SAM exon to the BEK exon. As expected, in cells transfected with pCI-neo or the hnRNP C expression vector, the endogenous pre-mRNA was spliced using the BEK exon: the RT-PCR products obtained using primer pair 7-8 (Fig. 2, lanes 1–3 for pCI-neo and lanes 4–6 for phnRNP C) corresponded to the R2-B product whose structure is shown in Fig. 1. The BEK and K-SAM exons can be distinguished by restriction enzyme mapping: the BEK exon contains an HpaI site (H in Fig. 1), while the K-SAM exon contains an AvaI site (A in Fig. 1). In cells transfected with the TIA-1 expression vector, a very small amount of product R2-SB (Fig. 1) corresponding to splicing of the K-SAM exon to the BEK exon was observed (Fig. 2, lane 7) in addition to the R2-B product. Thus TIA-1 overexpression does not lead to significant activation of K-SAM exon splicing on the endogenous pre-mRNA, while such overexpression does activate K-SAM exon splicing on pre-mRNA from a transiently transfected minigene (see Fig. 8 in Ref. 11). While one possible explanation for this difference is that TIA-1 is not involved in splicing of the endogenous FGFR-2 pre-mRNA, there are other possible explanations. For example, K-SAM exon splicing is repressed in cells that normally splice the BEK exon (13). If this repression is more effective on pre-mRNA transcribed from the endogenous gene, TIA-1 overexpression could tip the balance in favor of K-SAM exon splicing from transiently transfected minigene pre-mRNA, but not from endogenous pre-mRNA.

Exons 3 and 4 of the FGFR-2 gene are optional exons. When primers in exons 1 and 7, as shown in Fig. 1, were used to test for splicing of exons 3 and 4, three RT-PCR products were obtained from cells transfected with the control vector pCI-neo (Fig. 2, lane 10). These products (for structures, see Fig. 1) are R2, representing splicing of exons 3 and 4; Δ3, representing skipping of exon 3; and Δ34, representing skipping of both exons 3 and 4. Neither hnRNP C nor TIA-1 overexpression had any significant effect on the relative amounts of these products (compare lanes 11 and 12 with lane 10).

The CD44 gene contains 10 alternative exons, marked v1–10 in Fig. 1 (35). In 293-EBNA cells, none of these are usually spliced, and the RT-PCR product (data not shown) obtained from untransfected cells corresponds to the H form of CD44, whose structure is shown in Fig. 1. The single RT-PCR product obtained from cells transfected with the control vector pCI-neo also corresponds to the H form (Fig. 2, lane 13). Neither hnRNP C nor TIA-1 overexpression had any significant effect on CD44 pre-mRNA splicing (compare lanes 14 and 15 with lane 13); inclusion of v exons would have resulted in the appearance of products larger than the H form.

In as far as TIA-1 has been linked to apoptosis (20, 21), it seemed worthwhile to analyze the effect of TIA-1 overexpression on splicing of some pre-mRNAs that code for proteins involved in apoptosis and that are known to undergo alternative splicing (36). The bcl-x gene exon 2 has two alternative 5′ss
Caspase-2, optional exon (39), allowing generation of two forms with significant effect on the isoform ratio (compare lanes 20 and 21). Overexpression of TIA-1 had no significant effect on the L/S ratio (compare lanes 21 and 22). These were indeed observed in cells transfected with pCI-neo or phnRNP C (Fig. 3A). RNA from cells transfected with pTIA-1 and selected as above was analyzed by RT-PCR using primers covering different parts of the TIAR sequence. With primers covering exons 2–8, two RT-PCR products are expected, corresponding to splicing (+3) or skipping (−3) of optional exon 3 (Fig. 3A). These were indeed observed in cells transfected with pCI-neo or phnRNP C (Fig. 3B, lanes 1 and 2, respectively; we have not identified the third, shorter product seen in lane 2). However, in cells transfected with pTIA-1, two pairs of additional products were observed (lane 3, marked α and β). They were identified by cloning and sequencing of the products, followed by comparison of the obtained sequences with that of the TIAR gene.

**TIA-1 Alternative Exons**—We also analyzed the effect of TIA-1 overexpression on splicing of the TIAR pre-mRNA. The TIAR gene contains 13 exons, one of which, exon 3 (21), is optional (Fig. 3A). RNA from cells transfected with pTIA-1 and selected as above was analyzed by RT-PCR using primers covering different parts of the TIAR sequence. With primers covering exons 2–8, two RT-PCR products are expected, corresponding to splicing (+3) or skipping (−3) of optional exon 3 (Fig. 3A). These were indeed observed in cells transfected with pCI-neo or phnRNP C (Fig. 3B, lanes 1 and 2, respectively; we have not identified the third, shorter product seen in lane 2). However, in cells transfected with pTIA-1, two pairs of additional products were observed (lane 3, marked α and β). They were identified by cloning and sequencing of the products, followed by comparison of the obtained sequences with that of the TIAR gene.

**TIA-1 Overexpression Leads to Inclusion of Novel TIAR and TIA-1 Overexpression Induces Splicing of Human TIAR Gene Novel Alternative Exons.** A, schematic representation of the TIAR gene. Constitutive exons are represented by empty boxes and optional exon 3 by a shaded box. Positions of primers used in the RT-PCR analyses shown in B are marked by arrows. For each primer pair, the predicted RT-PCR products from normal 293-EBNA cells are shown below the gene map. B, 293-EBNA cells were transfected and recovered as described in the legend to Fig. 2 and RT-PCR carried out on RNA purified from them using the TIA-1 gene primers shown in A. For each primer pair, products appearing in cells transfected with pCI-neo correspond to products shown in A. Products appearing specifically in pTIA-1-transfected cells are identified as α and β (lane 3) or γ (lane 9). C, origin of RT-PCR products α, β, and γ. Sequences of novel alternative exons are available on request.

**FIG. 3.** TIA-1 overexpression induces splicing of human TIAR gene novel alternative exons. A, schematic representation of the TIA-1 gene. Constitutive exons are represented by empty boxes and optional exon 5 by a shaded box. Positions of primers used in the RT-PCR analyses shown in B and C are marked by arrows. For each primer pair, the predicted RT-PCR products from normal 293-EBNA cells are shown below the gene map. B, 293-EBNA cells were transfected with pHook-1 and expression vectors as marked and transfected cells recovered. RT-PCR was carried out on RNA purified from them using the TIAR gene primers shown in A. For each primer pair, products appearing in cells transfected with pCI-neo correspond to products shown in A. Products appearing specifically in pTIA-1-transfected cells are identified as α (lane 6 in B and lane 4 in C), β (lane 9 in B and lane 6 in C), γ (lane 12 in B and lane 8 in C), or δ (lane 15 in B and lane 10 in C). D, origin of RT-PCR products α, β, and γ. Sequences of novel alternative exons are available on request.

**FIG. 4.** TIA-1 and TIAR overexpression induces splicing of human TIAR gene novel alternative exons. A, schematic representation of the TIAR gene. Constitutive exons are represented by empty boxes and optional exon 3 by a shaded box. Positions of primers used in the RT-PCR analyses shown in B and C are marked by arrows. For each primer pair, the predicted RT-PCR products from normal 293-EBNA cells are shown below the gene map. B, 293-EBNA cells were transfected with pHook-1 and expression vectors as marked and transfected cells recovered. RT-PCR was carried out on RNA purified from them using the TIAR gene primers shown in A. For each primer pair, products appearing in cells transfected with pCI-neo correspond to products shown in A. Products appearing specifically in pTIA-1-transfected cells are identified as α (lane 6 in B and lane 4 in C), β (lane 9 in B and lane 6 in C), γ (lane 12 in B and lane 8 in C), or δ (lane 15 in B and lane 10 in C). D, origin of RT-PCR products α, β, and γ. Sequences of novel alternative exons are available on request.
gene. The longer member of each pair was found to include optional exon 3, while the shorter member did not (no significant effect of TIA-1 overexpression on splicing of optional exon 3 was detected). In addition the “α” product pair includes two novel exons (6A and 6B, Fig. 3C) spliced between exons 6 and 7. In the other pair of products (β), the intron between exons 6A and 6B was retained.

With primers covering exons 8–11, no effect of TIA-1 or hnRNP C overexpression was detected (a single RT-PCR product was seen; Fig. 3B, lanes 4–6). With primers covering exons 11–13, one major RT-PCR product was obtained in cells transfected with pCI-neo (lane 7) or phnRNP C (lane 8), corresponding to splicing of exons 11, 12, and 13. Two minor products were also seen whose identities were elucidated by sequencing cloned fragments. One of these (marked by an asterisk in Fig. 3, A and B) corresponds to retention of the intron between exons 11 and 12. The other minor product in cells transfected with pCI-neo or phnRNP C became the major product in cells transfected with pTIA-1 (marked γ in Fig. 3B, lane 9). This product corresponds to splicing of a novel exon (11A) between exons 11 and 12 (Fig. 3C).

A similar analysis was carried out to investigate the effect of TIA-1 overexpression on splicing of the TIA-1 pre-mRNA. This analysis was possible as a mouse TIA-1 cDNA was used for overexpression, while the effect of the overexpression was tested on the human TIA-1 gene. The TIA-1 gene contains 13 exons, one of which, exon 5 (40), is optional, but spliced efficiently in 293-EBNA cells (Fig. 4A). RNA from cells overexpressing TIA-1 was analyzed by RT-PCR using primers covering different parts of the TIA-1 sequence. No effect of TIA-1 overexpression was detected using primers covering exons 1–4 (compare lanes 1–3, Fig. 4B). However, an additional RT-PCR product was observed in cells transfected with pTIA-1 relative to those transfected with pCI-neo or phnRNP C using either (i) primers covering exons 4–6 (product a, compare lane 6 with lanes 4 and 5), (ii) primers covering exons 6–8 (product b, compare lane 9 with lanes 7 and 8); (iii) primers covering exons 8–11 (product c, compare lane 12 with lanes 10 and 11), or (iv) primers covering exons 11–13 (product d, compare lane 15 with lanes 13 and 14).

We also tested the effect of TIA-1 overexpression on splicing of the TIA-1 pre-mRNA. Cells transfected with a TIA-1 expression vector have an ~10-fold overexpression of TIA-1 (data not shown). As shown in Fig. 4C, TIA-1 overexpression has much the same effect as TIA-1 overexpression, inducing the appearance of the same four additional RT-PCR products a, b, c, and d. Cloning and sequencing of these products demonstrated that product a corresponds to the splicing of a novel exon (5A) between TIA-1 exons 5 and 6, product b to splicing of a novel exon (6A) between exons 6 and 7, and product c to use of an alternative downstream 5′ss for exon 8 (Fig. 4D). The identity of product d, present in lower amounts than products a, b, and c, was not investigated. In conclusion, overexpression of TIA-1 or TIAR induces very efficient inclusion of a variety of additional exons in the TIAR and TIA-1 mRNAs.

The 5′ss of Novel Exons Are followed by U-rich Stretches—The sequences surrounding the 5′ss of these novel exons are shown in Fig. 5A. There are two notable features: first, the 5′ss
are in general poor matches to the consensus 5'ss sequence. The weakest sites, au/guuggc (TIA-1 exon 6A), au/guacuu (TIA-1 exon 8A), and gg/guugu (TIAR exon 11A) contain no more than 2 or 3 consecutive residues matching the consensus 5'ss sequence. Second, they are followed by a pyrimidine-rich sequence (Fig. 5). While TIA-1 has been shown to act on splicing via U-rich sequences, no such information is available concerning TIAR. To test if U-rich sequences are also needed for TIAR action on splicing, we placed a fragment of the TIA-1 or TIAR gene containing sequences covering exons 6–7 (and their use is enhanced by TIAR overexpression (Fig. 5B, lane 5). As seen also for the original 5'ss, use of the cryptic sites is decreased by hnRNP C overexpression (lane 6). These observations create a strong link between a U-rich stretch downstream from a 5'ss and activation of the splice site by TIAR.

**TIA-1 Activates In Vitro Use of the Newly Identified 5'ss**—The experiments described above show that TIA-1 overexpression induces the appearance of TIA-1 and TIAR mRNAs which include novel exons. These exons all have 5'ss which are fol-
E1A pre-mRNA (Fig. 6A) indicates RNA resulting from premature termination of transcription shown in Fig. 6A, with the DTIAR corresponding to the TIAR alternative (H11032/exon 6B). The migration of input RNA is shown (pre-mRNA), as well as the 3′ss use. When splicing was carried out in a 6:4 mixture of S100 and nuclear extract enriched with SR proteins, use of the exon 11A 5′ss could be detected, and was enhanced by addition of TIA-1, while TIA-1 addition had no significant effect on use of the cryptic adenosiral splice site (Fig. 6C, compare lanes 1 and 2). With the 6:4 mixture, no use of the exon 6A 5′ss was seen in the absence of added TIA-1 (Fig. 6C, lane 3). However, weak use of the exon 6A 5′ss was observed in the presence of added TIA-1 (lane 4). Note that the exon 6A 5′ss is weaker than the exon 11A 5′ss and followed by a shorter pyrimidine-rich sequence (Fig. 5A). When splicing was carried out in S100 without added nuclear extract, use of both the exon 11A (compare lanes 5 and 6, Fig. 6C) and the exon 6A 5′ss (compare lanes 7 and 8, Fig. 6C) was fully dependent on TIA-1 addition.

The above results show that TIA-1 can activate use of the novel exons’ 5′ss in vitro. In vivo, we expect use of these 5′ss will also be facilitated by cross-exon bridging interactions with the 3′ss, and so even a modest additional activation of the 5′ss by TIA-1 could suffice to tip the balance in favor of exon splicing. This strongly suggests that TIA-1 overexpression leads to inclusion of novel TIA-1/TIAR exons in vivo by directly activating use of their 5′ss.

Efficient Activation by TIA-1 Requires a Stretch of More Than 7 Pyrimidines—Some of the 5′ss activated by TIA-1 overexpression are associated with very long pyrimidine-rich stretches (Fig. 5A). For example, the TIA exon 6B 5′ss is followed by a stretch of 39 nucleotides, including only 5 pyrimidines. We set out to determine the approximate sequence requirements for TIA-1 action at the TIA exon 6B 5′ss in vivo. We started from a construct coding for a truncated adenosiral E1A pre-mRNA (Fig. 6A) in which the TIA exon 6B 5′ss and downstream sequence (shown in Fig. 7A, WT) is placed downstream from the competing adenosiral 12S 5′ss D2. We removed increasing numbers of pyrimidines from the WT construct to obtain constructs Δ1, Δ2, and Δ3 (Fig. 7A). All these constructs were used in parallel for in vitro splicing assays in the presence or absence of added TIA-1 (Fig. 7B). For the WT, Δ1, and Δ2 pre-mRNAs, the TIA 5′ss was preferred to the D2 5′ss, and its use was enhanced by added TIA-1 (compare lanes 1 and 2 for WT, lanes 3 and 4 for Δ1, and lanes 5 and 6 for Δ2). In the Δ3 pre-mRNA, however, the dramatic decrease in the strength of the cis-acting U-rich sequence resulted in very effective competition for the common 3′ss by the D2 and the cryptic 5′ss. Thus the TIA exon 6B 5′ss was scarcely used in the absence of added TIA-1, although its use did increase slightly in the presence of added TIA-1 (compare lanes 7 and 8), probably because it can still bind weakly to the short pyrimidine-rich stretch, which remains in the Δ3 pre-mRNA, downstream from the 5′ss. These results suggest that TIA-1 requires more than 7, and less than 18, downstream pyrimidines (predominantly Us) to activate splicing from a 5′ss under the conditions of competition applying to the splicing substrates we have used. In other individual cases, it is clear that these requirements may vary slightly in function of the relative strength of the particular 5′ss concerned and the precise sequence context in which it and the U-rich sequence are to be found.
FIG. 8. Splicing of novel exons leads to mRNAs with premature termination codons. The TIA-1/TIAR mRNA codes for a protein with three RRMs, each made up of a pair of RNP motifs. The positions of novel exons relative to the TIA-1/TIAR mRNA are shown above the mRNA. Exons 6A, 8A, and 11A contain in frame stop codons marked by asterisks. Inclusion of exon 5A leads to a frameshift and premature termination. The TIAR gene contains 13 exons. The position of the final exon/intron junction is marked 12/13 on the mRNA. Below the mRNA are given the structures of truncated TIA-1/TIAR proteins resulting from inclusion of the novel exons.

**DISCUSSION**

We have shown that overexpression of TIA-1 or the related protein TIAR has little effect on splicing of a number of endogenous pre-mRNAs, but induces inclusion of novel alternative exons during splicing of the endogenous TIA-1 and TIAR pre-mRNAs.

These exons all have 5′ss followed by U-rich stretches, and our *in vitro* and *in vivo* analyses strongly suggest that exon inclusion is the result of activation by TIA-1 or TIAR of 5′ss use. Our results show that, despite their limitations, previous results with TIA-1 obtained *in vitro,* or *in vivo* with minigenes (11, 26), do have relevance to what goes on during splicing of a *bona fide* pre-mRNA transcribed from an endogenous gene in the cell nucleus. Furthermore, they show that the TIA-1-related protein TIAR can exert an effect on splicing similar to that exerted by TIA-1. While these two proteins are very similar, they are nonetheless not completely functionally redundant: TIAR-deficient mice fail to develop spermatogonia or oogonia, but this is not the case for TIA-1-deficient mice (23, 41). It remains, however, to be determined which aspect of TIAR activity is important for primordial germ cell activity, both TIA-1 and TIAR being involved in translational control (23, 41) as well as in splicing control.

In contrast to previous approaches that singled out 5′ss linked to U-rich stretches for testing (11, 26), the approach described here would have identified any TIA-1/TIAR-activated 5′ss in the pre-mRNAs tested irrespective of its downstream sequence. Thus the observation that all six of the activated 5′ss we identified are followed by U-rich sequences is significant. Interestingly, when the U-rich sequence following one of the 5′ss activated by TIA-1 or TIAR is deleted, the 5′ss in question is no longer used, another 5′ss being used in its place. This other 5′ss is also followed by a U-rich sequence. Our results thus strongly suggest that an immediately downstream U-rich sequence is required for TIA-1 activation of a 5′ss. Note that the alternative exons carried by most of the pre-mRNAs we tested, which were not affected by TIA-1 or TIAR, do not have 5′ss followed by a U-rich stretch.

10 different TIA-1/TIAR 5′ss are now available for comparison (the 7 described here, the FGF2-2 K-SAM exon 5′ss (11), the FAS exon 5′ss (26), and the msl-2 gene 5′ss (26)). Except for the FAS exon 5′ss, these 5′ss are intrinsically weak, or in the case of the K-SAM exon 5′ss, subject to repression. The U-rich sequences, which are the putative binding targets for TIA-1/TIAR, start between 4 and 11 residues downstream from the exon/intron junction. It has been shown experimentally that displacing the U-rich sequence so that it begins 14 residues downstream from the exon/intron junction leads to a loss of splicing-enhancing function (26). The U-rich sequences vary in size between the different 5′ss from 10 to 37 residues, with a U content of 9–26 residues. We have shown experimentally that a stretch of only 7 pyrimidines, while retaining some activity, is not of an optimal length for activating a 5′ss. Taken together, the above observations suggest that a good U-rich target for TIA-1 or TIAR should be at least 10 residues long and start no more than 12 to 13 residues downstream from the exon/intron junction. Quite how TIA-1 or TIAR gain access to their sites in the presence of abundant other nuclear proteins with a similar binding preference such as U2AF (43), PTB (44), or hnRNP C (45) is not yet clear. However, it is possible that access may be facilitated by snRNP U1, with coordinated recognition of the 5′ss by snRNP U1 and its associated U-rich sequence by TIA-1 or TIAR. Indeed, we have shown that TIA-1 cross-linking to the U-rich sequence of the K-SAM exon’s 5′ss is snRNP U1-dependent (11).

What significance should be ascribed to the observation that all the TIA-1- and TIAR-activated 5′ss identified here were found on the TIA-1 or TIAR gene pre-mRNAs? The regions of the CD44, FGF2-2, bcl-x, caspase-2, and caspase-9 genes investigated total of over 200 kilobase pairs, and no TIA-1/TIAR-activable exon was found. In contrast, the regions of the TIA-1 and TIAR genes investigated cover, respectively, 37 and 13 kilobase pairs, yet each gene contains three TIA-1/TIAR-activable exons. This suggests that TIA-1/TIAR overexpression will not lead to novel exon inclusion in the majority of genes, although it is likely that further 5′ss activated by TIA-1/TIAR would be detected if a larger sample of genes was investigated. Activation of the novel alternative TIA-1 and TIAR exons by TIA-1/TIAR overexpression may thus have some significance. In this respect it is important to note that one or more expressed sequence tags can be identified that contain each of the novel exons described here spliced to a downstream constitutive exon using a TIA-1/TIAR-activable 5′ss. These 5′ss are thus used normally, even if the corresponding mRNAs are not abundant (although this may be because mRNAs carrying them are potentially subject to nonsense-mediated decay, see below).

Splicing of the novel exons results in mRNAs with premature termination codons, either by introduction of an in frame stop codon (for TIA1 exons 6A and 11A and TIA1 exons 6A and 8A, Fig. 8), or by inducing a frameshift (for TIA1 exon 5A). This could participate in controlling TIA-1/TIAR activity in two different ways, either directly, by leading to synthesis of a truncated protein, or indirectly, by inducing nonsense-mediated decay. Normally, TIA-1 and TIAR contain three RNA recognition motifs (RRM1–3, Fig. 8) and a glutamine-rich domain. The second RRM is particularly important for recognition of U-rich...
sequences (31). Splicing of the TIA-1 5A exon is predicted to lead to synthesis of TIA-1 molecules containing only RRM1, while splicing of TIA-1 or TIAR exon 6A should lead to production of TIA-1 or TIAR with RRM1 and only the RNP2 motif of RRM2 (Fig. 8). Splicing of TIA-1 exon 8A should result in protein containing RRM1 and RRM2 (but not RRM3) being made, while splicing of TIAR exon 11A should lead to production of TIAR lacking the glutamine-rich domain (Fig. 8). We have tested the effect of overexpression of truncated TIA-1 using a TIA-1 expression vector containing exon 6A. In contrast to results obtained with intact TIA-1, no effect on splicing was observed following overexpression of TIA-1 containing only an intact RRM1. This suggests that inclusion of at least two of the novel exons (5A and 6A) prevents production of functional protein.

In addition to the direct effect on the protein produced, the premature termination codons may also have an effect on mRNA stability. Thus the presence of stop codons upstream from what is normally the last exon/exon junction is known to trigger nonsense-mediated mRNA decay (46, 47). Splicing of each of the novel exons described here positions a stop codon at least 179 nucleotides upstream from the final exon/intron junction (marked 12/13 in Fig. 8), and so the mRNAs carrying the novel exons could potentially be exposed to nonsense-mediated mRNA decay. Thus splicing of these exons could both lower the total amount of TIA-1 and TIAR mRNAs and render some of these mRNAs incapable of producing a functional protein. We recognize that it can be difficult to interpret the significance of overexpression data in terms of the normal function of the protein under investigation. It is nevertheless tempting to speculate that there is a role for TIA-1/TIAR-induced splicing of the alternative exons described here in limiting TIA-1/TIAR levels in cells: above a certain level of net activity, a feedback mechanism intervenes to stop further synthesis. Although this remains to be tested, should it prove to be the case TIA-1 and TIAR would be behaving like several other splicing activators, such as Drosophila tra-2 (48) and SWAP (49), and mammalian SRp20 (50) and SC35 (51), which autoregulate the splicing of their own pre-mRNAs through activation of alternative splicing events leading to the expression of a truncated protein or an unstable mRNA.

Finally, it is interesting to note that a protein family (the CELF proteins, for CUG-binding protein and ETR-3-like factors) has recently been identified, members of which bind to muscle-specific enhancers of the cardiac troponin exon 5 pre-mRNA and activate exon inclusion (52). CELF family members are built along the same lines as TIA-1 and TIAR: all these proteins have three RNA-binding domains, and an additional glutamine-rich domain, although this latter domain is to be found between the second and third RNA-binding domains of CELF family members, but after the third RNA-binding domain of TIA-1 and TIAR. There are further similarities between the CELF family members and TIA-1/TIAR. Like TIA-1 and TIAR (23, 24), CUG-binding protein can have either cytoplasmic or nuclear localization (53), and a protein related to CUG-BP has been implicated in translational control of certain mRNAs (42).

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TIA-1 and TIAR Activate Splicing of Alternative Exons with Weak 5′ Splice Sites followed by a U-rich Stretch on Their Own Pre-mRNAs
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