An association between viral genes and human oncogenic alterations: The adenovirus E1A induces the Ewing tumor fusion transcript EWS–FLI1

RICARDO SANCHEZ-PRIETO[1], ENRIQUE DE ALAVA[2], TERESA PALOMINO[1], JUAN GUINEA[1], VICTOR FERNANDEZ[1], SILVIA CEBRIAN[1], MATILDE LLEONART[1], PABLO CABELLO[3], PILAR MARTIN[1], CARLOS SAN ROMAN[3], RAFAEL BORNSTEIN[4], JAVIER PARDO[2], ANA MARTINEZ[1], FERNANDO DIAZ-ESPADA[5], YVE BARRIOS[5] & SANTIAGO RAMON Y CAJAL[1]

[1]*Department of Pathology,* [5]*and Immunology, Clinica Puerta de Hierro, San Martin de Porres* 4, *28035 Madrid, Spain*
[2]*Department of Pathology, Clinica Universitaria de Navarra, Avda Pio XII 36, 31008 Pamplona, Spain*
[3]*Department of Genetics, Hospital Ramon y Cajal, Cra Colmenar Km 9.100, 28034 Madrid, Spain*
[4]*Department of Hematology, Hospital 12 de Octubre, Km 5,4, 28041 Madrid, Spain*
R.S.-P. present address: NIH/NIDR, Bldg 30, Bethesda, Maryland 20892, USA
Correspondence should be addressed to: S.R.C.; email: srcajal@ctv.es

Malignant transformation of human cells requires the accumulation of multiple genetic alterations, such as the activation of oncogenes and loss of function of tumor suppressor genes or those related to genomic instability. Among the genetic alterations most frequently found in human tumors are chromosomal translocations[1] that may result in the expression of chimeric products with transforming capability or are able to change the expression of oncogenes. We show here that the adenovirus early region 1A (E1A) gene can induce a specific human fusion transcript (EWS–FLI1) that is characteristic of Ewing tumors[2]. This fusion transcript was detected by RT–PCR in normal human fibroblasts and keratinocytes after expression of the adenovirus E1A gene, as well as in human cell lines immortalized by adenoviruses. Cloning and sequencing of the RT–PCR product showed fusion points between EWS and FLI1 cDNA identical to those detected in Ewing tumors. In addition, we detected a chimeric protein by western blot analysis and immunoprecipitation and a t(11,22) by fluorescent *in situ* hybridization. This association between a single viral gene and a specific human fusion transcript indicates a direct link between viral genes and chromosome translocations, one of the hallmarks of many human tumors.

Early region 1A (E1A) of human adenovirus type 5 encodes two main proteins, 289R and 243R, that exert myriad cellular effects[3–8]. Tumors formed by diverse malignant cells, expressing the E1A protein, have a distinctive cell growth pattern characterized by a mainly uniformly round cell population[9–11]. These histological characteristics are also found in the Ewing family of tumors, which constitute a spectrum of aggressive bone and soft tissue neoplasias. Most Ewing tumors show fusion transcripts; 90% of these correspond to the EWS–FLI1 fusion gene, used for clinical diagnosis[2,12,13]. In addition, the EWS–FLI1 gene product transforms NIH3T3 cells, which acquire the appearance of polygonal cells[14].

Given the morphological similarities between Ewing tumors and E1A-expressing tumors, we investigated whether expression of the E1A gene in human cells could elicit the specific fusion transcript EWS–FLI1. We first transfected HeLa (cervix carcinoma cells) with the plANeo E1A plasmid. The transcript EWS–FLI1 was detected by RT–PCR only in the HeLa–E1A cells, and not in nontransfected HeLa cells (Fig. 1*a*). The amplified product was simultaneously recognized by the EWS and FLI1 probes in Southern blot analysis (Fig. 1*d*). Because HeLa cells contain multiple genetic alterations, we expressed the E1A gene in normal IMR90 human fibroblasts (Fig. 2*b*) and in the nontumorigenic human keratinocyte cell line HaCaT, and achieved a high level of E1A protein expression. RT–PCR and Southern blot analysis also detected the specific EWS–FLI1 fusion transcript in E1A-expressing IMR90 and HaCaT cells (Figs. 1 and 2). Given these results, we next investigated whether human cells immortalized by adenovirus also produced this specific fusion transcript. We used HEK 293 cells, a human embryonic kidney cell line immortalized with human adenoviruses, as well as cells derived from them, such as the Phoenix and BOSC-23 cell lines. These cell lines also contain the EWS–FLI1 fusion transcript (Figs. 1 and 2). In all cases, the amplified PCR products were also recognized by the EWS and FLI1 probes in Southern blot analysis.

To ensure that the RT–PCR bands were specific for that chimeric gene product, we cloned and sequenced the PCR products. The EWS–FLI1 fusion proteins detected in the E1A-expressing IMR90 fibroblasts and HeLa cells, as well as in the HEK 293 cells, had sequences identical to the type 1 EWS–FLI1 fusion gene (Fig. 1*c*), or, in other cases, to the type 2 EWS–FLI1 hybrid transcript previously reported in Ewing tumors. Next, we did northern blot analysis with a probe corresponding to the type 2 EWS–FLI1 junction cDNA using total RNA extracted from these cell lines. Fusion transcripts were again detectable only in the E1A-expressing cells (Fig. 2*a*).

To ensure that the chimeric fusion transcript EWS–FLI1 was not an artifact of RT–PCR, we again infected the IMR90 cells with the amphotropic retrovirus containing the ecotropic receptor and also infected cells with ecotropic E1A retroviruses. We then did RT–PCR analysis of the new populations of E1A-expressing cells. We detected a hybrid transcript of higher molecular weight than in the previous infections (Fig. 1 and 2*c*). Furthermore, after immunoprecipitation with antibody against FLI1 and subsequent western blot analysis with antibody against EWS, we detected a specific band in HEK 293 cells as well as in E1A-expressing IMR90 cells, and not in control IMR90 cells (Fig. 2*b*). In addition, western blot analysis with a polyclonal antibody against FLI1 demonstrated an immunoreactive band of approximately 70 kDa in HEK 293 cells and in a Ewing tumor cell line (RD-ES), and a band of ap-

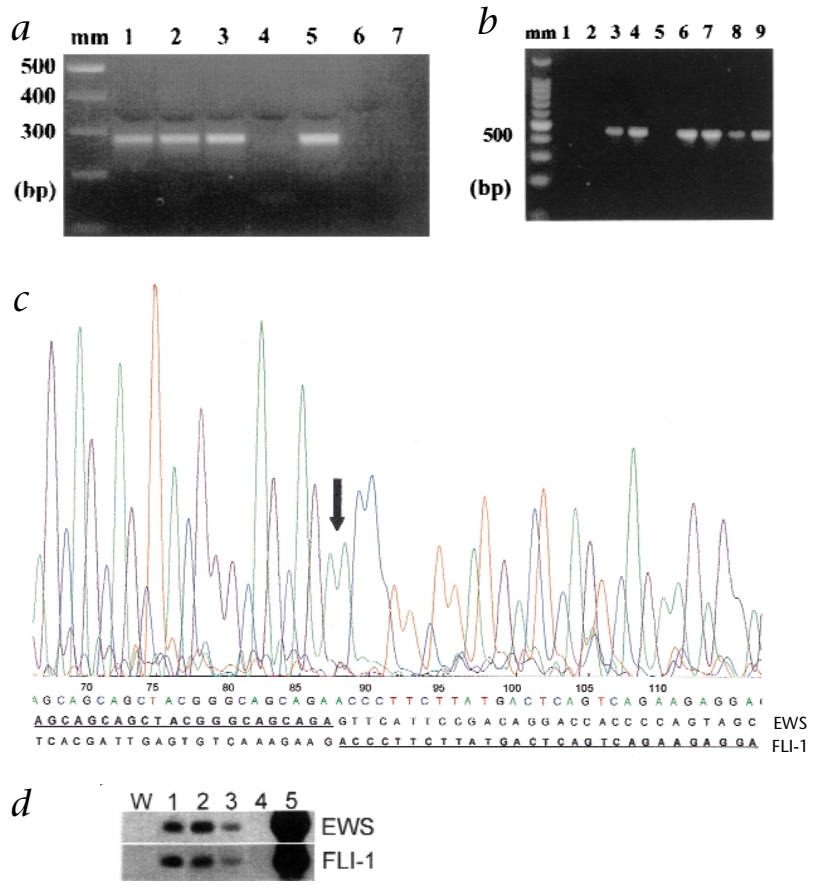

Fig. 1 RT–PCR and sequencing of the fusion transcript EWS–FLI1. ***a***, RT–PCR analysis of total RNA. Lane 1, HEK 293 cells; lane 2, E1A-expressing HeLa cells; lane 3, E1A-expressing IMR90 cells; lane 4, IMR90 control cells; lane 5, Ewing tumor; lanes 6 and 7, RT–PCR controls. The EWS–FLI1 fusion transcript was amplified with primers 11.4 and 22.4. A band of a size identical to that in a control Ewing tumor (280 bp) is detectable in E1A-expressing cells. mm, molecular markers (left margin, sizes). ***b***, Several clones of RT–PCR products from *a* were tested for the presence of the EWS–FLI1 cDNA fragment. Of the E1A-expressing HeLa cells (lanes 1,2 and 3), only the clone in lane 3 amplified the specific product; of the IMR90-E1A cells (lanes 4,5 and 6), the clones in lanes 4 and 6 were positive. Clones in lanes 7, 8 and 9 were derived from Ewing cell lines. mm, molecular markers (left margin, size). ***c***, Sequence corresponding to the EWS–FLI1 fusion transcript obtained from the clone in *b*, lane 3 (HeLa–E1A). Underlining, homologous sequences in the EWS and FlI1 genes; arrow, breakpoint. ***d***, Southern blot analysis of the chimeric products. After hybridization of the RT–PCR products with internal oligonucleotides for EWS and FLI1 cDNA sequences, a strong signal is detectable in the E1A-expressing cells. Lane 1, HEK 293 cells; lane 2, E1A-expressing HeLa cells; lane 3, E1A-expressing IMR90 cells; lane 4, IMR90 control cells; lane 5, Ewing tumor. W, water.

proximately 75 kDa in the E1A-expressing IMR90 cells (Fig. 2*c*). These molecular sizes correlate with those of the RT–PCR products obtained from these cell lines (Fig. 2*d*). There were no substantial changes in the basal levels of EWS and FLI1 messages in E1A-expressing cells, as shown by both northern and western blot analysis (data not shown). The difference in sizes of the fusion transcripts in the different cell lines is in keeping with the large heterogeneity of chimeric proteins detected in Ewing tumors. In fact, in these tumors the chromosomal translocation breakpoint can occur at introns 7–10 of the EWS gene and can be dispersed among introns 3–9 of the FLI1 gene.

Finally, we assessed the presence of translocations in HEK293 and IMR90 cells by fluorescent *in situ* hybridization. We used whole probes of chromosomes 11 and 22 and the cosmids cos1d1 and cosG9 to study the t(11:22) translocations. A t(11:22) translocation could be detected in the HEK293 cells (Fig. 3*a*) and in the E1A-expressing IMR90 cells as well as in the control Ewing cells. To determine whether the translocations were related to the specific genomic integration of the E1A gene, we also did *in situ* hybridization using a probe of the 12S E1A cDNA plasmid. As expected, we detected multiple gene copies in many chromosomes, with no specific chromosomal site of integration (Fig. 3*b*).

These findings demonstrate that a single viral gene, the adenovirus E1A gene, can induce the tumor-specific EWS–FLI1 fusion transcript in both normal and malignant human cells, as we could detect the chimeric product by RT–PCR, northern and western blot analysis, immunoprecipitation and *in situ* hybridization. The size of the chimeric product was different in the various cell lines studied as well as in the E1A-expressing IMR90 cells obtained from different infections. Although RT–PCR did not detect other fusion transcripts, found in Ewing tumors such as EWS-ERG1 or in human leukemias like the translocations t(11;22)(q23,q13), t(11;16)(q23;p13.3), t(14;18)(q32;q21), t(9;22) (q34;q11) or t(4;11)(q21;q23), we cannot conclude that E1A gene expression induces only the EWS–FLI1 fusion transcripts.

The molecular mechanisms involved in this intriguing and specific phenomenon could be related to a selection of cells in which the EWS–FLI1 translocation occurs spontaneously or to an inducible *trans*-splicing event, or could be mediated by genomic instability. The fact that we did not detect the EWS–FLI1 fusion transcript in the control IMR90 cells indicates that expression of the E1A gene is involved in the induction of that chimeric product.

The finding that EWS–FLI1 fusion transcript is induced after E1A gene expression creates a challenge for future studies concerning the chemistry of the recombination events. One possibility is that a *trans*-splicing alteration of the RNA precursors produces a hybrid mRNA molecule. However, *trans*-splicing is a rare phenomenon in mammalian cells and is tightly regulated, involving Ig genes or leader-sequence shuffling. Moreover, the simultaneous hybridization detected in some cells affecting chromosomes 11 and 22 indicated the existence of a translocation event involving the EWS and FLI1 genes. Although the induction of translocation events by viral products is unprecedented, to our knowledge, translocations by transpositional mechanisms in lymphoid cells may be possible[15]. The expression of RAG1/2 proteins, which occurs for a short time in lymphocyte development and is required for VDJ rearrangements, can precipitate a DNA transesterase reaction leading to aberrant translocations. Whether E1A proteins can induce the activity of some unknown recombinase mechanism that affects specifically the EWS and FLI1 genes requires further studies and could open new perspectives in the understanding of the relationships between virus and neoplastic alterations.

Genetic instability can arise through distinct pathways, including induction of fragile sites, microsatellite instability, chromosome instability, altered DNA methylation, homologous re-

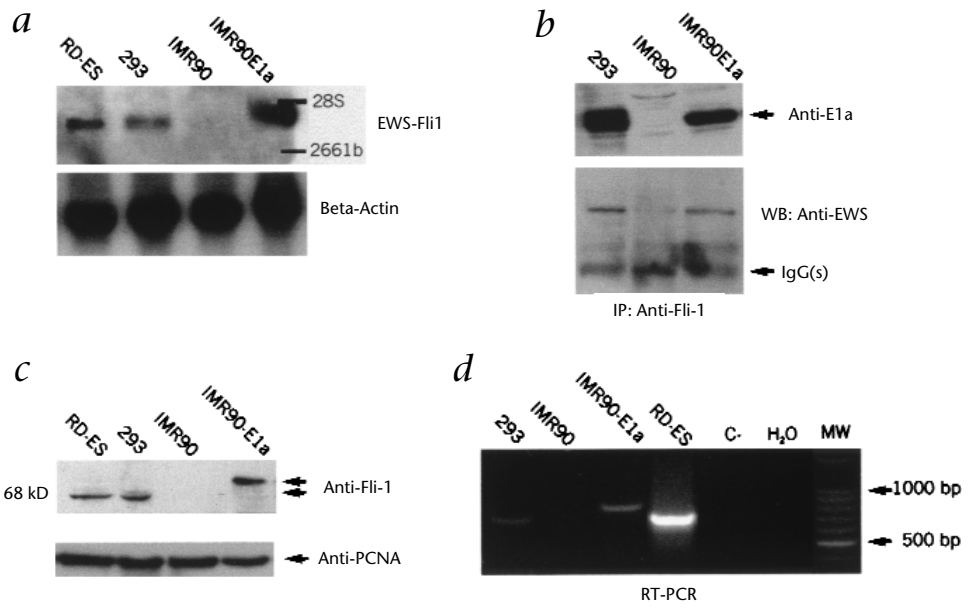

Fig. 2 Northern and western blot analysis and immunoprecipitation of the chimeric products. ***a***, Northern blot analysis with a probe corresponding to a EWS–FLI1 fusion transcript 1. A distinct signal is detectable in the HEK293 cells, in E1A-expressing IMR90 cells and in the control Ewing tumor cell line. β-actin (below), control for equal loading of RNA. ***b***, Western blot analysis, demonstrating a high level of E1A protein in HEK 293 cells as well as in E1A-expressing IMR90 cells. Immunoprecipitation with a monoclonal antibody against FLI1 and subsequent western blot analysis with an antibody against EWS, identifying specific immunoprecipitates containing the fusion protein in HEK 293 and in E1A-expressing IMR90 cells. IgG(s), immunoglobins G. ***c***, Western blot analysis with a polyclonal antibody against the C-terminal portion of FLI1, demonstrating the presence of a protein of about 70 kDa in HEK 293 and RD-ES cells and of approximately 80 kDa in IMR90-E1A cells. Anti-PCNA (below), antibody against PCNA (control). ***d***, Semi-nested RT–PCR, showing the differences in the sizes of the EWS–FLI1 transcripts, in agreement with the different molecular weights of the fusion proteins detected.

combination and other poorly understood mechanisms[16,17]. Indeed, infection with adenovirus can produce multiple chromosomal alterations and the induction of fragile sites[18]. In fact, HEK293 cells have many chromosomal abnormalities, and adenoviruses are associated with common fragile sites at regions 17q21–22,1p36, 1q21, 1q42–43 and with random fragile sites[18]. However, the most frequent common fragile sites are not related to the t(11:22) translocation in Ewing tumors. Furthermore, previous work with mutated adenoviruses has indicated that expression of the E1A protein is not sufficient for the induction of fragile sites, and that the E1B-55K protein is also necessary after infection with the adenoviruses[19]. Thus, as the IMR90 and HeLa cells expressing E1A do not express the E1B-55K protein, that molecular mechanism seems unlikely.

We analyzed microsatellite instability with several microsatellites in both IMR90 and HeLa cells, and detected no substantial differences among the control and E1A-expressing cell lines. Moreover, flow cytometry analysis of the IMR90 and HeLa cells expressing E1A also showed no substantial differences (data not shown). Thus, it seems that the specific fusion transcript induced by the adenovirus E1A is probably not related to a background of microsatellite or chromosome instability

Adenovirus E1A can influence histone acetylase activity and affect cellular chromatin remodeling processes[6], and chromosome translocations in Ewing tumors are mediated through a genuine illegitimate recombination mechanism[17]. The transcription function of some chimeric proteins can be cell-type-specific and may be related to chromatin configuration or to the availability of cofactors that vary in different cellular contexts[1]. The EWS–FLI1 fusion transcripts detected here in the different cell types expressing the E1A gene were of different sizes, as reported in Ewing tumors.

Given that a viral gene is associated with a specific fusion transcript found in human tumors, we propose a previously unknown involvement of viral genes in eliciting fusion transcripts and in the development of human tumors. Thus, specific viral sequences in human tumors should be investigated. The induction of fusion transcripts or chimeric products is probably an early event in tumor formation[1], and subsequent or concomitant oncogenic accumulation would drive cells to a fully malignant phenotype.

We did detect the EWS–FLI1 fusion transcript in human cells expressing the adenovirus E1A gene after transfection or retroviral infection. The fact that E1A expression is associated with a tumor suppressor effect *in vivo* and that it very substantially increases the cellular susceptibility to DNA-damaging agents should be balanced with the remote possibility of E1A integration into human cells after infection with human adenoviruses, a circumstance that could induce specific fusion transcripts.

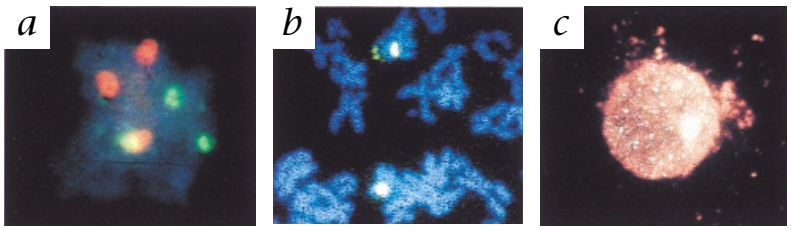

Fig. 3 Fluorescent *in situ* hybridization. ***a***, Simultaneous hybridization of chromosomes 11 (green) and 22 (red), demonstrating a t(11,22) translocation (yellow) in HEK 293 cells. These cells are aneuploid. ***b***, Simultaneous hybridization, on the same metaphase of the chromosome 11 (specific centromeric probe) and the cosmid probe G9 (chromosome 22). In the telomeric region of chromosome 11, there are two spots corresponding to specific hybridization of regions of chromosome 22. ***c***, In E1A-expressing HaCaT cells, many green spots corresponding to genomic integration of the E1A gene are detectable after *in situ* hybridization using the E1A 12S cDNA plasmid probe.

Methods

Cell cultures, plasmids and retroviral vectors. IMR90 human primary fibroblasts, HaCaT keratinocytes, HeLa carcinoma cells, HEK 293, Phoenix and BOSc-23 cell lines were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Sigma). The IMR90 cells, the amphotropic retrovirus packaging cell line (BING), and the ecotropic virus packaging line (Phoenix) were obtained from M. Serrano (Centro Nacional de Biotecnologia, Madrid, Spain). Human fibroblasts IMR90 were used between population doubling levels 20 and 50. HaCaT keratinocytes were obtained from M. Quintanilla (Instituto de Investigaciones Biomedicas, Madrid, Spain). The RD-ES and MHH-ES-1 human Ewing cell lines were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The p1Aneo plasmid, with an internal CMV promoter, and a puromycin-resistance gene driven by the LTR and containing the adenovirus-5 12S cDNA were transfected by the lipofectamine method (Life Technologies) into HeLa cervix carcinoma cells, as described[8]. To infect the human cells with ecotropic retrovirus carrying the adenoviral E1A gene, we 'murinized' the various human cells as described[20]. IMR90 fibroblasts and HaCaT keratinocytes were infected with the amphotropic vector PWZL-puro-EcoR produced in BING cells. Subsequently, cells were selected with puromycin and infected with ecotropic viruses containing the E1A 12S cDNA (PWZL 12S hygro), E1A 13S (MD 13S neo) and the respective vectors without E1A genes (controls). Infected cells were subjected to the appropriate antibiotic selection (2 μg/ml puromycin, 75 μg/ml hygromycin or 200 μg/ml G418).

RT–PCR. Total RNA was isolated from the cells with the RNazol extraction kit (Cinna/Biotecx Laboratories, Houston, Texas). Total RNA (1 μg) was reverse-transcribed with either random hexamers or oligo-dT using Moloney murine leukemia virus reverse transcriptase (Promega). The resulting cDNA was amplified by 40 polymerase chain reaction cycles with an annealing temperature of 58 °C. Negative controls (samples without the transcriptase enzyme or with water in place of RNA and RNA from non-infected cells) were included at every step of sample preparation. RNA from different Ewing cell lines served as a positive control. Amplification of β-actin was used as control. To prevent 'carryover' of amplified cDNA sequences, separate rooms and strict biosafety conditions were used for sample preparation and PCR. Primers included 11.3Fli1 (5´–ACTCCCCGTTGGTC-CCCTCC–3´), 22.3EWS (5´–TCCTACAGCCAAGCTCCAAGTC-3´), 11.4FLI1 (5´–CAGGTGATACAGCTGGCG–3´), 22.4EWS (5´–CCAACAGAGCAGCAGC-TAC–3´), P-14EWS (5´–TATGGCACTGGTGCTTATGATACC–3´) and M-25FLI1 (5´–GAAGCTGGAGGAAGTGACAGG–3´). Nested PCR used primers 11.3FLI1, 22.3EWS, 11.4FLI1 and 22.4EWS; semi-nested PCR used primers P-14 EWS, 22.3EWS and M-25FLI1. The expected sizes of the nested amplification products were 279 bp for the type 1 EWS–FLI1 translocation, 340 bp for type 2 and 531 bp for type 3. After semi-nested PCR, the sizes were 649 bp for the type 1 EWS–FLI1 fusion transcript, 712 bp for type 2 and 901 bp for type 3. Amplified products were analyzed by 1.5% agarose gel electophoresis.

Cloning and sequencing. The RT–PCR products were subcloned into the pCR(2.1 vector (Invitrogen, Carlsbad, California) and the resulting recombinant plasmids were sequenced using the Cy5 TM Autocycle Sequencing kit (Pharmacia) on a Pharmacia Biotech ALF express TM automated DNA Sequencer.

Southern, northern and western blot analysis and immunoprecipitation. For Southern blot analysis, RT–PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, blotted onto nylon membranes (Hybond; Amersham), and detected with an internal probe (EWS 22.6 and FLI1 11.6) labeled with chemoluminiscence (3′ oligolabeling system; Amersham). For northern blot analysis, 35 μg total RNA from each cell line was separated by 1.2% agarose gel electrophoresis in formaldehyde–MOPS buffer. The RNA was transferred to a Nytran membrane (Schleider & Schuell, Keene, New Hampshire) by capillary action and fixed by ultraviolet cross-linking. The blot was blocked and hybridized at 65 °C overnight with a PCR probe to a EWS–FLI1 fusion fragment previously cloned and randomly labeled with ^{32}P-dCTP. Western blot analysis of whole-cell extracts (obtained as described[8]) was accomplished using standard procedures; the membranes were incubated with either M73 antibody (Oncogene Sciences, Cambridge, Massachusetts) against E1A protein, or an antibody against EWS (N-18; Santa Cruz Biotechnology, Santa Cruz, California), or antibodies against FLI1 (C-19 Fli1, Santa Cruz Biotechnology, Santa Cruz, California; or anti-FLI1, PharMingen, San Diego, California). For immunoprecipitation assays, extracts of two almost-confluent 100-mm^2 dishes of HEK 293 or IMR90 cells or IMR90 cells expressing E1A were collected in Gannon's buffer. Immunoprecipitation used the monoclonal antibody against FLI1 (PharMingen, San Diego, California) and the subsequent western blot analysis used with the antibody against EWS. Immunoreactive bands were detected using ECL (Amersham).

Fluorescent in situ hybridization. To study chromosomes at metaphase, cells were incubated with colcemid for 4 h at 37 °C. Then the cells were trypsinized and centrifuged with complete medium at 400 g for 10 min. The cell pellets were incubated with KCl , centrifuged and washed with progressively increasing concentrations of Carnoy's fixative. Finally, the nuclei were extended on slides. 'Painting probes' (several cosmids which hybridite with almost the whole chromosome) specific for chromosomes 11 and 22, centromeric probes of chromosomes 11 and 22, and the cosmids cos1d1 and cosG9 labeled with green and red fluorophores were used for chromosome translocation analysis (WCP; Vysis, Downer's Grove, Illinois). The 12S E1A cDNA plasmid was labeled by nick translation (Boehringer) with spectrum green fluorophore, and 100 ng of this plasmid DNA was hybridized to the HEK293 and HaCaT cells to detect genomic integration. Cells were counterstained with DAPI and analyzed by photomicroscopy using the appropriate filters.

Acknowledgments

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