

Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by *FLII* for transformation

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ABSTRACT The 11;22 chromosomal translocation specifically linked to Ewing sarcoma and primitive neuroectodermal tumor results in a chimeric molecule fusing the amino-terminal-encoding portion of the *EWS* gene to the carboxyl-terminal DNA-binding domain encoded by the *FLII* gene. We have isolated a fourth *EWS-FLII* fusion cDNA that is structurally distinct from the three forms previously described. To determine the transforming activity of this gene, alternative forms of the *EWS-FLII* fusion were transduced into NIH 3T3 cells. Cells expressing either type 1 or type 4 fusion constructs formed foci in culture and colonies in soft agar, indicating that *EWS-FLII* is a transforming gene. *EWS-FLII* deletion mutants were created to map functionally the critical regions within the chimera. Deletion of either the *EWS* domain or the *FLII* corresponding to the DNA-binding domain totally abrogated the ability for *EWS-FLII* to transform 3T3 cells. These data indicate that the oncogenic effect of the 11;22 translocation is caused by the formation of a chimeric transcription factor. Formation of chimeric transcription factors has now been demonstrated to promote tumors of both neuroectodermal and hematopoietic origin, suggesting that this may be a common mechanism in human carcinogenesis.

Structural alteration or aberrant expression of transcription factors is often a critical event in neoplastic transformation (for review, see ref. 1). Indeed, many known transcription factors were initially identified as a result of their activation by oncogenic retroviruses of lower vertebrates. Insertion of retroviral enhancers in proximity to transcription factor genes can result in inappropriate expression and cellular transformation. Alternatively, replication-deficient retroviruses that harbor structurally altered transcription factors directly result in the expression of oncogenic proteins.

Structural alteration or aberrant expression of transcription factors is also common in human malignancies but usually results through somatic genomic mutation (for reviews, see refs. 2 and 3). Karyotypic analyses have revealed a tumor-specific t(11;22)(q24;q12) chromosomal translocation in 86% of both Ewing sarcoma and primitive neuroectodermal tumor (PNET), suggesting that the product of this rearrangement is necessary for the formation of both these malignancies (4, 5). Recently it has been shown that this rearrangement juxtaposes the *FLII* gene on chromosome 11 with a previously uncharacterized gene of unknown function, termed *EWS*, on chromosome 22 (6).

FLII (named for Friend leukemia integration site 1) is a member of the ETS family of transcription factors (7). These proteins directly bind to target DNA sequences through

related structural motifs in their DNA-binding regions, usually located at their carboxyl terminus (8). Divergent transcriptional activation domains at the amino terminus participate in protein-protein interactions with other transcription molecules to activate gene expression at particular targets. The 11;22 translocation joins the 5' portion of the *EWS* locus to the 3' (DNA-binding) region of the *FLII* gene and results in the replacement of the transcription activation domain of *FLII* with *EWS* sequences. This chimeric product has the potential to promote tumorigenesis by acting as an aberrant transcription factor. We demonstrate here that (i) *EWS-FLII* is a transforming gene and (ii) that both *EWS* and *FLII* domains of the chimera are necessary for transforming activity.

MATERIALS AND METHODS

PNET cDNA Library Construction and Isolation of *EWS-FLII* Chimeras. TC-32, a PNET tumor cell line containing the 11;22 translocation, was grown in RPMI medium/10% fetal calf serum, as described (5). Total RNA was harvested by lysis with guanidine isothiocyanate and purified over cesium chloride (9). Poly(A)⁺ RNA was obtained by using columns packed with oligo(dT)-cellulose (Collaborative Research) and used for construction of cDNA libraries.

A TC-32 cDNA library was made according to previously published procedures (10). Briefly, first-strand synthesis was accomplished using methyl mercury-denatured poly(A)⁺ RNA primed with oligo(dT) and murine leukemia virus reverse transcriptase (GIBCO/BRL). Second-strand synthesis was done by using RNase H and polymerase I (GIBCO/BRL), and synthesized products were purified over a Sephadex G100 column (Pharmacia). cDNAs were blunted by using T4 polymerase (GIBCO/BRL) and ligated to a molar excess of *EcoRI* adaptors (Invitrogen, San Diego). The adaptor ends were phosphorylated with T4 polynucleotide kinase (United States Biochemical), and cDNAs were fractionated over a 6% acrylamide gel. DNA species >600 bp were recovered from gel slices by electroelution, purified over an Elutip-D column (Schleicher & Schuell), ligated into λ GT-10 vector, and packaged (Gigapack; Stratagene).

Approximately 700,000 primary phage were screened with a cDNA fragment from the carboxyl-terminal region of murine *Fli-1* (amino acids 259–450, see ref. 7). Three positive clones were identified ranging from 1.5 to 2.8 kb. The longest cDNA contained a t(11;22) fusion point and was subcloned into pBluescript KS(+) (Stratagene), and its coding regions were sequenced by using reagents and instruction prepared

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Abbreviations: PNET, primitive neuroectodermal tumor; *FLII*, Friend leukemia integration site 1.

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by United States Biochemical. In similar fashion, the 5' end of this *EWS-FLII* cDNA was used as a probe to isolate germ-line *EWS* clones from the TC-32 cDNA library.

The *EWS-FLII(alt)* construct junction point was isolated by reverse transcriptase PCR amplification of TC-32 mRNA and contained an additional 66 bp of *FLII*. First-strand synthesis was accomplished using *epi-1* (5'-TCTCGAAT-TCTAAGCGTAATCTGGCACATCGTATGGGTAG-TAGCTGCCTAAGTGTA) or *ESBP2* (5'-CCGTTGCTCT-GTATTCTTACTGA) oligonucleotides to prime poly(A)⁺ TC-32 RNA with murine leukemia virus reverse transcriptase (GIBCO/BRL), according to manufacturer's recommendations. Portions of these reactions were amplified by *Pfu* DNA polymerase (Stratagene) with *ESBP1* (5'-CGACTAGTAT-GATCAGAGCAGT) and *ESBP2* primers in a thermocycler using the following parameters: denature, 94°C; anneal, 65°C; extend, 72°C; 35 cycles. The resultant fragment was digested with *Bam*HI and *Eco*RI, gel-purified, subcloned into pBlue-script KS(+) (Stratagene), and sequenced.

Retroviral Constructs and Agar Assays. *EWS-FLII* constructs and their derivatives were made as follows.

EWS-FLII1. A full-length *EWS-FLII* cDNA was constructed by ligating the 5' end of a germ-line *EWS* cDNA to the chimeric t(11;22) cDNA at an overlapping *Pst* I site. Digestion with *Eco*RI and *Hind*III yielded a 1.4-kb fragment that contained 123 bp of 5' untranslated sequence and no 3' untranslated sequence, which was subcloned into pBlue-script KS (Stratagene). This construct was tagged with a 9-amino acid epitope from the influenza hemagglutinin molecule (11) by PCR amplification using *Pfu* DNA polymerase (Stratagene) and the 3' primer *epi-1* (see above). The resultant fragment was cloned into the *Eco*RI site of pSR α MSV(Δ *Hind*III, Δ *Cla* I), a derivative of the retroviral vector pSR α MSV (12) that lacks a neomycin-resistance gene.

EWS-FLII(alt). A 112-bp *Bam*HI-*Eco*RI fragment containing the *EWS-FLII(alt)* fusion point was sequentially ligated to a 5' *EWS Eco*RI-*Bam*HI fragment and a *Eco*RI-*Hind*III fragment from the carboxyl-terminal domain of human *FLII* (13). This construct was epitope-tagged and placed into the retroviral vector pSR α MSV(Δ *Hind*III, Δ *Cla* I) as described for *EWS-FLII1*.

EWS-FLII(Δ 22). An *Eco*RI-*Rsa* I fragment from the 5' end of *EWS* was ligated to a blunted internal *Eco*RI site of *EWS-FLII(alt)*, deleting all but the amino-terminal 6 amino acids of *EWS*. This molecule was cloned into the *Eco*RI site of pSR α MSV(Δ *Hind*III), a retroviral vector containing the neomycin-resistance gene under a thymidine kinase promoter.

EWS-FLII(Δ ETS). A 54-amino acid internal deletion was created in *EWS-FLII* by ligating a 1-kb *Eco*RI-*Pvu* II *EWS-FLII* fragment to a blunted *Hpa* II-*Hind*III carboxyl-terminal *FLII* fragment. An epitope tag was added by PCR amplification, and the construct was placed into the pSR α MSV(Δ *Hind*III) vector.

Replication-deficient retroviral stocks were created by transiently transfecting COS cells with *EWS-FLII* constructs together with a ψ^- packaging plasmid (12). Conditioned medium containing virus was harvested and used to infect NIH 3T3 cells. Infectants, either unselected or selected in G418 for 2 weeks, were plated in agar in duplicate at various serum concentrations and cell-seeding densities (14). Agar colonies were enumerated after 2 weeks growth.

***EWS-FLII* RNA and Immunoprecipitation Analyses.** RNA analyses of *EWS-FLII*-infected NIH 3T3 cells were performed as follows. Total RNAs were size-fractionated through a 1% formaldehyde-agarose gel (Seakem LE; FMC), transferred to nitrocellulose (Micron Separations, Westboro, MA), and probed with the 600-bp carboxyl-terminal *Eco*RI-*Hind*III fragment from human *FLII*.

A glutathione-*S*-transferase-*EWS-FLII1* polypeptide was created to be used as an immunogen to generate antibodies against *EWS-FLII1*. A 189-bp *Bam*HI-*Pvu* II fragment containing the *EWS-FLII* fusion point (see Fig. 3) was subcloned into the vector pGEX-2T (Pharmacia), and the protein was expressed in *Escherichia coli*. The glutathione-*S*-transferase fusion polypeptide was purified from bacterial cell lysates using glutathione-agarose (Sigma), as described (15). Purified protein was injected s.c. into rabbits to create specific antisera.

EWS-FLII1 proteins were detected by sequential immunoprecipitation with two different antibodies. [³⁵S]Methionine-labeled whole-cell lysates were prepared from the same cell populations described in the RNA analysis plus *EWS-FLII1* polypeptide translated *in vitro* using rabbit reticulocyte lysate (Promega). The first immune precipitation was done by using monoclonal antibody 12CA5, which was raised to the hemagglutinin epitope placed at the carboxyl terminus of *EWS-FLII1*-encoding constructs (11). Immune complexes were recovered by using protein G-Sepharose (Pharmacia), denatured, and hybridized with rabbit heteroantisera generated to glutathione-*S*-transferase-*EWS-FLII1* polypeptides. Secondary immune complexes were precipitated with protein A-Sepharose, fractionated by SDS/PAGE, and autoradiographed.

RESULTS

Multiple Forms of the *EWS-FLII* Are Expressed in the Same PNET Tumor Cell Line. A cDNA clone containing a t(11;22) fusion transcript was isolated by screening a library constructed from TC-32, a PNET tumor cell line (5), with a murine *Fli-1* cDNA probe. Nucleotide sequence analysis revealed an in-frame joining between the *EWS* and *FLII* genes that differs from those previously described. The fusion contained the same amount of *EWS* sequences described in one of the previously characterized t(11;22) rearrangements (6) but lacked 66 bp of 5' *FLII* sequences (Fig. 1). This cDNA represents a fourth type of *EWS-FLII* junction. The previous three t(11;22) rearrangements could be categorized by the amount of *EWS* and *FLII* retained in the fusion transcript. In those species, as in ours, the *FLII-ETS* DNA-binding motif was preserved, and there were no differences from germ-line in either *EWS* or *FLII* sequences.

A PCR assay was used to determine whether other *EWS-FLII* fusion species were being expressed in the TC-32 PNET cell line. *EWS* and *FLII* oligonucleotide primers were used to specifically amplify t(11;22) fusion fragments from TC-32 poly(A)⁺ RNA and our *EWS-FLII* fusion cDNA. Amplification of TC-32 RNA resulted in a single detectable product 66 bp larger than that seen with the cDNA (Fig. 1). Nucleotide sequence analysis of this fragment showed it to be identical to a type 1 *EWS-FLII* junction. These results suggest that both type 1 and type 4 *EWS-FLII* species may be simultaneously expressed in TC-32 cells, perhaps through alternative splicing of RNA intermediates. The fact that type 4 *EWS-FLII* species is not seen in our PCR assay may reflect lower levels of this species, compared with type 1.

***EWS-FLII* Chimeras Transform NIH 3T3 Cells.** Because type 1 and type 4 *EWS-FLII* fusions appear to be coexpressed in TC-32 cells, we wished to determine whether they both could act as transforming genes. Full-length *EWS-FLII* constructs containing either type 1 or 4 junctions were made by ligating fragments from PCR products and overlapping t(11;22) and germ-line *EWS* cDNA clones at common restriction sites. Nucleotide sequence analysis and *in vitro* translation of constructs demonstrated correct open reading frames. To facilitate detection of the protein products generated by these cDNA constructs, a 9-amino acid epitope from the influenza hemagglutinin molecule, recognized by

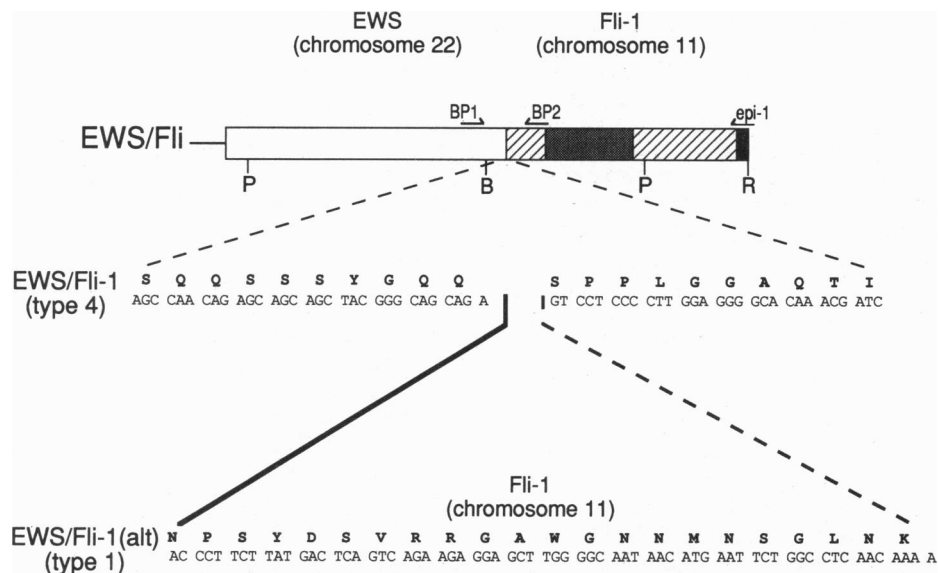


FIG. 1. Comparison of different *EWS-FLII* fusion junction points. *EWS-FLII* (type 4) nucleotide sequence was generated directly from a clone isolated from a TC-32 PNET cDNA library. The *EWS-FLII*(alt) junction point was isolated by reverse transcriptase PCR amplification of TC-32 mRNA and contained an additional 66 bp of *FLII*. The small arrows, labeled BP1, BP2, and epi-1, in the cDNA schematic indicate the position of PCR primers referred to in text.

the monoclonal antibody 12CA5 (11), was added to their carboxyl termini by PCR amplification. To make retroviruses that could be used in transformation assays, both native and epitope-tagged *EWS-FLII* constructs were placed in correct orientation downstream to the 5' long terminal repeat of the retroviral vector pSR α MSV(Δ HindIII, Δ Cla I) (12). Replication-deficient recombinant retroviruses were generated by expressing each construct, together with a ψ^- packaging plasmid, in COS cells.

To determine whether the fusion genes could transform fibroblasts, retroviruses containing type 1 or type 4 *EWS-FLII* constructs were assessed for their ability to alter growth of NIH 3T3 cells. Unselected primary infectants were plated both in liquid culture and in soft agar at various serum concentrations and cell-seeding densities. In three independent experiments, *EWS-FLII* retroviruses consistently induced transformation in 3T3 cells. *EWS-FLII* infectants formed foci in liquid culture after 7 days of growth, and macroscopic colonies in agar were apparent within 8–10 days. Both type 1 and type 4 *EWS-FLII* constructs transformed 3T3 cells (Fig. 2). Under all conditions cells infected with either native or epitope-tagged t(11;22) retroviruses consistently formed colonies in agar where mock infectants formed none.

Both *EWS* and *FLII* Domains Are Necessary for *EWS-FLII* Transforming Activity. Because the 11;22 translocation fuses two normally distinct genes, it is possible that the *EWS* and *FLII* components each provide functions that are necessary for transformation. The *FLII* component present in the t(11;22) protein contains a Trp-Xaa₁₇-Trp-Xaa₁₈-Trp motif

present in most *ETS* family genes (16). Deletions involving this region in *ETS1* result in decreased DNA-binding activity (17). The *FLII* portion of the t(11;22) fusion gene also retains a sequence that encodes basic amino acids similar to a motif necessary for the efficient nuclear localization of *ETS1* (17). To determine whether these domains of *FLII* were necessary for *EWS-FLII* transformation, an epitope tagged *EWS-FLII*(Δ ETS) recombinant retrovirus was created that deleted 54 amino acids within the *ETS*-common domain, removing these critical motifs (Fig. 3).

The normal function of the germ-line *EWS* gene is unknown. Nucleotide sequence analysis of its carboxyl terminus-encoding domain demonstrates regions of similarity between *EWS* and the RNA-binding domain of several proteins (6). The amino terminus that is fused to *FLII* in the t(11;22) chimera consists of a series of degenerate glutamine-rich repeats, with few predicted secondary structures. These characteristics are consistent with the notion that *EWS* functions as an alternative transcription activation domain, replacing the *FLII* domain lost in the t(11;22) rearrangement. To assess whether this region is essential for transformation, an epitope-tagged *EWS-FLII*(Δ 22)-producing retrovirus was constructed in which all but the six amino-terminal amino acids of the *EWS* domain were deleted (Fig. 3).

To evaluate the transforming properties of the mutated *EWS-FLII* constructs, NIH 3T3 cells were infected with either *EWS-FLII*(Δ ETS) or *EWS-FLII*(Δ 22) and compared with unaltered *EWS-FLII*. Agar assays were done both on unselected primary infectants as well as on G418-resistant polyclonal outgrowths. Regardless of the populations used,

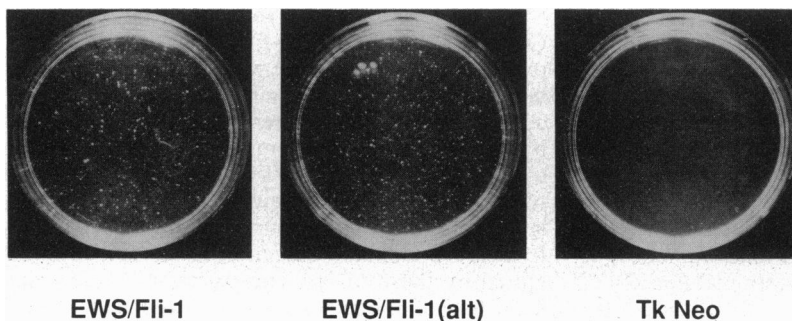


FIG. 2. Agar assays demonstrate NIH 3T3 transformation by two different *EWS-FLII* fusions. Polyclonal primary infectant NIH 3T3 populations of either *EWS-FLII* or *EWS-FLII*(alt) created large numbers of colonies when plated in soft agar. Mock-infected 3T3 cells using an empty thymidine kinase (Tk) Neo vector generated no colonies in agar.

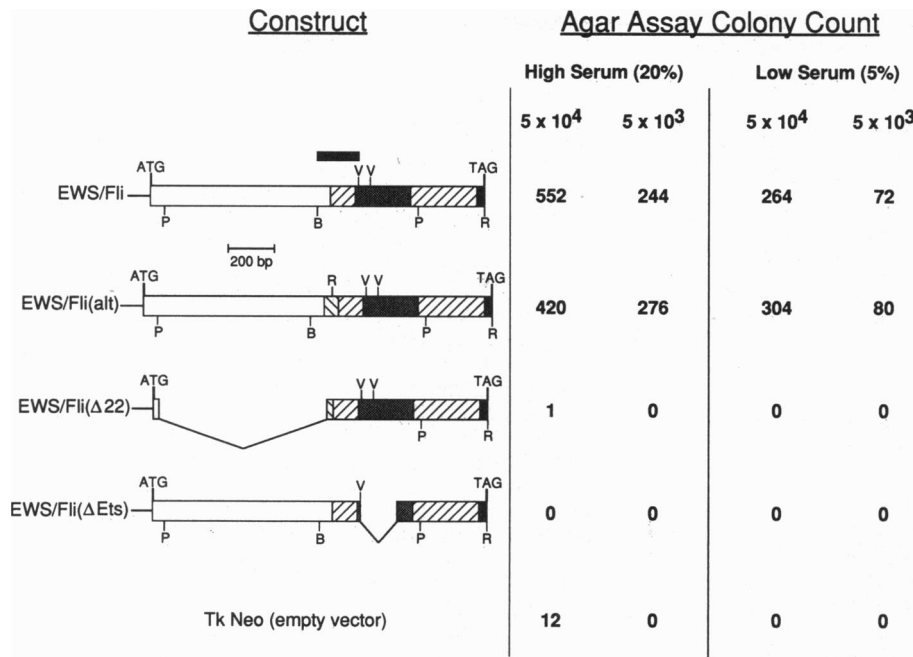


FIG. 3. Growth in agar of NIH 3T3 cells expressing *EWS-FLII* or mutated *EWS-FLII* constructs. Colony counts of unselected primary 3T3 cells expressing either *EWS-FLII* or *EWS-FLII(alt)* constructs are enumerated. Under the same conditions, G418-selected polyclonal populations of the deletion constructs *EWS-FLII(Δ22)* and *EWS-FLII(ΔETS)* failed to form any colonies in agar over background empty vector (Tk Neo). Colony counts represent the mean of duplicate experiments done at each serum concentration and cell-seeding density. Structures of each *EWS-FLII* construct are schematically displayed: □, *EWS* sequences; ▨, *FLII* sequences; ▩, DNA-binding domain of *FLII*; ■, influenza hemagglutinin epitope tag. Black bar delineates the *Bam*HI-*Pvu* II fragment expressed as a GST fusion polypeptide in *E. coli*. P, *Pst* I; B, *Bam*HI; R, *Eco*RI; V, *Pvu* II.

3T3 cells expressing either of the deleted t(11;22) fusion cDNA constructs failed to form colonies in agar (Fig. 3). Northern and immune precipitation analyses of the G418-resistant outgrowths from both deleted constructs demonstrated fusion mRNA and protein expression equal to or greater than those seen in *EWS-FLII*-transformed clones (Fig. 4). Therefore, the lack of transforming activity by the mutated constructs was due to loss of biologic function and not from underexpression or instability of the deleted products.

DISCUSSION

We have determined that two distinct *EWS-FLII* chimeras are coexpressed in the same PNET tumor-derived cell line. The three different *EWS-FLII* fusion genes previously described are thought to arise from variation in the t(11;22) genomic breakpoints (18). Because the TC-32 PNET cell line contains only one t(11;22) allele, alternative mRNA splicing seems a likely explanation for formation of the type 4 *EWS-FLII* fusion; this provides a second mechanism for the observed heterogeneity of t(11;22) products.

These studies show that *EWS-FLII* is a transforming gene and are consistent with the chimeric protein playing a crucial

role in Ewing sarcoma and PNET oncogenesis. The efficient and rapid agar growth of NIH 3T3 primary infectants suggests that, at least in this cellular background, *EWS-FLII* is a potent single-step transforming agent and that this system can be used to explicitly define the function of *EWS-FLII*. This was not true for all fibroblast cell lines. RAT1 cells or a RAT1 cell line that stably expressed the *c-MYC* oncogene (19) failed to form foci or agar colonies when infected with the same *EWS-FLII* retroviral stocks (data not shown). These data suggest that *EWS-FLII* transformation activity may depend on other cellular factors present in NIH 3T3 cells but lacking in both RAT1 cell lines. Despite being structurally distinct, both *EWS-FLII* fusion genes transform NIH 3T3 cells. This result contrasts with the *E2A-PBX* chimera produced by the t(1;19) translocation found in acute lymphoblastic leukemia, where different fusion species varied markedly in their ability to transform NIH 3T3 cells (20).

Our experiments with *EWS-FLII* deletion mutants indicate that both *EWS* and *FLII* domains are necessary for transformation by the 11;22 translocation product. This relationship is also seen with *ETS1*, which encodes a transcription factor, but when fused to the *MYB* protooncogene in the

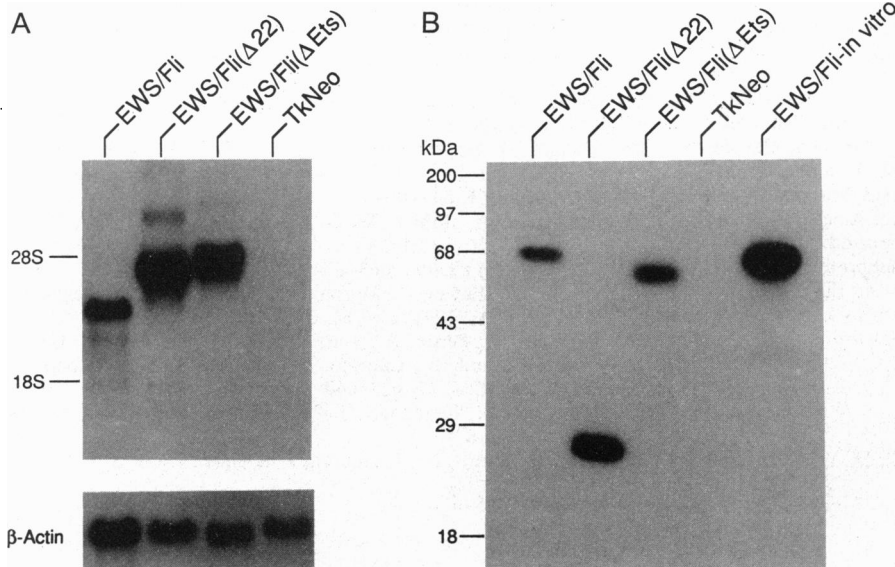


FIG. 4. Expression of *EWS-FLII* mRNAs and proteins in NIH 3T3 cells. (A) RNA analysis shows high levels of *EWS-FLII* transcript in both a clonal NIH 3T3 transformant and polyclonal G418-selected populations from nontransforming *EWS-FLII* mutants. (Upper) Hybridization to a 600-bp (carboxyl-terminal domain) human *FLII* cDNA probe. (Lower) Rehybridization of the same blot with a β -actin probe detects approximately equal amounts of intact RNA in each lane. (B) Two-cycle immune precipitation analysis detects *EWS-FLII* protein products in retrovirally infected NIH 3T3 populations. Mutant *EWS-FLII* proteins in nontransformed G418-selected populations are expressed at levels greater than or equal to wild-type *EWS-FLII* in a NIH 3T3 transformant. NIH 3T3 cells infected with empty vector (TkNeo) and *in vitro* translated *EWS-FLII* are included as negative and positive controls.

E26 virus causes chicken erythroleukemia (21, 22). Though *v-ETS1* alone inefficiently transforms avian bone marrow cells, fusion to *MYB* is required for full tumorigenicity. Chimeric transcription factors have also been linked to human hematopoietic malignancies that, like Ewing sarcoma, are caused by chromosomal rearrangement. The t(15;17) translocation found in acute promyelocytic leukemia fuses *PML*, a gene of unknown function, to the retinoic acid α receptor (*RARA*) (23, 24). Similarly, a subclass of acute lymphoblastic leukemia contains a t(1;19) translocation that joins the transcriptional activation domain of the immunoglobulin κ enhancer binding gene *E2A* to the DNA-binding region of *PBX*, a homeobox gene (25–27).

The mechanisms by which oncogenic transcription factors mediate their effects vary. In the cases of *v-Erb-A* and *v-Rel*, “dominant negative” interference of their normal cellular counterparts, the thyroid hormone receptor and *c-Rel*, respectively, seems to be active (28, 29). A similar mechanism has been implicated in the function of the *PML-RARA* fusion (30–32). However, this type of mechanism involving *EWS-FLII* seems unlikely in Ewing sarcoma and PNET. Germ-line *FLII* from the untranslocated allele is not expressed in these tumors, though germ-line *EWS* is present. The fact that the *FLII* DNA-binding region is necessary for transformation argues against a simple inhibition of *EWS* function as the only mechanism of action. These data suggest that *EWS-FLII* may be acting as an aberrant transcription factor.

FLI1 is normally expressed primarily in hematopoietic tissues and is not expressed in brain (7). Part of the oncogenic effect of the 11;22 translocation could be to express a protein equivalent to *FLI1* in cells where *FLI1* is normally transcriptionally silent. Alternatively, the primary effect of the 11;22 translocation could be to produce an aberrant transcription factor that is qualitatively different from *FLI1*. Such a product would be expected to bind to the same consensus DNA sequences as *FLI1* but possibly with different avidity. Regions within the transcriptional activation domain of *ETS1* have been shown to inhibit binding of target sequences (33, 34). In addition, replacement of the normal *FLI1* transcriptional activation domain with *EWS* sequences could alter specific protein-protein interactions with transcription complexes and result in the modulation of a different repertoire of genes. In this sense the 11;22 translocation may share functional similarity with the t(1;19) *E2A-PBX* rearrangement (20). The fact that generation of chimeric transcription factors can promote tumorigenesis in both hematopoietic and neuroectodermal malignancies suggests that this may be a common mechanism in human carcinogenesis.

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