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Identification of Target Genes for the Ewing's Sarcoma EWS/FLI Fusion Protein by Representational Difference Analysis

BENJAMIN S. BRAUN,[1] RICHARD FRIEDEN,[2] STEPHEN L. LESSNICK,[1]
WILLIAM A. MAY,[2] AND CHRISTOPHER T. DENNY[1,2]*

Molecular Biology Institute[1] and Department of Pediatrics, Division of Hematology/Oncology, Gwynne Hazen Cherry Memorial Laboratories, and Jonsson Comprehensive Cancer Center, School of Medicine,[2] University of California, Los Angeles, California 90024

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The *EWS/FLI-1* fusion gene results from the 11;22 chromosomal translocation in Ewing's sarcoma. The product of the gene is one of a growing number of structurally altered transcription factors implicated in oncogenesis. We have employed a subtractive cloning strategy of representational difference analysis in conjunction with a model transformation system to identify genes transcribed in response to EWS/FLI. We have characterized eight transcripts that are dependent on EWS/FLI for expression and two transcripts that are repressed in response to EWS/FLI. Three of the former were identified by sequence analysis as stromelysin 1, a murine homolog of cytochrome P-450 F1 and cytokeratin 15. Stromelysin 1 is induced rapidly after expression of EWS/FLI, suggesting that the stromelysin 1 gene may be a direct target gene of EWS/FLI. These results demonstrate that expression of EWS/FLI leads to significant changes in the transcription of specific genes and that these effects are at least partially distinct from those caused by expression of germ line FLI-1. The representational difference analysis technique can potentially be applied to investigate transformation pathways activated by a broad array of genes in different tumor systems.

Somatic mutation plays a primary role in human carcinogenesis. Tumor-associated rearrangements can produce chromosomal deletions, amplifications, or translocations. Chromosomal translocations juxtapose two previously distinct genomic domains and frequently result in the formation of novel fusion genes. In many cases, at least one translocation partner encodes a transcription factor, suggesting that the resultant fusion product may function as an aberrant transcription factor (reviewed in references 22 and 43).

EWS/FLI is a fusion gene that is formed by an 11;22 chromosomal translocation characteristically found in Ewing's sarcomas and primitive neuroectodermal tumors of childhood (7). EWS/FLI transforms NIH 3T3 cells, suggesting that this molecule plays an active role in tumorigenesis (21, 32). Normal FLI-1, on the other hand, does not transform NIH 3T3 cells (33). The fusion gene encodes a chimeric protein that consists of the amino terminus of EWS, a putative RNA-binding protein, fused to the carboxyl terminus of FLI-1, a member of the ETS family of transcription factors. EWS/FLI also displays the biochemical attributes of a transcription factor: (i) EWS/FLI localizes to the cell nucleus (1, 33), (ii) EWS/FLI can bind DNA in a site-specific manner (30, 33, 39), and (iii) the amino terminus of EWS can function as a strong transcriptional activator (1, 21, 33, 39, 40). Accumulating evidence suggests that EWS/FLI may transform cells by acting as an aberrant transcription factor that inappropriately activates and/or represses the expression of target genes.

This hypothesis is further supported by observations of other tumor-specific rearrangements involving the *EWS* gene. In each case, the corresponding fusion protein includes the amino terminus of EWS and the DNA-binding domain of a transcription factor. Approximately 10% of Ewing's sarcomas contain a t(21;22) instead of the more common t(11;22). In these cases, EWS is fused to another ETS protein, ERG, that displays extensive similarity to FLI-1 (6, 8, 11, 49, 56). Fusion to a more distantly related ETS family member, ETV1, has also been documented in Ewing's sarcoma (16). EWS has been found joined to ATF1 in malignant melanomas of soft parts (55) and to WT1 in desmoplastic small round cell tumors (20). Finally, a molecule similar to EWS and known as TLS (or FUS) is fused to the transcription factor CHOP in myxoid liposarcoma (5, 42, 44) and to ERG in some acute myeloid leukemias (15). The fact that EWS and its analog TLS are consistently fused to sequence-specific DNA binding domains further suggests that inappropriate regulation of transcription contributes to their transforming potential.

In order to define which genes are modulated by EWS/FLI, we have used a modified form of representational difference analysis (RDA). The technique of using RDA to detect differentially expressed genes was first developed by Hubank and Schatz (14). We demonstrate that RDA can be productively applied to the isolation of cDNA fragments of differentially expressed genes from cells stably expressing EWS/FLI and cells expressing normal FLI-1. We report here the cloning of 10 cDNA fragments from genes identified by this procedure. Using an inducible EWS/FLI construct, we found that the stromelysin 1 gene is transcriptionally up regulated within 8 h after *EWS/FLI* expression, indicating that this gene may be a direct target of EWS/FLI. These data present a list of candidate genes that may mediate or influence transformation by EWS/FLI and also demonstrate that RDA can be used to identify genes that are differentially expressed in two physiologically distinct cell populations.

MATERIALS AND METHODS

cDNA synthesis. Total RNA was prepared from NIH 3T3 derivative cell lines by guanidinium isothiocyanate lysis and CsCl gradient centrifugation (4). Polyadenylated RNA was purified from total RNA with oligo(dT) cellulose columns (Collaborative Research) or the polyATtract system (Promega) according to the manufacturer's instructions. Polyadenylated RNA (3 to 5 μg) was heated to 70°C

* Corresponding author. Mailing address: Department of Pediatrics, A2-312 MDCC, UCLA Medical Center, Los Angeles, CA 90095. Phone: (310) 825-2020.

for 10 min in a total volume of 20 μl and then was incubated with 1× first-strand buffer (GIBCO BRL), 5 U of human placenta ribonuclease inhibitor (GIBCO BRL), 1 mM dithiothreitol, 100 μg of bovine serum albumin (BSA) per ml, 50 mM deoxynucleoside triphosphates (dNTPs), 500 μg of T_{12-18} per ml, and 600 U of Superscript II reverse transcriptase (GIBCO BRL) in a total volume of 50 μl at 37°C for 1 h. Second-strand synthesis was accomplished by adding 10 μl of 5× DS buffer [250 mM Tris (pH 7.5), 250 mM KCl, 200 mM $(NH_4)_2SO_4$, 35 mM $MgCl_2$), 25 U of *Escherichia coli* DNA polymerase I (GIBCO BRL), 2.25 U of RNase H (GIBCO BRL), 5 U of *E. coli* ligase (GIBCO BRL), and dithiothreitol to 10 mM in a final volume of 100 μl and then by incubating the mixture at 16°C for 3 h. The final cDNA product was extracted with phenol-chloroform, ethanol precipitated, and resuspended in 20 μl of water. Tester and driver cDNA samples were prepared in parallel at all times.

RDA oligonucleotides. The following oligonucleotides were used for RDA: RBgl24, 5′-AGCACTCTCCAGCCTCTCACCGCA-3′; RBgl12, 5′-GATCTGCGGTGA-3′; JBgl24, 5′-ACCGACGTCGACTATCCATGAACA-3′; JBgl12, 5′-GATCTGTTCATG-3′; NBgl24, 5′-AGGCAACTGTGCTATCCGAGGGAA-3′; and NBgl12, 5′-GATCTTCCCTCG-3′. Underlined bases indicate complementary regions for each pair of oligonucleotides.

Amplicon preparation. Approximately one-half of a cDNA synthesis reaction mixture was digested with *Dpn*II (New England Biolabs), extracted with phenol-chloroform, and ethanol precipitated. After resuspension, ligation buffer was added (to a final concentration of 50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, 25 μg of BSA per ml, and 1 mM ATP), along with the oligonucleotides RBgl24 (8 μg) and RBgl12 (4 μg) in a total volume of 56 μl. The partially complementary RDA oligonucleotides were annealed by heating the reaction mixture to 55°C for 1 min and then by cooling it slowly to 14°C, leaving a *Dpn*II-compatible 5′ GATC overhang at one end. Four microliters (1,600 U) of T4 DNA ligase (New England Biolabs) was added, and the final mixture was incubated overnight at 16°C and diluted to 200 μl. In the first RDA experiment described below, a 1.3-kb human *FLI-1* cDNA was digested with *Sau*3AI and added to *Dpn*II-digested total cDNA prior to ligation so that the *FLI-1* cDNA contributed 1% of the total DNA.

Ligated *Dpn*II cDNA fragments were amplified by PCR, with oligonucleotide RBgl24 being used as a primer. Twelve identical PCRs were performed, each with 2 μl of the ligation mixture described above being used as a template in a final volume of 200 μl [67 mM Tris (pH 8.8), 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 20 μg of BSA per ml, 0.25 mM dNTPs, and 10 μg of RBgl24 per ml]. The reaction mixtures were heated to 72°C prior to the addition of 5 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus) and mineral oil, after which they were incubated at 72°C for another 5 min to fill the 5′ overhanging ends of the RBgl12 and -24 adapters. This was immediately followed by 20 cycles of PCR (95°C for 1 min and 72°C for 3 min) and a final extension at 72°C for 10 min. The reaction mixtures were pooled, extracted with phenol-chloroform, ethanol precipitated, and resuspended in 450 μl of water.

RDA subtractive hybridization and PCR. Driver DNA was prepared by digestion of 300 μl of amplicons with *Dpn*II followed by phenol-chloroform extraction and ethanol precipitation. Tester DNA was prepared by electrophoresis of 1/15 of the driver amplicons in 1.5% agarose–1× Tris-acetate-EDTA followed by excision and purification of DNA between 200 bp and 1,600 bp (QiaEX; Qiagen) and ligation to JBgl12 and -24 adapters as described above. Approximately 40 μg of driver DNA from one cell line was mixed with varying amounts of tester DNA from another cell line. Typical amounts of tester DNA were 0.4 μg (1:100), 0.1 μg (1:400), and 0.01 μg (1:4,000) for the first, second, and third iterations of RDA, respectively. In one experiment (see Results), four previously generated fragments were suppressed by adding 150 to 300 ng of each fragment, without adapters, to the hybridization at each iteration. The mixture was ethanol precipitated and resuspended in 4 μl of 3× EE buffer (30 mM EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid] and 3 mM EDTA [pH 8]) and covered with mineral oil. After the mixture was heated to 99°C for 4 min, 1 μl of 5 M NaCl was added and the solution was incubated at 67°C for 21 h. After hybridization, the reaction mixture was diluted to 400 μl with water plus tRNA carrier.

Tester-specific PCRs were performed in duplicate. Twenty microliters of the hybridization reaction mixture was used in a 200-μl reaction with oligonucleotide JBgl24. Unhybridized tester DNA (1 μl) was used in a control reaction to verify efficient adapter ligation and amplification. Reaction mixtures were heated to 72°C for 3 min prior to the addition of 5 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was maintained at 72°C for 5 min to fill the 5′ overhanging ends, providing a sequence complementary to JBgl24 at the ends of the molecules that had hybridized to the tester fragments. Oligonucleotide JBgl24 (2 μg) was then added, and the reaction mixtures were covered with mineral oil. Ten cycles of PCR were performed as described above, after which the duplicate reaction mixtures were pooled.

Following phenol-chloroform extraction and ethanol precipitation, the initial PCR product was resuspended in 36 μl of water. Four microliters of 10× MBN buffer (New England Biolabs) and 20 U of mung bean nuclease (New England Biolabs) were added, and the reaction mixture was incubated at 30°C for 30 min. A 160-μl amount of 50 mM Tris (pH 8.8) was added prior to the heating of the mixture to 97 to 99°C for 5 min, after which the reaction mixture was maintained on ice.

A second PCR was performed, also in duplicate. Twenty microliters of the mung bean nuclease-digested reaction mixture was added to 2 μg of oligonucleotide JBgl24 in the PCR buffer described above. After the mixture was heated to 95°C for 1 min and cooled to 80°C, 5 U of Amplitaq was added. Eighteen cycles of PCR were performed as described above. Like products were pooled, extracted with phenol-chloroform, ethanol precipitated, and resuspended in 40 μl of water. Control reactions were performed with 1 μl of the previous control reaction mixtures.

After PCR, the products were digested with *Dpn*II and ligated to a new adapter, NBgl12 and -24 (after the first iteration) or JBgl12 and -24 (after the second), and the RDA process was repeated with this DNA being used as the tester and with the corresponding oligonucleotides (NBgl24 and JBgl24) being used as PCR primers. Alternatively, products were gel purified in 1.0 to 1.4% agarose–0.5× Tris-borate-EDTA and subcloned into pBluescript KS II+ (Stratagene) previously digested with *Bam*HI and dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim). Transformed XL-1 Blue (Stratagene) colonies were screened by colony PCR followed by polyacrylamide electrophoresis.

Plasmids. Expression constructs containing *EWS/FLI*, *TLS/FLI*, and *EWS-(mut9)/FLI* have been previously described (21, 32). The *EWS/ETV1* fusion gene was constructed by ligating an 823-bp *Eco*RI-*Bam*HI fragment containing the 5′ region of *EWS* to a 500-bp fragment of *EWS/ETV1* (16) that had been generated by PCR amplification with *Pfu* DNA polymerase (Stratagene) and primers ESBP1 (32) and ETV-3 (5′-GGTCTAGATTAATACACGTAGCCTTCGTTG T-3′) followed by digestion with *Bam*HI and *Xba*I. The *Eco*RI-*Bam*HI fragment containing 5′ *EWS* and the *Bam*HI-*Xba*I fragment containing the fusion point and 3′ *ETV1* were ligated to the *Eco*RI and *Xba*I sites of the retroviral vector pSRαΔXba (21). *EWS/ERG* was generated similarly. A portion of the *EWS/ERG* mRNA had previously been amplified from cell line TTC-466 by reverse transcriptase PCR with primers ESBP1 and ERG-1 (49). The product was digested partially with *Bam*HI and completely with *Hin*dIII, and a 750-bp product extending from the *Bam*HI site in *EWS* to the *Hin*dIII site immediately following the stop codon of *ERG* was gel purified. This fragment, together with the *Eco*RI-*Bam*HI fragment from the 5′ end of *EWS*, was ligated with pSRαΔXba previously digested with *Eco*RI and *Hin*dIII.

The plasmid pMTCB6+EWS/FLI was created by excision of *EWS/FLI* with *Not*I and *Hin*dIII followed by ligation into pMTCB6+ (kindly provided by Frank Rauscher III), placing the *EWS/FLI* cDNA immediately 3′ of the ovine metallothionein promoter and 5′ of splicing and termination sequences from the human growth hormone gene.

The π3-luciferase reporter construct was created by excising the trimeric π element and c-*fos* minimal promoter from J12(μE2-π) (45) with *Hin*dIII and *Bam*HI and ligating the approximately 300-bp fragment into pT81Luc (38), which had been digested with *Hin*dIII and *Bgl*II. The resulting plasmid contains two upstream polyadenylation signals, three repeats of the μE2-π element, and a minimal promoter from c-*fos* (−71 to +109) followed by the firefly luciferase structural gene and simian virus 40 termination sequences.

Cell lines and tissue culture. Helper-free retrovirus stocks were prepared as previously described (32). NIH 3T3 cells expressing *EWS/FLI*, *EWS(mut9)/FLI*, and *TLS/FLI* fusion genes have been described previously (21, 32). Cells expressing *EWS/ERG* and *EWS/ETV1* were generated similarly, with the retrovirus expression constructs described above being used. All cell lines were cultured in Dulbecco modified Eagle medium with 10% calf serum.

To achieve metal-dependent transcription of *EWS/FLI*, plasmid pMTCB6+ EWS/FLI was linearized with *Pvu*I and electroporated into NIH 3T3 cells (960 μF, 260 V). Stable transfectants were selected in 450 μg of G418 (GIBCO BRL) per ml for 14 days. Polyclonal populations were incubated with 50 μM or 100 μM $ZnSO_4$ for 24 h and then seeded in soft agar (32) that had been supplemented with 50 μM or 100 μM $ZnSO_4$. Ten colonies were isolated from soft agar, expanded, and analyzed. Clones were screened for inducible *EWS/FLI* mRNA expression and a reproducible dependency on $ZnSO_4$ for growth in soft agar. Clone MT-EWS/FLI(12.7) was selected for further experiments on the basis of its observed low levels of constitutive *EWS/FLI* expression, high levels of induced expression, and vigorous but strictly zinc-dependent proliferation in soft agar. The cell line MT46 was derived by electroporation of NIH 3T3 cells with pMTCB6+ followed by G418 selection as described above.

Transcriptional activation of the π3-luciferase reporter construct was measured by lipofection of NIH 3T3 and MT-EWS/FLI(12.7) cells as described previously (21). Plates (diameter, 100 mm) that were 70 to 90% confluent were cotransfected with 2 μg of the reporter construct, 6 μg of the *EWS/FLI* expression construct pSRαMSV EWS/FLI tk neo(ΔHindIII) (33), 6 μg of the E12 expression construct pJ3ΩE12 (45), 4 μg of the plasmid CMV-βgal (identical to pON260) (50), and pBluescript KS II+ to bring the final DNA content to 20 μg. Control samples lacked EWS/FLI, E12, both E12 and EWS/FLI, or E12, EWS/FLI, and π3-luciferase. CMV-luciferase was used as a positive control (courtesy of Charles Sawyers). Each plate was incubated overnight, trypsinized, and split into four 60-mm-diameter plates and incubated overnight again. Fresh medium was added to all plates simultaneously. $ZnSO_4$ was added to a concentration of 100 μM at time zero, 11 h, and 16 h or not at all, and the cells were harvested at 22 h. Assays for luciferase and β-galactosidase were performed as described previously (21). Luciferase activity was standardized to β-galactosidase activity to account for any variation in transfection efficiency.

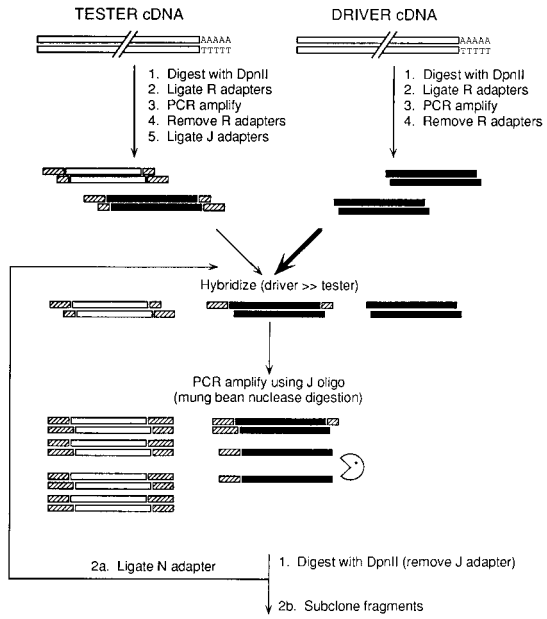

FIG. 1. Schematic depicting cloning by RDA of sequences unique to tester DNA. Tester and driver amplicons are generated by ligating R adapters to *Dpn*II-digested oligo(dT)-primed cDNAs. After PCR amplification, R adapters are removed by *Dpn*II digestion and J adapters (hatched boxes) are ligated to the tester fragments only. The tester population is hybridized to an excess of driver. Fragments present predominantly in the tester (open rectangles) will form homodimers with an adapter at each end. Fragments present in both driver and tester (filled rectangles) will form heterodimers with an adapter only at one end. The hybridization mix is amplified by PCR with the J24 oligonucleotide. Homodimers with adapters amplify efficiently. Amplification of heterodimers results in single-stranded species that are destroyed by mung bean nuclease digestion. J adapters are then removed by *Dpn*II digestion, and fragments are either subcloned into plasmids or ligated to N adapters and subjected to another round of RDA subtraction. Three to four iterations are performed with increasing driver-to-tester ratios.

Northern (RNA) blots. RNA purification and Northern blotting was performed as described previously (32). To quantitate the expression levels of EAT-2, 600-bp fragments from β-actin or EAT-2 were gel purified and labelled by random priming (Boehringer Mannheim) to specific activities of 1.4×10^9 cpm/μg and 1.3×10^9 cpm/μg, respectively. After simultaneous hybridization with both probes, the blot was quantitated with a Phosphorimager (Molecular Dynamics).

RESULTS

RDA detects unique fragments from heterogeneous cDNA populations. RDA was originally developed to identify fragments unique to one of two populations of genomic DNAs (2, 27–29). The sample containing sequences of interest is termed the tester, and the sample used for comparison is the driver. DNA from each population is digested with a restriction endonuclease and then ligated to short adapter molecules used for PCR amplification. The products of amplification, termed amplicons, contain fragments that are between 200 and 1,500 bp in length. To isolate amplicons unique to the tester pool, tester DNA is ligated to new adapters and mixed with the driver amplicon in a subtractive hybridization. PCR with primers for the new adapters preferentially amplifies tester-tester homoduplexes. This process is reiterated several times, with hybridization taking place with increasing ratios of driver to tester, until only fragments unique to the tester remain (see Fig. 1 for a summary and Materials and Methods for a more detailed description).

To test if this procedure would work with cDNA being used as the substrate, a model system was constructed. Poly(A)$^+$ RNA isolated from NIH 3T3 cells was used to make oligo(dT)-primed cDNA. After digestion with *Dpn*II, cDNAs were split into two pools, and *Sau*3A-digested *FLI-1* cDNA fragments were added to one pool only, which became the tester. The tester pool was subtracted with the driver pool, and the difference was selectively amplified by PCR. This difference product was then sequentially subjected to two more cycles of subtraction followed by PCR amplification. The final difference product contained the *FLI-1* cDNA test fragments to the exclusion of other cDNA species (Fig. 2). This demonstrated that RDA could be used to isolate unique fragments from heterogeneous populations of cDNAs and suggested that it could be applied to the detection of differentially expressed genes.

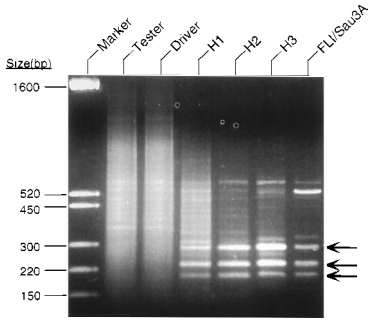

FIG. 2. Gel demonstrating recovery of FLI-1 test fragments by RDA. The tester amplicons consisted of FLI-1 test fragments mixed with total NIH 3T3 cDNA and amplified by PCR. Driver amplicons were derived from NIH 3T3 cDNA. Tester and driver amplicons were hybridized to each other at molar ratios of 1:100, 1:800, and 1:8,000, respectively, for each successive round of RDA depicted in lanes H1, H2, and H3. All three of the FLI-1 *Sau*3A-*Sau*3A fragments (arrows) were enriched almost to the exclusion of other cDNA species. The one 520-bp FLI-1 fragment that was not enriched was a *Hin*dIII-*Sau*3A fragment and therefore not able to be amplified by RDA under these conditions.

RDA identifies differentially expressed genes from NIH 3T3 cells containing *EWS/FLI* or normal *FLI-1*. EWS/FLI transforms NIH 3T3 cells, whereas normal FLI-1 does not (33). The different biologic activities of these two molecules may reflect their abilities to transcriptionally activate particular target genes in NIH 3T3 cells. To test this hypothesis, RDA was performed on polyclonal NIH 3T3 populations that stably expressed either *EWS/FLI* or normal *FLI-1*. Oligo(dT)-primed cDNAs made from both cell lines were digested with *Dpn*II and used to generate the respective amplicons. To identify genes up regulated by EWS/FLI but not by FLI-1, an RDA subtracting EWS/FLI amplicons with FLI-1 amplicons (EWS/FLI minus FLI-1) was performed. To identify potential genes transcriptionally repressed by EWS/FLI, we performed the reciprocal subtraction in parallel (FLI-1 minus EWS/FLI).

The initial EWS/FLI–FLI-1 RDA yielded five apparent bands (Fig. 3) that were individually subcloned (EF.33, EF.35, EF.323, EF.41, and EF.4L) (Table 1). Northern blot experiments revealed that each of these fragments hybridized to distinct RNA species that were present in *EWS/FLI*-expressing NIH 3T3 cells but absent in either the parent or *FLI-1*-expressing NIH 3T3 cell lines (Fig. 4A and Table 1). RDA fragments were recovered from genes expressed at both high and low copy numbers as estimated by Northern analysis. For example, EF.41 was derived from a transcript present at 10% of the level of β-actin transcripts (data not shown). In an effort to recover more fragments from differentially expressed genes, RDA was repeated after four of the initial RDA-derived fragments (EF.33, EF.35, EF.323, and EF.41) were added to the FLI-1 amplicons to increase their representation in the RDA driver. This effectively suppressed the appearance of these four bands during three cycles of RDA. Two additional fragments were generated (EF.1171 and EF.1172); both hybridized to differ-

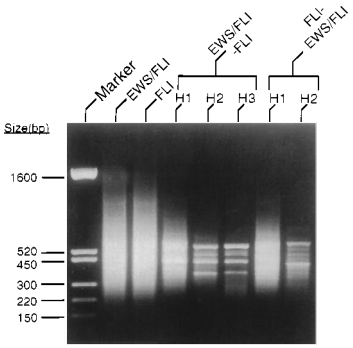

FIG. 3. Gel showing RDA of cDNA populations generated from NIH 3T3 cells expressing *EWS/FLI* or normal *FLI-1*. After three rounds of RDA subtracting EWS/FLI with FLI-1 cDNAs, five apparent fragments were recovered and subcloned. The reciprocal FLI-1-minus-EWS/FLI RDA yielded two fragments. Only the major bands seen in the H2 lane persisted by the third round of RDA (data not shown).

entially expressed transcripts. Finally, the last cycle of this RDA was repeated with a reduced driver-to-tester ratio (500:1 rather than 4,000:1). This resulted in many low-intensity bands that were subcloned and hybridized to Northern blots. Only 1 of 12 of these fragments detected a differentially expressed transcript (EF.119), indicating that this change decreased the signal-to-noise ratio. We termed differentially expressed genes *EAT* genes, for EWS/FLI-activated transcript.

The reciprocal RDA, subtracting FLI-1 cDNAs with EWS/FLI, resulted in two major bands (FE.1173 and FE.1174). This pattern was reproduced with independently derived amplicons in a repeated RDA procedure. As before, each fragment detected transcripts that were present at higher levels in NIH 3T3 cells expressing *FLI-1* than in those expressing *EWS/FLI* (Table 1). These were termed *ERT*, for EWS/FLI-repressed transcript.

Overall, 3 of 10 RDA probes detected additional transcripts that were present in NIH 3T3 cells expressing either *FLI-1* or *EWS/FLI*. Nucleotide sequence analysis of all RDA fragments revealed the identities of the parent genes in 3 of 10 cases (Table 1).

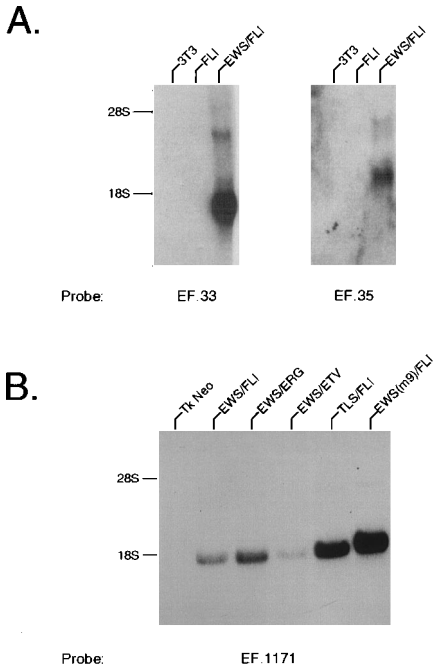

FIG. 4. Northern blots demonstrating that RDA probes detect differentially expressed transcripts. (A) Two representative RDA fragments recovered from the EWS/FLI-minus-FLI-1 subtraction were used to probe Northern blots containing RNAs from NIH 3T3 cells or NIH 3T3 cells expressing either *FLI-1* or *EWS/FLI*. All RDA fragments derived from the transcripts were expressed only in *EWS/FLI* NIH 3T3 cells. (B) An RDA fragment detects a transcript induced by the transforming variants of EWS/FLI but only minimally detects that of a nontransforming variant, EWS/ETV1. For more complete data, see Table 1. Positions of ribosomal RNAs are shown as a size reference.

Most genes detected by RDA are also up regulated by variant *EWS/FLI* fusion genes. Our RDA results indicate that expression of *EWS/FLI* increased the transcript levels of several genes. In an effort to further discern which of the EWS/FLI target genes were associated with the transformed phenotype, we performed Northern experiments with NIH 3T3 cell

TABLE 1. Summary of cDNA fragments generated from stable cell lines by RDA

Probe	Size (bp)	Gene[a]	mRNA (kb)[b]	Expression in[c]:							
				NIH 3T3	tk neo	FLI	EWS/FLI	EWS/ERG	EWS/ETV	TLS/FLI	mut9/FLI
EWS/FLI minus FLI-1											
EF.33	430	(*EAT-1*)	1.4	−	−	−	+	+	+	−	+
EF.35	500	P-450	2.3	−	−	−	+	+	−	+	+
EF.323	470	*CK 15*	1.7	−	−	−	+	+	−	+	+
EF.41	390	(*EAT-2*)	1.5	−	−	−	+	+	−	+	+
EF.119	360	(*EAT-2*)	1.5	−	−	−	+	+	−	+	+
EF.4L	240	(*EAT-3*)	2.3[d]	−	ND[e]	−	+	ND	ND	ND	ND
EF.1171	500	Stromelysin 1	1.7	−	−	−	+	+	−	+	+
EF.1172	310	(*EAT-4*)	1.4[d]	−	−	−	+	+	±	−	+
FLI-1 minus EWS/FLI											
FE.1173	510	(*ERT-1*)	1.7[d]	+	+	+	±	±	+	−	−
FE.1174	360	(*ERT-2*)	3.2	ND	+	+	±	±	±	±	±

[a] Sequence identity, if known, or provisional name in parentheses.
[b] Size of the major differentially expressed species.
[c] Relative expression data are based on Northern blot hybridization to RNA from parental cells (NIH 3T3), cells infected with an empty retroviral vector (tk neo), and NIH 3T3 cells expressing the indicated fusion proteins (see the text for details). Evaluations of mRNA levels (+, readily detected; −, little to no mRNA detected; ±, intermediate levels detected) are consistent for each probe but cannot be compared among probes.
[d] The probe hybridized with one or more additional transcripts that were not differentially expressed.
[e] ND, not determined.

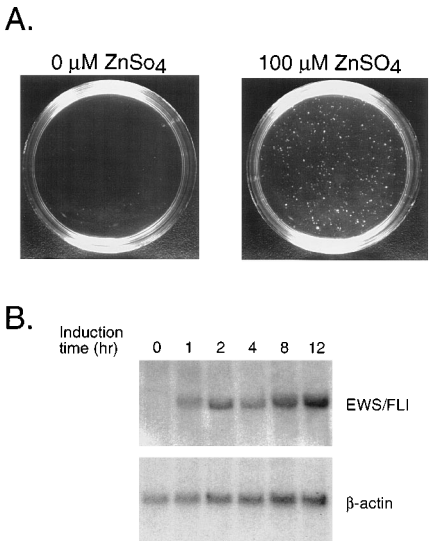

FIG. 5. Control of *EWS/FLI* expression with the metallothionein promoter construct. (A) Agar assay showing polyclonal NIH 3T3 populations containing the MT-EWS/FLI construct. Under standard plating conditions, cells are unable to form colonies. Adding Zn^{2+} to the medium induces expression of *EWS/FLI* and results in the formation of macroscopic colonies. (B) Induction of *EWS/FLI* transcripts by Zn^{2+} in MT-EWS/FLI(12.7) cells. Northern blots from MT-EWS/FLI(12.7) cells exposed to Zn^{2+} for various time periods were hybridized to a *FLI-1* cDNA probe. EWS/FLI transcripts are evident within 2 h and reach maximal levels by 8 h. Hybridization of the same membrane with a β-actin probe was used to verify equal loading and transfer among gel lanes.

lines expressing fusion genes structurally related to *EWS/FLI*. Four cell lines were generated: two expressing *FLI* domain mutations and two containing *EWS* domain mutations. *EWS/ERG* and *EWS/ETV1* fusions are found in certain Ewing's sarcomas and contain the DNA binding domains of the ETS genes, *ERG* and *ETV1*, instead of *FLI-1*. *EWS*(*mut 9*)*/FLI* contains only the amino-terminal 80 amino acids of *EWS*—the smallest portion of *EWS* we have found that confers near full-length transforming activity (21). *TLS/FLI* is a recombinant fusion of the 3′ end of *FLI-1* and the 5′ portion of *TLS*, a gene that is structurally related to *EWS*. With the exception of *EWS/ETV1*, all of these chimeric mutants transform NIH 3T3 cells (reference 21 and unpublished data).

RDA fragments were used to probe Northern blots containing RNAs from NIH 3T3 cell lines expressing mutant chimeric fusions. Target genes that were up regulated by EWS/FLI could also be induced by other chimeric fusions. With the exception of the gene *EAT-1*, EWS/ERG, EWS(mut 9)/FLI, and TLS/FLI increased transcript levels of EWS/FLI target genes. The converse was also true for *ERT-1*, which is a gene repressed by EWS/FLI and which was down regulated in these mutant cell lines. EWS/ETV1 caused little or no change in *EAT* or *ERT* transcript levels, again with the exception of *EAT-1*. Thus, the ability of each mutant fusion to modulate most of the EWS/FLI target genes correlated with its ability to transform NIH 3T3 cells (Fig. 4B and Table 1).

The stromelysin 1 gene is up regulated soon after the induction of EWS/FLI expression. EWS/FLI may mediate its effect on target genes either by directly activating transcription or by inducing a transcription factor cascade. In order to bias the screen in favor of genes that EWS/FLI directly regulates, RDA was performed with cell lines containing an inducible *EWS/FLI* construct. A full-length *EWS/FLI* cDNA was placed under the transcriptional control of the ovine metallothionein promoter in the vector pMTCB6+ (generously provided by Frank Rauscher III) and transfected into NIH 3T3 cells. Clones requiring Zn^{2+} for transformation were selected by a single passage in soft agar (Fig. 5A). Northern analysis of MT-EWS/FLI clone 12.7 demonstrated a greater than 10-fold increase in *EWS/FLI* mRNA within 4 to 8 h of the addition of Zn^{2+} to the medium (Fig. 5B).

An optimal EWS/FLI induction time that would be just long enough for transcription of direct target genes was empirically determined. A reporter gene assay was used to estimate the time between the addition of Zn^{2+} and the induction of a direct EWS/FLI target gene. We had shown previously that EWS/FLI can transcriptionally activate a model reporter gene consisting of three copies of the π immunoglobulin enhancer element upstream of a luciferase gene (21). This construct was transiently transfected into MT-EWS/FLI(12.7) cells together with an E12 expression plasmid. (The E12 transcription factor is required for maximal stimulation of this reporter.) After transfection, EWS/FLI was induced by the addition of Zn^{2+} at fixed time points, and the cells were later harvested and assayed for luciferase activity. Maximal induction of this model reporter gene occurred within 11 h of the induction of *EWS/FLI* transcription (Fig. 6).

To enrich for primary EWS/FLI target genes, RDA was performed after a short induction course. MT-EWS/FLI(12.7) cells and NIH 3T3 cells stably transfected with the empty pMTCB6+ vector (MT46) were expanded, exposed to Zn^{2+} for 8 h, and harvested. RDA subtractions searching for genes that were both up regulated [MT-EWS/FLI(12.7) minus MT46] and down regulated [MT46 minus MT-EWS/FLI(12.7)] by EWS/FLI were performed in parallel. After four cycles, only the MT-EWS/FLI(12.7)-minus-MT46 RDA yielded three bands, all of which detected differentially expressed transcripts by Northern analysis (Table 2). Sequence analysis revealed the identities of two of these RDA fragments. The first (ZN.501)

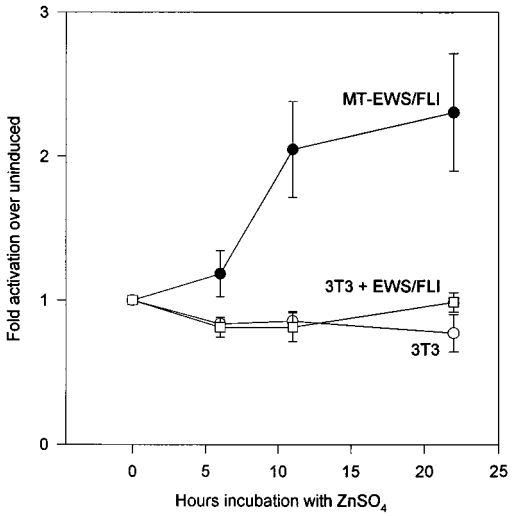

FIG. 6. Induction of a model reporter gene by EWS/FLI. A luciferase reporter construct was cotransfected with an E12 expression plasmid into NIH 3T3 cells or into MT-EWS/FLI(12.7) cells. A retroviral *EWS/FLI* expression vector (32) was also added to NIH 3T3 cells. Cells were then exposed to Zn^{2+} for various time periods, harvested, and assayed for luciferase activity. Exposure of MT-EWS/FLI(12.7) cells to Zn^{2+} resulted in a transcriptional up regulation of the reporter gene, as measured by the increasing luciferase activity that reached a plateau by 11 h. Luciferase activity was unchanged by Zn^{2+} in NIH 3T3 cells in the presence or absence of EWS/FLI. In NIH 3T3 cells, absolute luciferase levels were approximately twofold higher with EWS/FLI than without (data not shown). Four separate transfections were performed, and the results were averaged. The error bars show standard errors of the means.

TABLE 2. Summary of cDNA fragments generated from an inducible EWS/FLI cell line by RDA[a]

Probe	Size (bp)	Gene[b]	mRNA (kb)[c]	Expression in[d]:				
				tk neo	EWS/FLI	MT-EWS/FLI		MT46 +Zn
						−Zn	+Zn	
ZN.501	770	*EWS*	2.0[e]	−	+	−	+	−
EF.1171	500	Stromelysin 1	1.7	−	+	−	+	−
ZN.503	440	(*EAT-5*)	2.7[e]	−	−	−	+	−

[a] MT-EWS/FLI(12.7) cells (MT-EWS/FLI) that express *EWS/FLI* in response to zinc and cells transfected with the corresponding empty vector (MT46) were incubated in the absence (−Zn) or presence (+Zn) of 100 μM zinc sulfate for 8 h. The products of the MT-EWS/FLI(12.7)-minus-MT46 RDA are shown.
[b] Sequence identity, if known, or provisional name in parentheses.
[c] Size of the major differentially expressed species.
[d] Relative expression data are as described in footnote c to Table 1.
[e] The probe hybridized with one or more additional transcripts that were not differentially expressed.

was from the 5′ end of the transfected *EWS/FLI* fusion gene. The second (EF.1171) matched the reported sequence of murine stromelysin 1 and was identical to a previously identified RDA fragment. Northern analysis comparing induced and uninduced MT-EWS/FLI(12.7) cells demonstrated a >10-fold increase in stromelysin mRNA after an 8-h incubation with Zn^{2+} (Fig. 7).

DISCUSSION

Though RDA was originally developed to isolate differences in genomic DNA populations, our data provide the second demonstration that RDA can be used to detect differentially expressed genes. Using this same technique, Hubank and Schatz have recently identified several genes that are up regulated in pre-B cells after exposure to caffeine (14). We have used RDA to identify potential target genes of EWS/FLI, an oncogenic transcription factor. This technique could readily be applied to study the effectors of other transcription factors for which transcriptional targets remain to be identified. In fact, this strategy is not limited to nuclear transcription factors but could easily be used to characterize the genetic response to cytoplasmic oncoproteins or a wide range of exogenous stimuli.

RDA holds particular advantages when compared with other subtraction methodologies. First, in comparison with the time needed for the generation of subtracted cDNA libraries, RDA is fast. A complete analysis can be performed within 1 month. Second, while standard subtraction libraries favor the isolation of highly expressed genes, we show that RDA is able to capture genes expressed at low levels as well. Third, RDA can capture coding portions of expressed genes. A sequence analysis of 4 of 11 RDA fragments was sufficient to identify the parent gene, obviating the need for the subsequent cloning of full-length cDNAs. This contrasts with differential display (23–25), which favors the isolation of 3′ untranslated regions. Finally, our experience to date indicates that RDA is highly reproducible.

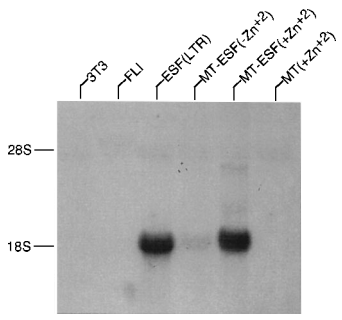

FIG. 7. Northern blot demonstrating early induction of stromelysin 1 by EWS/FLI. The stromelysin 1 RDA fragment was hybridized to a Northern blot containing the following NIH 3T3 populations: 3T3, parent cells; FLI-1, cells constitutively expressing normal *FLI-1*; ESF(LTR), cells constitutively expressing *EWS/FLI*; MT-ESF(−Zn^{+2}), MT-EWS/FLI(12.7) cells without Zn^{2+}; MT-ESF(+Zn^{+2}), MT-EWS/FLI(12.7) cells after an 8-h Zn^{2+} exposure; and MT(+Zn^{+2}), MT46 cells after an 8-h Zn^{2+} exposure. Rehybridization with a β-actin probe verified equal loading and transfer (data not shown).

While RDA appears to be a promising technique, its limits remain to be defined. While the specificity of RDA is high—virtually all the major RDA bands detected differentially expressed mRNA species—its sensitivity is unclear. The relative differences in transcript levels between tester and driver populations may be one potential determinant of detectability. Most of the genes identified by RDA were induced by EWS/FLI at least 10-fold. Further experience is necessary to determine how effectively RDA may recover genes that are up regulated at more modest levels. Other factors may also limit the ability of RDA to detect differentially expressed genes. Small differences in amplification efficiency may be important, since over 100 cycles of DNA synthesis during amplicon preparation and three rounds of RDA are performed. The current protocol may place an absolute limit on the number of fragments generated. Finally, detection of a transcript requires appropriately spaced restriction sites for a chosen enzyme.

Two observations suggest that we are approaching a limit of detection by this protocol. First, two independent RDA fragments (EF.41 and EF.119) were later discovered to derive from the same cDNA (Table 1) (unpublished data). Interestingly, RDA produced fragment EF.41 at much higher yield than that of fragment EF.119. Second, RDA performed while suppressing appearance of the previous major products resulted in detection of only two additional fragments. One of these (EF.1171) comigrated with an earlier fragment (EF.33) and may have in fact been present in the original RDA. The relative lack of new bands indicates that the procedure must be modified if more differentially expressed genes are to be detected. We are currently investigating the effects of adjusting hybridization conditions, changing the restriction enzyme used for representation, and increasing the fold difference in gene expression by using higher-expression cell lines.

EWS/FLI probably does not up regulate all target genes directly. It is more likely that EWS/FLI directly interacts with the regulatory sequences of a minority of target genes whose products subsequently modulate the activities of other genes. Identifying such primary target genes is a crucial step in determining the biochemical mechanism of action of EWS/FLI. By coupling RDA to an inducible EWS/FLI system, we demonstrate that the stromelysin 1 gene is up regulated within 8 h

of EWS/FLI expression. This suggests that the stromelysin 1 gene may be a primary target that responds to EWS/FLI but not normal FLI-1. Interestingly, a tandem ETS binding motif that is responsive to c-*ets1* and c-*ets2* has been found in the rat and human stromelysin 1 promoters (53, 54). Studies are in progress to determine whether this element is also responsive to EWS/FLI.

EWS/FLI probably up regulates a variety of genes which may not all directly contribute to cellular transformation. For example, cytokeratin 15 is an intermediate filament protein that is up regulated by EWS/FLI. At present, however, its contribution to dysregulating cell growth is uncertain. It is also unclear whether this observation relates to the cytoskeletal and morphologic changes frequently observed in transformed cells. Dominant negative alleles (41) may help answer these questions. Variable expression of epithelial cytokeratins has been documented in Ewing's sarcoma by immunohistochemistry (9, 12, 37, 48), although antibodies specific to cytokeratin 15 have not been tested. A related gene encoding cytokeratin 8 (*mK8*, also called *endo A*) contains an enhancer that confers responsiveness to ETS family members (10, 13, 47, 52).

Cytochrome P-450s encode a large family of monooxygenases that modify a diverse array of both exogenous and endogenous substrates. EWS/FLI induces the expression of a murine gene closely related to rat cytochrome P-450-4F1 (*CYP4F1*) that was originally isolated from various chemically induced hepatic tumors (3). Unlike those of most P-450 genes that were repressed during tumorigenesis, *CYP4F1* mRNA levels increased, suggesting a possible role in oncogenesis in this model system. CYP4F1 and related human proteins CYP4F2 and CYP4F3 act as ω-hydroxylases of the arachidonate derivatives leukotriene B_4, lipoxin A_4, and lipoxin B_4 (18, 19, 35, 36, 51). These pleiotropic inflammatory mediators are inactivated by P-450-mediated oxidation. It is unclear whether this enzyme contributes to transformation by EWS/FLI, though it is conceivable that it could do so by modulating the immune function or modifying the substrates in signal transduction pathways.

Finally, our initial RDA has identified genes previously implicated in transformation. EWS/FLI up regulates stromelysin 1, a member of the metalloproteinase family that digests a variety of extracellular matrix proteins. While metalloproteinases function normally in tissue remodelling during repair and embryogenesis, the ectopic expression of these proteins has also been linked to tumor invasion and metastasis (reviewed in references 26 and 31). In particular, metalloproteinase activity has been shown to be required (34, 46) or sufficient (17) for tumor invasion in some systems. We are currently investigating whether this and other transcripts cloned by RDA influence the phenotype of murine fibroblasts or Ewing's sarcoma cells.

Our data demonstrate that at least in part, EWS/FLI and FLI-1 modulate distinct target genes in NIH 3T3 cells. This is consistent with the notion that EWS/FLI acts as an aberrant transcription factor. Genes affected by EWS/FLI were also modulated in the same way by EWS/ERG, a fusion found in approximately 10% of Ewing's sarcomas. This suggests that in addition to their structural similarities, EWS/FLI and EWS/ERG are probably playing equivalent functional roles in cellular transformation. In contrast, only one of eight EWS/FLI target genes was also up regulated by EWS/ETV1. This suggests at least two possibilities: (i) EWS/ETV1 is not involved in Ewing's sarcoma tumorigenesis, and (ii) EWS/ETV1 activates only a portion of the EWS/FLI transformation pathway, and other complementing genetic alterations are needed in tumors harboring this fusion. With the methodologies developed here, the biochemical mechanisms and biologic pathways of EWS/FLI transformation are accessible for molecular study.

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