EWS/FLI Alters 5′-Splice Site Selection*

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The chimeric gene EWS/FLI is present in at least 85% of Ewing’s sarcomas as a result of chromosomal translocations. The resulting fusion protein contains the N terminus of the RNA-binding protein EWS and the ETS DNA-binding domain of the transcription factor FLI-1. Although EWS/FLI binds DNA and activates transcription, both EWS and EWS/FLI also interact with SF1 and U1C, essential components of the splicing machinery. Therefore, we tested the ability of EWS and EWS/FLI to alter 5′-splice site selection using an EIA gene in vivo splicing assay. We found that EWS/FLI, but not EWS, interfered with heterogeneous nuclear ribonucleoprotein A1-dependent splice site selection of EIA. Mutational analysis of EWS/FLI revealed that the ability to affect pre-mRNA splicing coincided with transforming activity. Therefore, EWS/FLI has the ability to influence splicing as well as transcription.

Cancer results from the accumulation of multiple genetic mutations, eventually leading to deregulated growth and/or differentiation. Chromosomal anomalies such as translocations are a common mechanism to introduce genetic changes. Many human sarcomas are characterized by specific chromosomal translocations, which result in the creation of novel fusion genes; these new genes are thought to be critical for tumorigenesis. EWS/FLI is a fusion gene created by the translocation t(11;22)(q24;q12) found in >85% of all Ewing’s sarcomas (1). The resulting fusion protein combines the N terminus of EWS, an RNA-binding protein of unknown function, with the C terminus of the ETS family transcription factor FLI-1 (2). EWS is fused to related transcription factors of the ETS family in the remaining cases of Ewing’s sarcoma (3–6). This consistent combination suggests that both the retained region of EWS, which is capable of transcriptional activation, and the ETS DNA-binding domain of the fusion protein contribute critical functions in the genesis of these tumors. TLS and TAF12-68, EWS-related genes, are also translocated and fused to transcription factor gene partners in human sarcomas (7–12).

The transcriptional activity and cellular transformation functions of EWS/FLI are not completely concordant. Although most of the transformation potential appears to reside in the N-terminal 82 amino acids of EWS, the entire EWS domain is required to provide full transformation efficiency. Interestingly, the region of EWS that contributes most to transactivation by EWS/FLI does not coincide with the domain that confers the most efficient transforming activity (13). In addition, an introduced mutation in EWS/FLI that ablates ETS-specific DNA binding only partly diminishes transformation (14), suggesting an ETS transcription-independent aspect of transformation.

Transcription and post-transcriptional processing are closely coupled processes in vivo (15), and it is likely that EWS and TLS participate in both transcription and pre-mRNA splicing. TLS has the capacity to alter 5′-splice site selection of the adenoviral EIA gene in certain cell types (16, 17). Both EWS and TLS interact with the serine/arginine-rich splicing proteins TARS-1 and TARS-2 (16, 18) and can be copurified with other components of the splicing machinery such as hnRNPA1 and hnRNPC1/C2 (19). EWS interacts with the essential splicing factors SF1 (20) and U1C (21) as well as with CUG-BP, a protein that is involved with the alternative splicing of the APP gene and the human cardiac troponin T gene. The binding of CUG-BP to cardiac troponin T-pre-mRNA is thought to disrupt splicing and to contribute to the pathogenesis of myotonic dystrophy (22). Interestingly, EWS and TLS both copurify with the splicing factor PSF (22), which is commonly involved in chromosomal translocations found in human papillary renal cell carcinomas (23).

Interaction with the splicing factor U1C represses EWS/FLI-mediated transactivation (21). The ability of a splicing factor to influence the transcriptional activity of EWS/FLI suggested that, conversely, EWS/FLI may play a direct role in splicing or have the ability to alter the composition of functional splicing complexes. To address this question, we used an in vivo splicing assay based on the alternative splicing of the adenoviral EIA gene. This assay is a well established method to show the ability of proteins such as hnRNPA1 and SF2 to influence splice site selection. The relative concentrations of these two proteins to one another determine which splice site in the EIA gene is favored. High concentrations of hnRNPA1 relative to SF2 lead to use of distal 5′-sites, whereas higher concentrations of SF2 favor proximal 5′-splice sites (24). Utilization of different 5′-splice sites leads to the subsequent formation of different amounts of alternatively spliced isoforms of EIA. We used this assay to show that although EWS and EWS/FLI have no direct effect on the splicing of EIA when expressed alone, the tumor-associated EWS/FLI, but not wild-type EWS, can oppose the change in splicing pattern induced by expression of hnRNPA1.

EXPERIMENTAL PROCEDURES

Plasmids—The epitope-tagged constructs pCFLAG-EWS and pCFLAG-EWS/FLI were generated as previously described (21).

1 The abbreviations used are: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.
coding sequence was amplified by PCR from HL-60 cDNA and cloned in-frame into pCFLAG at the EcoRI site. hnRNP A1 coding sequence was amplified by PCR from pGA1 (a gift from Dr. Adrian Krainer) (25) and cloned in-frame into pCFLAG at the EcoRI site. EWS/FLI point mutation constructs were made by PCR-based strategy (14), and the deletion construct was generated as described previously (26). These inserts were cloned into pCFLAG at the EcoRI site. Amino acid numbers for point mutations refer to the FLI-1 sequence. The absence of PCR errors in all plasmids was verified by automated sequence analysis. The clone containing the E1A minigene, pBSV-E1ATFN, was a generous gift from Dr. James Manley and has been previously described (27).

**Splicing**—293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. 3

**Results**

**EWS and EWS/FLI Do Not Directly Affect 5’-Splice Site Selection of E1A**—We performed in vivo splicing assays to see if EWS or EWS/FLI could affect 5’-splice site selection of the adeno viral E1A gene. Alternative splicing of E1A pre-mRNA generated three major isoforms (13 S, 12 S, and 9 S) and two minor isoforms (11 S and 10 S) (Fig. 1) (25). Proteins such as hnRNP A1, SF2, and TLS have the ability to generate these isoforms in differing amounts by altering 5’-splice site selection (16, 17, 24). In this established assay, the proteins of interest are coexpressed with an E1A minigene. Alternatively spliced isoforms of E1A are detected by RT-PCR using the primers shown in Fig. 1. 293T cells are human embryonic kidney cells immortalized with the adeno viral E1A gene (29) and therefore contain endogenous E1A. To analyze alternative splicing of E1A in 293T cells, we amplified E1A mRNA by RT-PCR (Fig. 2A) and quantitated the relative proportions of the three major isoforms (Fig. 2B). The endogenous representation of the three E1A isoforms in these cells comprised 51% of the 13 S isoform, followed by the 12 S isoform at 41% and then the 9 S isoform at only 5% (Fig. 2, A and B). Introduced expression of EWS, EWS/FLI, or FLI-1 did not significantly affect the alternative splicing of E1A (Fig. 2, A, lanes 2 and 3; and B) (data not shown). In contrast, overexpression of hnRNP A1 caused increased usage of the distal 5’-splice site, resulting in greatly increased levels of the 9 S isoform and decreased levels of the relative proportion of the 13 S isoform, consistent with previous reports (Fig. 2, A, lane 4; and B). Therefore, we concluded that EWS, EWS/FLI, and FLI-1 did not directly affect E1A splice site selection in these cells, despite expression at levels comparable to hnRNP A1, as shown by Western blotting (Fig. 2C) (data not shown). All PCRs were repeated at differing cycle numbers and with varying amounts of input cDNA to verify that results were reproducible and quantitative.

One potential limitation of this assay was that the low levels of the 9 S isoform in 293T cells could preclude detection of an increase in proximal splice site selection and an associated decrease in the 13 S isoform. Therefore, we repeated the assay in the Ewing’s sarcoma cell line A673. These cells were cotransfected with the E1A minigene and empty vector or EWS, EWS/FLI, or hnRNP A1 expression plasmid, and subsequent results were compared with those seen in 293T cells. In vivo splicing of E1A in this cell background gave increased levels of the 9 S isoform relative to 293T cells. However, overexpression of EWS or EWS/FLI still did not affect splice site selection. As before, expression of hnRNP A1 increased distal splice site selection,

**Fig. 1. E1A isoforms generated by in vivo splicing.** Shown is a diagrammatic representation of the alternatively spliced isoforms of E1A pre-mRNA. The major isoforms (9 S, 12 S, and 13 S) were generated by alternative selection of the 5’-splice site. The minor isoforms (10 S and 11 S) involved usage of an additional internal 3’-splice acceptor site. Dashed lines represent sequences removed by splicing. Sizes, in base pairs (bp), of the corresponding RT-PCR products obtained using the E1A569 and E1A1315 primers (represented by arrows) are shown.

eraldehyde-3-phosphate dehydrogenase primers and probe (Applied Biosystems) according to the manufacturer’s recommendations.

**Allele-specific RT-PCR**—PCR primers were designed for E1A isoforms relative to 293T cells. However, overexpression of EWS or EWS/FLI still did not affect splice site selection. As before, expression of hnRNP A1 increased distal splice site selection,
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resulting in increased levels of the 9 S isoform (Fig. 2D). Thus, EWS and EWS/FLI do not directly affect alternative splicing of E1A in either cell background. The E1A in vivo splicing assay was used first to demonstrate that the relative concentrations of hnRNPA1 and SF2 to one another determined splice site selection. These relative concentrations were manipulated by experimental overexpression (24). To detect an effect on splice site selection, the introduced proteins must be expressed at levels higher than the endogenous proteins to increase the relative concentration within the cell. Under our experimental conditions, EWS/FLI was expressed at ~6-fold higher levels than endogenous EWS/FLI protein in a Ewing’s sarcoma cell line, and hnRNPA1 was expressed at 2–3-fold higher levels than endogenous protein in 293T or Ewing’s sarcoma cells (data not shown).

EWS/FLI, but Not EWS, Alters hnRNPA1-dependent Splice Site Selection—Because EWS has been shown to co-immunoprecipitate with hnRNPA1 (19), we considered the possibility that EWS or EWS/FLI may influence hnRNPA1-dependent splice site selection by changing the composition of splicing complexes. As expected, cotransfection of hnRNPA1 with empty vector gave a distinct increase in the 9 S isoform of E1A as compared with levels in untransfected 293T cells (Fig. 3, A, lanes 1 and 2; and B). Cotransfection of EWS with hnRNPA1 did not change the pattern of isoforms seen with hnRNPA1 and empty vector (Fig. 3, A, lanes 2 and 3; and B). However, the addition of EWS/FLI to the transfection with hnRNPA1 significantly decreased the relative amount of 9 S isoform generated by 25%, with a concomitant increase in the 12 S and 13 S isoforms (Fig. 3, A, lanes 2 and 4; and B). The interference with hnRNPA1-mediated splice site selection was likely to come from the FLI-1 portion of the fusion protein since wild-type EWS did not demonstrate this activity. To confirm this, a plasmid containing the full coding sequence of FLI-1 was co-transfected with hnRNPA1, and the resulting alternative splicing pattern was analyzed. FLI-1 expression at levels similar to EWS/FLI did not affect the E1A splicing pattern (Fig. 3, A, lanes 2 and 5; and B). However, elevated FLI-1 expression opposed the hnRNPA1-dependent effect on E1A differential splicing in the same manner as EWS/FLI, with a dramatic decrease in the 9 S isoform and a compensatory increase in both the 12 S and 13 S isoforms (Fig. 3, A, lanes 2, 4, and 6; and B). The effects seen with EWS/FLI and FLI-1 were not a result of less hnRNPA1 expression in these transfected cells, as Western blot analysis showed that hnRNPA1 levels were consistent in all samples (Fig. 3C).

EWS/FLI Mutants Show Association between Ability to Alter Splice Site Selection and Transforming Activity—Several EWS/FLI mutant proteins were previously analyzed for their ability to bind DNA and to transform NIH-3T3 cells (14). The activities of the mutants and the wild-type counterparts are reviewed in Table I. The del54 mutant contains a gross deletion of the 5′-splice site selection, the introduced proteins must be expressed at levels higher than the endogenous proteins to increase the relative concentration within the cell. Under our experimental conditions, EWS/FLI was expressed at ~6-fold higher levels than endogenous EWS/FLI protein in a Ewing’s sarcoma cell line, and hnRNPA1 was expressed at 2–3-fold higher levels than endogenous protein in 293T or Ewing’s sarcoma cells (data not shown).

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of 54 of the 80 amino acids comprising the ETS domain of EWS/FLI. This deletion ablates DNA-binding activity and should abolish protein-protein interactions or any other processes mediated by the ETS domain. Two point mutations that ablate ETS DNA-binding activity, replacement of tryptophan 321 with arginine (W321R) and replacement of isoleucine 347 with glutamic acid (I347E), were also tested in transformation. Although the del54 and W321R mutant proteins lack transforming activity, I347E retains a somewhat reduced transforming activity. The differential ability of I347E to transform cells may be related to the structural integrity of the ETS domain. Tryptophan 321 is absolutely conserved in all ETS family members, and this substitution may disrupt protein structure. The substitution of I347E, however, occurs naturally in the ETS protein PU.1. Therefore, this change can be accommodated within the structural constraints of the ETS domain. The transforming activity of the I347E mutant demonstrates that a component of EWS/FLI-mediated transformation is independent of ETS DNA binding. For this reason, we looked at the effects of this mutant protein, as well as the del54 and W321R mutant proteins, on the alternative splicing pattern of E1A mediated by hnRNPA1. 293T cells were cotransfected with hnRNPA1 and the various forms of EWS/FLI, and the resulting splicing patterns were analyzed. As expected, cotransfection of hnRNPA1 and empty vector gave a large increase in the 9 S isoform, with compensatory decreases in the 13 S and 12 S isoforms compared with untransfected cells (Fig. 4, A, lanes 1 and 2; and B). This effect was opposed by the addition of EWS/FLI (Fig. 4, A, lanes 2 and 3; and B). The EWS/FLI del54 mutant protein failed to affect the splicing pattern induced by hnRNPA1, giving the same isoform profile as the addition of empty vector (Fig. 4, A, lanes 2 and 4; and B). The EWS/FLI W321R mutant protein significantly altered this profile (p = 0.02), decreasing the amount of the 9 S isoform by only 12%, whereas the I347E mutant protein more dramatically decreased the 9 S isoform by 25% (p = 0.002) (Fig. 4, A, compare lanes 2, 5, and 6; and B). The effect of the I347E mutant on alternative splicing did not differ significantly from that seen with the wild-type EWS/FLI protein. Therefore, there was a correlation between transforming activity and the ability to influence alternative splicing in the absence of DNA-binding activity (Table I). Western blotting confirmed that hnRNPA1 was made at comparable levels in all samples (Fig. 4C, lower panel) and that EWS/FLI mutants were expressed at least as highly as the wild-type EWS/FLI protein (upper panel). To ensure that changes in splicing were not a secondary effect from changes in the levels of E1A transcript, we quantitated the levels of E1A using TaqMan real-time PCR. The transfected constructs did not affect levels of E1A transcription (data not shown).

**DISCUSSION**

The recurrent examples of splicing proteins associated with EWS, EWS/FLI, and the related TLS protein (16, 17, 20–22) suggest that alterations in pre-mRNA splicing may contribute to EWS/FLI-mediated cellular transformation. We used an in vitro system to study the effects of transforming versions of EWS/FLI (Table I). Western blotting confirmed that hnRNPA1 was at comparable levels in all samples (Fig. 4C, lower panel) and that EWS/FLI mutants were expressed at least as highly as the wild-type EWS/FLI protein (upper panel). To ensure that changes in splicing were not a secondary effect from changes in the levels of E1A transcript, we quantitated the levels of E1A using TaqMan real-time PCR. The transfected constructs did not affect levels of E1A transcription (data not shown).

**TABLE I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>DNA binding</th>
<th>Transformation</th>
<th>Effect on splice site selection</th>
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<tr>
<td>EWS</td>
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<tr>
<td>EWS/FLI</td>
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<td>EWS/FLI W321R</td>
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<td>EWS/FLI I347E</td>
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**FIG. 3.** EWS/FLI and FLI-1 oppose hnRNPA1-mediated 5′-splice site selection. A, shown is the in vivo alternative splicing of endogenous E1A pre-mRNA in 293T cells. 293T cells were transiently cotransfected with 12 μg of pCFLAG-hnRNPA1 and 8 μg of pCFLAG vector (lane 2), pCFLAG-EWS (lane 3), or pCFLAG-EWS/FLI (lane 4); 0.3 μg of pCFLAG-FLI-1 and 5.7 μg of pCFLAG vector (lane 5); or 6 μg of pCFLAG-FLI-1 (lane 6). Untransfected cell lysate is represented in lane 1. Resolution of radiolabeled PCR fragments corresponding to E1A isoforms was performed on polyacrylamide gel and detected by autoradiography. B, the E1A isoforms were quantified with ImageQuant on a PhosphorImager as described for Fig. 2B. C, 25 μg of total protein isolated from 293T cell lysates used in A was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was performed with the anti-FLAG M2 monoclonal antibody, and proteins were visualized by enhanced chemiluminescence and autoradiography. All proteins were FLAG-tagged and resolved on the same gel.

**Transforming versions of EWS/FLI interfere with hnRNPA1-mediated 5′-splice site selection**

“DNA binding” indicates the capacity to bind to the FLI-1-binding consensus sequence and activate transcription. “ Transformation” refers to the capacity of the protein to induce anchorage-independent growth of NIH-3T3 colonies in soft agar. “Effect on splice site selection” indicates the ability to interfere with the distal 5′-splice site selection favored by hnRNPA1. + + +, activity of EWS/FLI; +, diminished activity relative to EWS/FLI; –, no activity.
lysates used in A were analyzed by enhanced chemiluminescence and autoradiography. With the anti-FLAG M2 monoclonal antibody, and proteins were visualized and transferred to nitrocellulose. Immunoblotting was performed on polyacrylamide gel and detected by autoradiography.

**Fig. 4.** Transforming EWS/FLI mutant protein opposes hnRNPA1-mediated 5'-splice site selection, whereas non-transforming mutants do not. A, shown is the in vitro alternative splicing of endogenous E1A pre-mRNA in 293T cells. 293T cells were transiently cotransfected with 12 μg of pCFLAG-hnRNPA1 and 6 μg of pCFLAG vector (lane 2), pCFLAG-EWS/FLI (lane 3), pCFLAG-EWS/FLI del54 (lane 4), pCFLAG-EWS/FLI W321R (lane 5), or pCFLAG-EWS/FLI I347E (lane 6). Untransfected cells are represented in lane 1. Radiolabeled RT-PCR fragments corresponding to E1A isoforms were resolved on polyacrylamide gel and detected by autoradiography. B, the E1A isoforms were quantified with ImageQuant on a PhosphorImager as described for Fig. 2B. C, 25 μg of total protein isolated from 293T cell lysates used in A was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was performed with the anti-FLAG M2 monoclonal antibody, and proteins were visualized by enhanced chemiluminescence and autoradiography.

vivo splicing assay with the E1A gene to test the hypothesis that EWS or EWS/FLI may have the ability to affect alternative splicing. We showed that although EWS and EWS/FLI do not directly affect the splicing pattern of E1A, the tumor-associated EWS/FLI opposed alternative 5'-splice site selection induced by hnRNPA1. Furthermore, analysis of mutant proteins showed an association between the ability to affect alternative splicing and transforming activity, independent of ETS DNA binding. Thus, the splicing-associated functions of EWS/FLI may contribute to transformation.

Although the contribution of aberrant splicing to tumorigenesis remains unclear, abnormal splicing profiles have been associated with tumors. For example, two novel aberrantly spliced transcripts of fibroblast growth factor receptor 3 were identified in a large percentage of both primary tumors and colorectal carcinoma cell lines. It is thought that these may confer a selectable growth advantage to cells contributing to the progression of colorectal tumors (30). Similarly, a spliced variant of vascular endothelial growth factor is associated with greater tumorigenicity and increased angiogenic properties in breast carcinoma cells compared with other isoforms (31). Alternatively spliced forms of the key cell cycle regulators cyclin D1 and p53 have also been identified in human tumors and cell lines (32, 33).

There are several examples of splicing factor involvement in tumor-derived fusion proteins. DEK, which is fused to CAN in a subset of acute myeloid leukemia, associates with serine/arginine-rich splicing proteins in vitro and in vivo. DEK remains bound to exon-product RNA after prior removal of an intron, suggesting a role for DEK in pre-mRNA processing (34). In papillary renal cell carcinomas, the splicing proteins PSF and p54NRB/NonO are fused to DNA-binding domains from TFE3 (23). Additionally, EWS is fused to WT-1 in chimeric proteins from desmoplastic small round cell tumors (10). WT-1 exists as two different isoforms, one of which binds to DNA at specific promoter sequences, whereas the other binds more efficiently with splicing factors (35).

Studies on the PU.1 protein illustrate a precedent in which the ETS domain regulates both transcription and splicing. Fli-1 and Pu.1 are the two genes most frequently targeted by viral integration in Friend virus-induced mouse erythroleukemias (36). Pu.1 interacts with TLS and modulates TLS-mediated splice site selection (17). Pu.1 also interacts with the RNA-binding protein p54NRB and can inhibit splicing of β-globin mRNA. It is thought that Pu.1 may disturb gene regulation at the post-transcriptional level through sequestration of important RNA-binding proteins like p54NRB (37). It is important to note that the ETS domain of Pu.1 alone could inhibit splicing (37), suggesting that this domain is critical for mediating such sequestering interactions.

Our data support a similar role for EWS/FLI in altering 5'-splice site selection. Interactions between EWS/FLI and hnRNPA1, or other proteins required for hnRNPA1-mediated splice site selection, may alter the composition of the active spliceosome. EWS/FLI was more potent than Fli-1 in its effect on splice selection, perhaps due to the contribution of EWS interactions with splicing factors (20, 21). However, the ETS domain was required to influence splicing. Deletion of the ETS domain ablated the effect, and the point mutation W321R, which is likely to disrupt the structure of the ETS domain, decreased the effect substantially. The diminished activity of EWS/FLI W321R in the splicing assay may reflect a failure to interact with a subset of proteins involved in splice site selection. The EWS/FLI I347E substitution is found in wild-type PU.1 and therefore probably does not greatly alter the structure of the ETS domain. Thus, it is possible that EWS/FLI I347E maintains critical interactions with RNA-binding proteins mediated by this domain and therefore retains the full effect on hnRNPA1-dependent splice site selection.

An alternative possibility is that EWS/FLI may alter the composition of the spliceosome by influencing the transcription of other splicing factors. Although it is formally possible that the mutant proteins I347E and W321R retain the ability to bind a variant DNA sequence, they lack the expected ETS
DNA-binding activity that is well characterized for proteins containing this modular DNA-binding domain. Therefore, it seems quite unlikely that the I347E mutant would regulate specific transcriptional targets in a manner that produces splicing effects equivalent to those induced by EWS/FLI.

Consistent with our findings, EWS/FLI and TLS/ERG, a fusion protein found in acute myeloid leukemia, also interfere with the alternative splicing patterns of E1A mediated by serine/arginine-rich proteins. The RNA-binding regions of TLS and EWS interact with the serine/arginine-rich proteins TASR-1 and TASR-2, perhaps recruiting them to sites of transcription. EWS/FLI and TLS/ERG inhibit splicing mediated by TASR proteins, presumably due to loss of the interaction (18, 38). Unlike the effect on hnRNPA1-mediated splicing, wild-type Fli-1 does not affect splicing by TASR proteins. Our study illustrates that alterations in splicing can also be mediated by the ETS domain, adding another level of complexity and specificity to the involvement of EWS/FLI in splicing.

Altogether, the evidence indicates that EWS/FLI has the ability to influence the splicing process. The domains of the protein required and the precise effect depend on the complement of splicing proteins present and will almost certainly vary with the RNA substrate examined. EIA is an established experimental splicing substrate, but is not a physiological substrate for EWS/FLI in Ewing’s sarcoma. New technologies such as in vitro sequence information system (ISIS), an intron information system used to detect alternative splicing in the human genome (39), could help to identify such transcripts in the future. Transcriptional activity contributes substantially to the transforming activity of EWS/FLI, although it is not absolutely required. Identification of relevant target genes that are transcriptionally regulated by EWS/FLI may also provide key substrates to dissect the specific effects of EWS/FLI on splicing.

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