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Combinatorial generation of variable fusion proteins in the Ewing family of tumours

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Balanced translocations involving band q12 of human chromosome 22 are the most frequent recurrent translocations observed in human solid tumours. It has been shown recently that this region encodes EWS, a protein with an RNA binding homologous domain. In Ewing's sarcoma and malignant melanoma of soft parts, translocations of band 22q12 to chromosome 11 and 12 result in the fusion of EWS with the transcription factors FLI-1 and ATF1, respectively. The present analysis of 89 Ewing's sarcomas and related tumours show that in addition to the expected *EWS–FLI-1* fusion, the *EWS* gene can be fused to *ERG*, a transcription factor closely related to FLI-1 but located on chromosome 21. The position of the chromosome translocation breakpoints are shown to be restricted to introns 7–10 of the *EWS* gene and widely dispersed within introns 3–9 of the Ets-related genes. This heterogeneity generates a variety of chimeric proteins that can be detected by immunoprecipitation. On rare occasions, they may be associated with a truncated EWS protein arising from alternate splicing. All 13 different fusion proteins that were evidenced contained the N-terminal domain of EWS and the Ets domain of FLI-1 or ERG suggesting that oncogenic conversion is achieved by the linking of the two domains with no marked constraint on the connecting peptide.

Key words: *ERG*/Ewing's sarcoma/*EWS*/*FLI-1*/fusion proteins

Introduction

Specific chromosome translocations are frequently associated with human lymphomas and leukaemias. Molecular characterization of these translocations has led to the discovery of new mechanisms involved in neoplastic transformation (Rabbitts, 1991).

Although such translocations are less frequent in solid tumours, a recurrent t(11;22)(q24;q12) translocation has been described in a group of closely related tumours including Ewing's sarcoma (ES) of bone (Aurias *et al.*, 1983; Turc-Carel *et al.*, 1983), extraskeletal Ewing's sarcoma (Becroft *et al.*, 1984), peripheral neuroepithelioma (PN) (Whang Peng *et al.*, 1984) and Askin tumour (Whang Peng *et al.*, 1986). Increasing evidence suggests that these tumours share a common neural crest origin but may exhibit variable neural differentiation and tissue localization. This group of tumours is now referred to as the Ewing family of tumours (Horowitz *et al.*, 1993).

Recently, the cloning of the chromosome breakpoints of the t(11;22) translocation and analysis of a small series of tumours revealed that the breakpoints were localized within two regions, termed EWSR1 and EWSR2, on chromosome 22 and 11, respectively (Zucman *et al.*, 1992). On chromosome 22, EWSR1 is nested within the *EWS* gene, which encodes a 656 amino acids protein presenting two different domains (Delattre *et al.*, 1992). Its C-terminal portion shows homologies with RNA binding proteins. Its N-terminal portion (NTD-EWS) is composed of multiple repeats of a degenerated polypeptide which shares distant homology with the C-terminal polypeptide repeat of eukaryotic RNA polymerases II. On chromosome 11, the gene involved in the translocation was revealed to be the human homologue of the murine *Fli-1* gene, a member of the Ets family of transcription factors previously identified in mice at the insertion site of the Friend virus in induced erythroleukaemias (Ben-David *et al.*, 1991). In ES and PN, the t(11,22) translocation results in a chimeric *EWS–FLI-1* transcript that encodes a fusion protein in which the NTD-EWS is fused to the DNA binding domain of FLI-1 (Delattre *et al.*, 1992). The analysis of the genomic organization of both genes revealed that the coding sequence of *EWS* and *FLI-1* are encoded by 17 and nine exons and extend over 40 and 100 kb, respectively (Plougastel *et al.*, 1993; J.Zucman, T.Melot, B.Plougastel, L.Selleri, M.Giovannini, G.A.Evans, O.Delattre and G.Thomas, submitted).

We now report the analysis of the genomic rearrangements and the resulting abnormal transcripts in a large series of tumours from the Ewing family. This provides a detailed blueprint of the molecular consequences of the classical t(11;22) and of an unsuspected variant, a new (21;22) chromosome rearrangement.

Result

Position of breakpoints within EWSR1 and EWSR2

In order to map the position of the breakpoints in tumours with respect to the different exons of *EWS* and *FLI-1*, Southern blots made with *Eco*RI-digested DNA from 89

pPNETs tumours were successively hybridized with probes from EWSR1 and EWSR2 (Table I and Figure 1). It was thus possible, for each tumour DNA, to search an abnormal structure in the 3 and 11 contiguous *Eco*RI fragments that include EWSR1 and EWSR2, respectively. Since exons of both genes have been mapped precisely within *Eco*RI fragments (Plougastel *et al.*, 1993; J.Zucman, T.Melot, B.Plougastel, L.Selleri, M.Giovannini, G.A.Evans, O.Delattre and G.Thomas, submitted), the position of rearranged *Eco*RI fragments gave insight to the intron localization of breakpoints. For some *Eco*RI fragments that contain multiple exons and introns, the position of the breakpoint was further refined either by the analysis of a single *Eco*RI fragment with different probes or by the analysis of the tumour DNA with additional restriction enzyme digests. In no instance did different tumour DNAs demonstrate identical rearranged *Eco*RI fragments. This, together with the observation that abnormal *Eco*RI fragments from EWSR1 and EWSR2 have never been detected in DNA isolated from normal tissues, strongly suggest that these rearrangements were, in all instances, somatically acquired and not polymorphisms.

Within EWSR1, a rearranged fragment was observed in 80 cases, the position of the breaks being unambiguously located within introns 7, 8, 9 or 10 or *EWS* in 34, 27, three and five cases, respectively (Figure 1A). In 11 cases, the breakpoint could be localized within a 600 bp *Sac*I restriction fragment that contains exon 8 but its exact position with respect to this exon remained ambiguous. It might in some cases disrupt this exon. Within EWSR2, 66 breakpoints could be mapped, being localized in introns 3, 4, 5, 6 and 7 of *FLI-1* in two, 19, 37, three and two cases respectively

Table I. Probes used for the analysis of EWSR1 and EWSR2

Name of probe	Cosmid	Restriction fragment	Size (kb)	References
HP.5	B6	*Hin*dIII/*Pst*I	0.5	Zucman *et al.* (1993)
5.5 Sac	B6	*Sac*I	0.6	Zucman *et al.* (1992)
PR.8	B6	*Pst*I–*Eco*RI	0.8	Zucman *et al.* (1992)
RX.3	B6	*Eco*RI–*Xba*I	0.3	Zucman *et al.* (1993)
RX.4	B6	*Eco*RI–*Xba*I	0.4	This work
K	Cosco1.1	*Hin*dIII–*Not*I	1.8	This work
L (RR1.2)	Cosco1P3	*Eco*RI	1.2	Zucman *et al.* (1992)
M	Cosco1P3	*Eco*RI–*Hin*dIII	1.7	This work
N	Cosco1P3	*Eco*RI–*Xba*I	1.2	This work
O	Cosco1.1	*Eco*RI–*Xba*I	2	This work
P	Cosco1P3	*Xba*I–*Eco*RI	4	This work
Q	Cosco1.1	*Eco*RI–*Hin*dIII	1.8	This work
R (RR.9)	Cosco1P3	*Eco*RI	0.9	Zucman *et al.* (1992)
S	Cosco1.1	*Eco*RI	0.7	This work
T	Cosco1P3	*Hin*dIII–*Pst*I	0.8	This work
V	Cosco1.1	*Hin*dIII–*Pst*I	0.3	This work

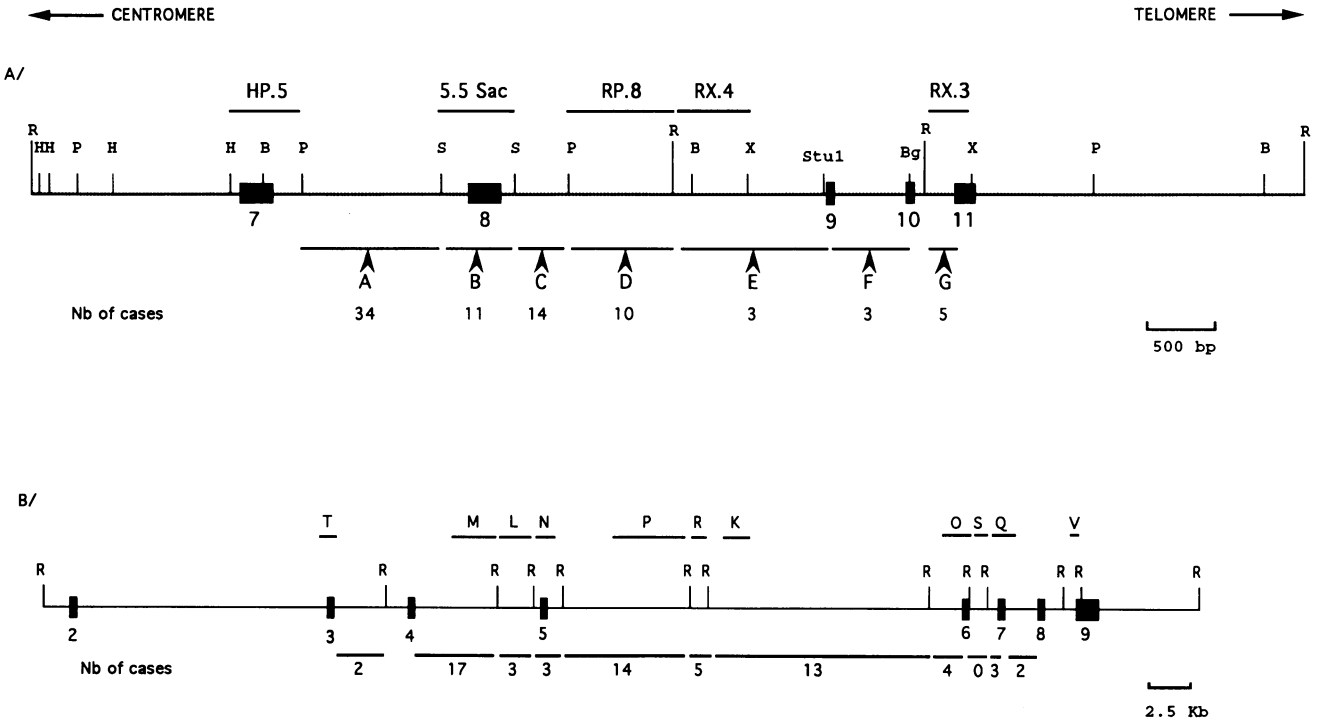

Fig. 1. Position of breakpoints with EWSR1 and EWSR2. (**A**) A restriction map of the three contiguous *Eco*RI fragments that contain EWSR1 with *Bam*HI(B), *Pst*I(P), *Xba*I(X), *Sac*I(S), *Stu*I, *Bgl*II(Bg) and *Eco*RI(R) is schematized. Positions of the different exons of EWS (plain boxes) are shown. Probes described in Table I are indicated on the top of the figure. Localization of breakpoints within regions A–D of the 5.5 *Eco*RI fragment that contains exons 7 and 8, was deduced from the hybridization pattern of both junction fragments generated by the translocation with probes HP.5, 5.5 Sac and RP.8, essentially as described by Plougastel *et al.* (1993). Within the 2 kb *Eco*RI fragment that contains exons 9 and 10, position of the breaks could be assigned to regions E and F when the rearranged fragment(s) could be detected with *Stu*I and *Bgl*II or with *Bgl*II but not with *Stu*I digested tumour DNA, respectively. For breaks assigned to region G, both derivative junction fragments were detected with the RX.3 probe. (**B**) *Eco*RI (R) restriction map of EWSR2. Positions of the different exons of FLI-1 (plain boxes) are shown. The positions of exons 2 and 3 within the 15 kb *Eco*RI fragment are only indicative. On the bottom of the figure, numbers beneath horizontal bars indicate the number of breakpoints detected within each single *Eco*RI fragment.

(Figure 1B). In three cases, a rearrangement of the 1.9 kb *Eco*RI fragment that contains exon 5 was observed but its precise positioning with respect to this exon was not determined. Altogether, both breakpoints within EWSR1 and EWSR2 were located for 64 tumours.

Different types of EWS–FLI-1 chimeric transcripts

RNAs could be obtained for 54 of 89 tumours. These RNAs were used as templates for RT-PCR experiments with primer 22.3 derived from *EWS* exon 7 and primer 11.3 derived from *FLI-1* exon 9 (Table II). RNAs from individual tumours promoted the amplification of a single fragment in 44 cases and of two different fragments in four cases. Altogether, 13 different sizes of the amplification products were observed. Control experiments performed with primer 22.6, homologous to *EWS* exon 1 and primer 11.11 derived from *FLI-1* exon 9 did not reveal additional hybrid transcript. This indicated that the sequence encoded by the seven first exons of *EWS* was always present in the fusion transcripts. The sequence of these amplified products revealed nine different *EWS–FLI-1* in-frame junctions and four different *EWS–FLI-1* out-of-frame fusions. The two most frequently observed in-frame fusions were the results of the junction between exon 7 of *EWS* with either the exon 6 or the exon 5 of *FLI-1*. They correspond to the type 1 and type 2 fusion transcripts previously described (Delattre *et al.*, 1992). The other in-frame *EWS–FLI-1* transcripts were the result of various junctions between *EWS* exons 7, 9 or 10 with *FLI-1* exons 4 to 8 (Figure 2A). In one additional case, sequence of the fusion transcript revealed an insertion of a previously unknown 44 bp sequence in-between *EWS* exon 8 and *FLI-1* exon 7 sequences. It restores an in-frame fusion between *EWS* and *FLI-1* coding sequences (Figure 2B). Hybridization experiments enabled the localization of this sequence within *FLI-1* intron 6 thus suggesting that it was derived from a cryptic exon. Finally, in four tumours, RT-PCR experiments revealed two different *EWS–FLI-1* cDNAs. In each case, one of the cDNA was in-frame as the other was not. These out-of-frame cDNAs were generated by a splice out of *EWS* exon 9 in three cases and by an *EWS* exon 8 to *FLI-1* exon 6 junction in one case. In this last case, the in-frame transcript was generated by a splice out of *EWS* exon 8 leading to an *EWS* exon 7 to *FLI-1* exon 6 junction. These out-of-frame fusion transcripts are predicted to encode truncated EWS proteins which do not contain the DNA binding domain of FLI-1 (Figure 2C).

Correlation between the genomic position of breaks and the types of EWS–FLI-1 transcript

When precisely mapped, the genomic position of breakpoints provides information on the exon and intron composition of the *EWS–FLI-1* chimeric gene encoded by the der(22) chromosome. Apart from gene fusion occurring within *EWS* intron 8, the in-frame chimeric transcript could be directly derived from the genomic structure of the *EWS–FLI-1* gene since no splicing out of internal exons was evidenced. In contrast, with one exception linked to the inclusion of a cryptic exon of *FLI-1*, gene fusion within *EWS* intron 8 always resulted in the efficient splicing out of *EWS* exon 8. This splicing out was partial, generating two transcipts, on a single occasion (Figure 2C, case No 54). The simultaneous expression of both in-frame and out-of-frame transcripts was observed more frequently for gene fusion localized within *EWS* exons 9 or 10 (Figure 2C, case Nos 53, 59 and 63).

EWS–ERG transcripts

In 13 cases, although a rearrangement within EWSR1 could be detected, EWSR2 appeared normal. In nine of these cases an *EWS–FLI-1* transcript was observed suggesting that the rearrangement within EWSR2 had escaped our detection procedure. We focused on the remaining four cases demonstrating rearranged EWSR1 but no evidence of *FLI-1* involvement. We hypothesized that another gene different from *FLI-1* might be fused to *EWS* in these cases. We further considered that this gene might be another member of the Ets family of transcription factors. In order to test this hypothesis, we took advantage of the strong homology of the region encoding the DNA binding domain observed throughout this family of genes. Primer 11.11 homologous to this conserved region was used together with primer 22.8 to PCR amplify oligo(dT) primed cDNAs from these four tumours. In the PCR reaction, the annealing temperature was lowered to 62°C, in order to provide less stringent conditions which could promote the hybridization of primer 11.11 to mismatched cDNA sequences. Under these conditions, a PCR product, each of different size, could be evidenced in these four cases. Sequence of these products revealed fusion transcript between *EWS* exons 7 or 10 with sequences which differed from that of *FLI-1*. Homology search revealed in each case a perfect match and an in-frame fusion with the previously described *ERG* cDNA sequence (Rao *et al.*, 1987). Analysis of the junctions between *EWS* and *ERG* together with that of the homology between *ERG* and *FLI-1* sequence revealed that *EWS–ERG* gene fusion occur at the exact homologous nucleotide position separating different exons of *FLI-1* strongly suggesting that *ERG* and *FLI-1* genomic organization are very similar (Figure 3). Demonstration that these chimeric *EWS–ERG* transcripts resulted from a gene fusion was provided by bicolour FISH on interphase nuclei from the EW18 cell line. Using ERG and EWS specific cosmids, these experiments showed that the proximal part of *EWS* was juxtaposed to the *ERG* locus

Table II. Primers used for RT-PCR experiments

Name	Gene/exon No	Sequence
22.6	*EWS*/ex 1	5′-GAACGAGGAGGAAGGAGAGA-3′
22.3	*EWS*/ex 7	5′-TCCTACAGCCAAGCTCCAAGTC-3′
22.8	*EWS*/ex 7	5′-CCCACTAGTTACCCACCCCAAA-3′
11.A	*FLI-1*/ex 9	5′-AGAAGGGTACTTGTACATGG-3′
11.3	*FLI-1*/ex 9	5′-ACTCCCCGTTGGTCCCCTCC-3′
11.11	*FLI-1*/ex 9	5′-TGTTGGGCTTGCTTTTCCGCTC-3′
11Cter1	*FLI-1*/ex 9	5′-GGGATCCTTTGACTTCCACGGCATTGC-3′
11Cter2	*FLI-1*/ex 9	5′-GGAATTCGTGAAGGCACGTGGGTGTTA-3′

thus demonstrating a rearrangement between chromosomes 21 and 22 in this cell line (Figure 4).

Combined results of DNA and RNA analysis on the 54 tumours are displayed on Table III. Among the five cases that demonstrated neither rearrangement of EWSR1 nor of EWSR2, an *EWS–FLI-1* transcript was detected in three cases and an *EWS–ERG* fusion in one case. Thus, only one case failed to demonstrate either genomic rearrangement or fusion transcript.

Detection of chimeric EWS–FLI-1 proteins

Serum from rabbits immunized against a 92 amino acid polypeptide from the C-terminal end of FLI-1 was used to immunoprecipitate EWS–FLI-1 proteins from 8 ES cell lines labelled with [^{35}S]methionine. In each ES cell line expressing an *EWS–FLI-1* transcript, one single specific protein was observed (Figure 5). Its molecular weight varied from tumour to tumour, the difference in the size of these proteins being fully concordant with the predicted differences

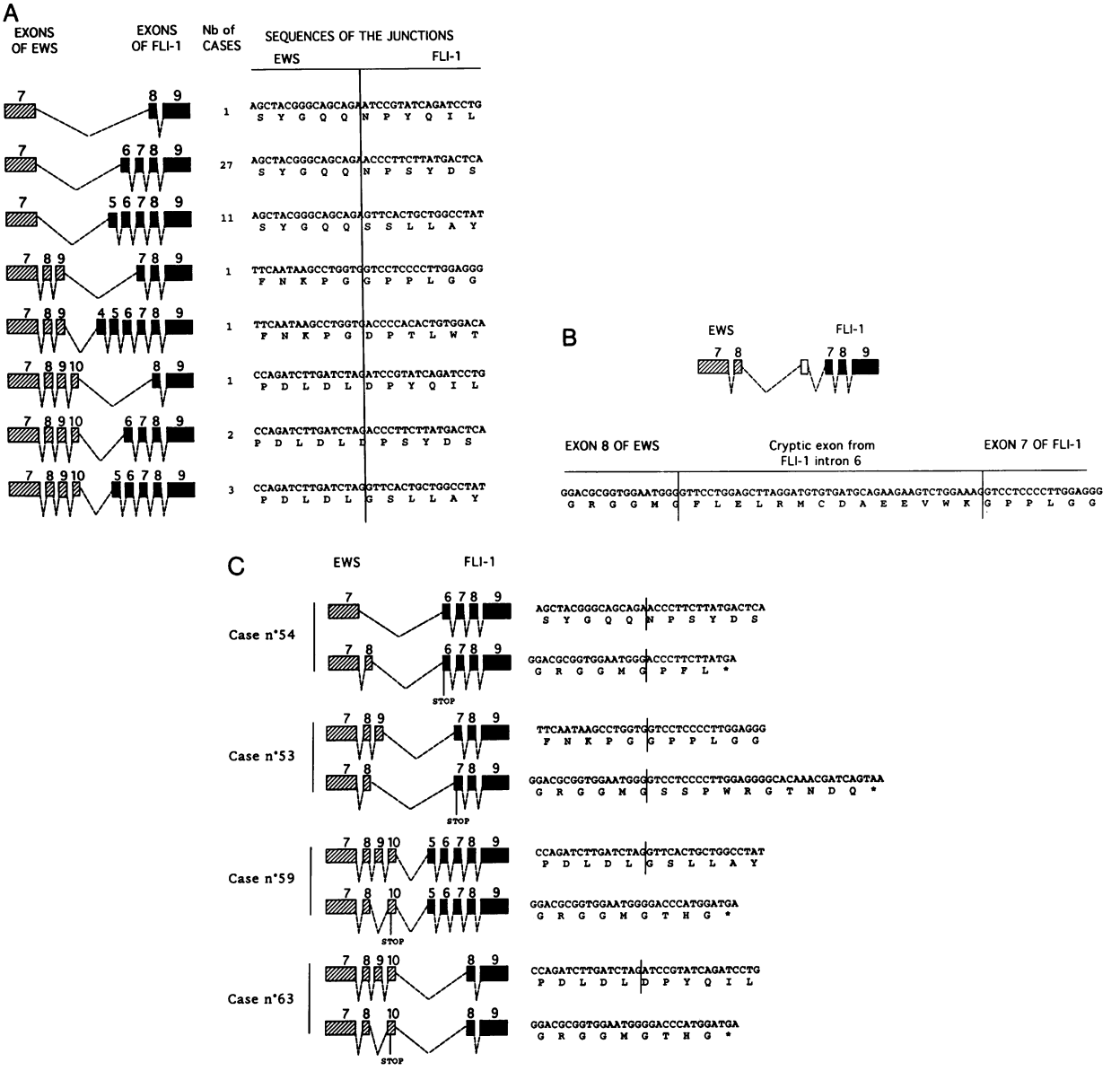

Fig. 2. *EWS–FLI-1* mRNA junctions. The different *EWS–FLI-1* junctions are schematized and their sequences are reported. Hatched boxes denote *EWS* exon sequence and black boxes represent *FLI-1* exon sequence. Numbers above boxes indicate the exon number of each gene. Vertical lines indicate the nucleotide position of the junction between the two genes. (**A**) Eight different *EWS–FLI-1* in-frame fusions are shown. The number of tumours which demonstrated each type of junction is reported. Nucleotide sequences and deduced amino acid sequences of the junctions are indicated. (**B**) Schematic representation and nucleotide sequence of a complex fusion. A new sequence (open box) that was shown by hybridization experiments to arise from *FLI-1* intron 6 is inserted in between *EWS* exon 8 and *FLI-1* exon 7. This sequence presents an open reading frame that links together the *EWS* and *FLI-1* reading frames. (**C**) Mechanisms of the generation of two different *EWS–FLI-1* junctions in the four tumours where two transcripts could be detected. The position of the stop codon (STOP) in the out-of-frame fusion is indicated. Both in-frame and out-of-frame junctions are indicated. In case No 54, breakpoints were localized within *EWS* intron 8 and *FLI-1* intron 5. The in-frame fusion resulted from a splice out of *EWS* exon 8. The out-of-frame fusion resulted from a 'correct' splicing event that joined together *EWS* exon 8 to *FLI-1* exon 6. In cases number 53, 59 and 63, the *EWS–FLI-1* in-frame fusion results from a 'correct' splicing event of *EWS* and *FLI-1* sequence as the out-of-frame results from a splice out of exon 9.

deduced from sequences of the respective chimeric transcripts.

Discussion

We report the mapping of 80 chromosome 22 breakpoints and 66 chromosome 11 breakpoints in ES and related tumours. Within *FLI-1*, the breakpoints are scattered along the 50 kb DNA region that extend from intron 3 to intron 7 and brings, in the resulting fusion transcript, any of exons 4 to 8 of *FLI-1* in direct contact with *EWS* sequences. In contrast, within *EWS*, the breakpoints are precisely located in a small 7 kb region bounded by exons 7 and 11 and most frequently occur in close proximity of exon 8. Although introns 7 and 8 tend to be split with an approximate equal frequency, this variability is not apparent on the fusion transcript since the exon 8 of EWS, when included in the chimeric gene on the der(22) chromosome, is almost systematically spliced out.

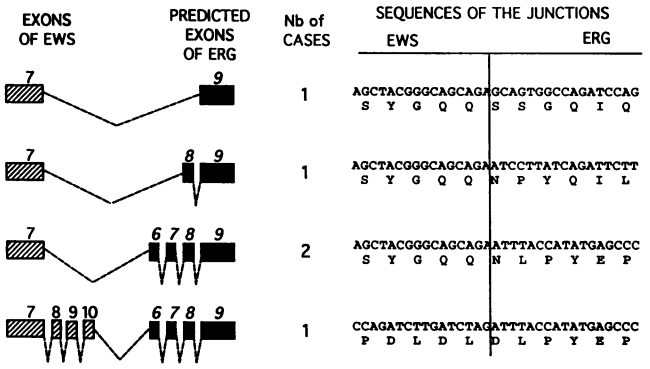

Fig. 3. Scheme and nucleotide sequence of *EWS–ERG* junctions. Exons numbers and symbols are the same as in Figure 2 except that black boxes refer to ERG sequences. The numbering of ERG exons is indicated in italic letters assuming an identical genomic organization for *ERG* and *FLI-1*.

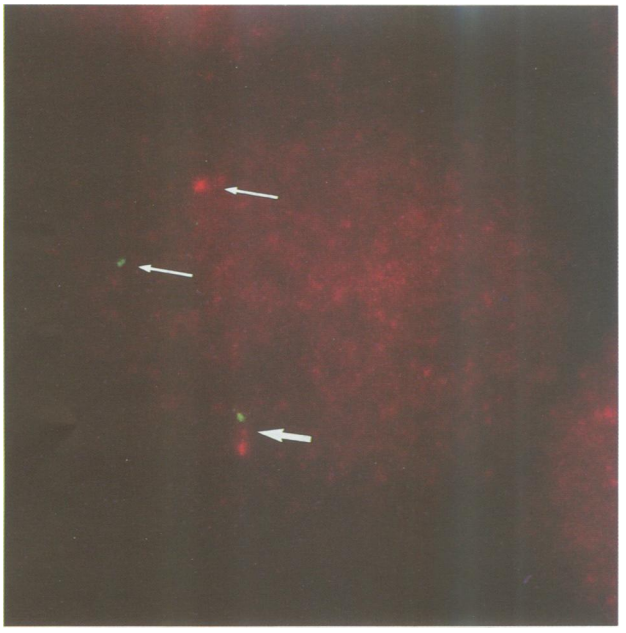

Fig. 4. Juxtaposition of *EWS* and *ERG* genes in the EW18 cell line demonstrated by bicolour FISH. Thin arrows point to a red and a green spot corresponding to the normal *EWS* and *ERG* loci, respectively. The thick arrow points to a red/green double spot corresponding to the fusion of the 5′ part of *EWS* with the 3′ part of *ERG*.

Strikingly, with the exception of *EWS* intron 8, any junction between introns contained within EWSR1 and EWSR2 is predicted to lead to a mature in-frame *EWS–FLI-1* transcript. Half (9/18) of these different possible combinations have been observed suggesting that as long as the reading frame is maintained in the fusion transcript, no strong constraint is placed on the exon composition of the median part of the *EWS–FLI-1* hybrid transcript. Although less documented, the same conclusion may tentatively be applied to the *EWS–ERG* fusion.

The exons included within EWSR1 encode a hinge region of the EWS protein located in between the NTD-EWS encoded by the first seven exons and the predicted RNA binding portion of the protein encoded by the last 7 exons (Plougastel *et al.*, 1993). None of the latter have ever been observed in the chimeric EWS–FLI-1 or EWS–ERG proteins although fusion of EWS introns 12, 13 and 15 with any intron contained in EWSR2 would maintain the reading frame. This observation suggests that the presence, in the hybrid protein, of part or whole of the predicted RNA binding domain may hinder its oncogenic role.

In contrast, the NTD-EWS appears to be required since, in spite of the variability of the EWS–FLI-1 or EWS–ERG chimeric proteins, it is systematically observed in them. This domain consists of multiple repeats of a degenerated polypeptide motif with the consensus SYGQQS. Recently, the NTD-EWS has been shown to contain a potent transcription activation domain (R.Bailly, R.Bosselut, J.Zucman, F.Cormier, O.Delattre, M.Roussel, G.Thomas, and J.Ghysdael, manuscript in preparation). In the hybrid protein, this peptide can be linked to various portions of either of two closely related members of the Ets family of transcription factors. These portions systematically contain the Ets-related DNA binding domain of either FLI-1 or ERG, two domains which differ by only two conservative amino acid changes and which may have very similar DNA target sequences. Thus the NTD-EWS and the Ets domain of either FLI-1 or ERG appear to be invariant features of the hybrid protein and may both be required for its oncogenic

Table III. Detection of rearranged EWSR1 or EWSR2 and of chimeric transcript in ES and related tumours

	No of cases	*EWS–FLI-1* transcript (No of cases)	*EWS–ERG* transcript (No of cases)
EWSR1 and EWSR2 rearranged	35	35	0
EWSR1 rearranged, EWSR2 normal	13	9	4
EWSR1 normal, EWSR2 rearranged	1	1	0
EWSR1 and EWSR2 normal	5	3	1
Total	54	48	5

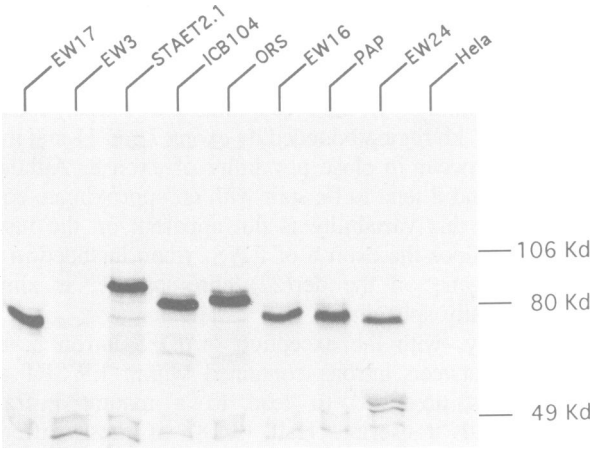

Fig. 5. Expression of EWS−FLI-1 proteins in ES cell lines. Lysates from nine different cell lines were immunoprecipitated with a polyclonal antibody directed against the C-terminus portion of FLI-1. EW24 and EW17 express type 1 (*EWS* ex 7−*FLI-1* ex 6) and PAP a type 2 fusion transcript (*EWS* ex 7−*FLI-1* ex 5). Exons of *EWS* and *FLI-1* involved in the junction for the other cell lines are indicated in parenthesis: EW16 (*EWS* ex 9−*FLI-1* ex 7), ORS (*EWS* ex 10−*FLI* ex 5), ICB 104 (*EWS* ex 10−*FLI* ex 6), STAET2.1 (*EWS* ex 9−*FLI-1* ex 4). EW3 expresses an EWS−ERG protein that is not detected with the anti FLI-1 antibodies used here.

properties. This hypothesis is strengthened by the recent observation that deletion mutants within the NTD-EWS or within the FLI-1 Ets domain have lost the ability to transform NIH3T3 cells (May *et al.*, 1993).

Over the rest of their sequence, ERG and FLI-1, although still similar, are less homologous. Interestingly sequences on the N-terminal side of the DNA binding domain which are believed to participate in normal transcription regulation by these factors (Klemz *et al.*, 1993; Murakami *et al.*, 1993; Wasylyk *et al.*, 1993; Zhang *et al.*, 1993), may not contribute or hinder significantly the oncogenic properties of the hybrid protein since they can be, in part or entirely, included or omitted. Conversely, the last 90 amino acids C-terminal sequence of ERG and FLI-1 are always present in the chimeric proteins. Although ERG and FLI-1 differ significantly in this C-terminal region, they remain the two most homologous members of the Ets family. No specific function has yet been assigned to this 90 amino acid domain. Mutant constructs in this domain should help to define its role in transformation, if any. Finally, since no evident association between tumour phenotype and specific rearrangements was found, the observed variability within the fusion proteins does not appear to be related to the variable neural differentiation and tissue localization observed in the Ewing family of tumours. This contrasts with the markedly different phenotype associated with a fusion between EWS and ATF1, a transcription factor of the bZIP family which is observed in malignant melanoma of soft parts, a rare neuroectodermal tumour which does not belong to the Ewing family of tumours (Zucman *et al.*, 1993).

This study demonstrates for the first time the implication of ERG in human carcinogenesis. In one well-documented case, this implication is mediated by a complex rearrangement between chromosomes 21 and 22. Although cytogenetic series of ES or related tumours have reported occasional chromosome 21 alteration (Gorman *et al.*, 1991), a balanced t(21;22) translocation has never been described. A recent physical map of the *ERG*-containing region on chromosome 21 suggests that the orientation of transcription of *ERG* might be from telomere to centromere (Crete *et al.*, 1993). The transcription of *EWS* is in the opposite orientation (Delattre *et al.*, 1992) precluding the generation of the *EWS−ERG* fusion gene through a simple and balanced translocation. It is noteworthy that cytogenetics has shown that ~20% of ES do not demonstrate typical t(11;22) (Turc-Carel *et al.*, 1988). Although *EWS−FLI-1* fusion transcript has been shown to occur in some of them, *EWS−ERG* fusion, which in the present series was observed in 10% of the cases, might account for a large portion of the remaining cases.

Interestingly, the characteristic t(12;16) translocation associated with myxoid liposarcoma has been shown recently to result in the fusion of the N-terminal domain of FUS/TLS, a novel RNA binding protein, with CHOP, a member of the C/EBP family of transcription factors (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993). Strikingly, FUS/TLS shows a strong homology with EWS, in particular its N-terminal portion presents very similar repeats of a degenerated polypeptide with consensus SYGQQS. Thus, the combinatorial process linking NTD-EWS to DNA binding domains of various transcription factors, which is described in detail here, may provide a prototypic mechanism for the oncogenic conversion of a subset of RNA binding proteins in human solid tumours.

Materials and methods

Tumours and cell lines

Surgical samples from 89 patients were collected immediately after surgery and frozen in liquid nitrogen. Clinical and histological evaluation diagnosed 68 ES of bone, eight extrasequeletal ES, 10 PN and three Askin's tumour. Permanent cell lines were established in 35 cases. DNA extraction and Southern blotting were performed according to standard procedures (Maniatis *et al.*, 1989).

Probes and oligonucleotides

The different probes and oligonucleotides used in this study are listed in Tables I and II, respectively.

Detection of fusion transcript and sequence analysis

Total RNA from tumours and cell lines were isolated using the RNAzol extraction kit (Bioprobe Systems, France). One microgram of total RNA was reverse transcribed with either oligonucleotide 11A or with oligo(dT) using the Gen Amp RNA PCR kit (Cetus). The resulting cDNAs were PCR amplified using either primers 11.3 and 22.3 as previously described by Delattre *et al.* (1992) or with primer 11.11 and 22.8. For this last set of primers, 30 cycles were performed with the following parameters: denaturation step at 94°C for 30 s, annealing at 68°C for 1 min and elongation step at 72°C for 1 min. Amplified products were analysed on 1.2% TBE−agarose gel. The amplified fragments were purified on Centricon 100 ultrafiltration devices (Amicon, Epernon, France) and direct sequencing was performed using a Taq polymerase Kit (PRISM, Applied Biosystems) with fluorescent primers or dideoxynucleotides. Sequences were analysed with an Applied Biosystems model 373A automatic sequencer. When multiple bands were observed, they were eluted from the gel and PCR amplified prior to sequence analysis.

Fluorescent in situ hybridization

Bicolour FISH experiments were performed as previously described by Desmaze *et al.* (1992) on nuclei from standard cytogenetic spreads obtained after short-term culture of the EW18 cell line. G9 cosmid corresponds to the proximal part of the *EWS* locus and was previously described by Zucman *et al.* (1992). The cosmid corresponding to the *ERG* locus was kindly provided by Nathalie Crete and Nicole Créau-Goldberg.

Generation of FLI-1-specific antibodies and immunoprecipitation analysis

The sequence encoding the 93 C-terminus amino acids of FLI-1 was PCR amplified using primer 11ter1 and primer 11ter2. The resulting PCR product was cloned in the *Bam*HI and *Eco*RI sites of the pGEX-2T vector. The GST fusion polypeptide was gel purified and injected subcutaneously into rabbits to create specific antiserum. EWS–FLI-1 proteins were detected by immunoprecipitation as previously described by Ghysdael *et al.* (1986).

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