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Lack of matrix metalloproteinase (MMP)-1 and -3 expression in Ewing sarcoma may be due to loss of accessibility of the MMP regulatory element to the specific fusion protein in vivo

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Abstract

Ewing sarcoma is a malignant bone and soft tissue tumor of children and young adults, which is known to be highly aggressive and invasive. It expresses specific chimeric genes (EWS-FLI-1, EWS-ERG, EWS-ETV1, and EWS-E1AF), the 3′ portions of which are all members of the ETS family. ETS-related proteins, such as FLI-1, ERG, and E1AF, transactivate the promoters of matrix metalloproteinase (MMP) genes, which play important roles in the processes of invasion and metastasis. Therefore, we hypothesize that the Ewing sarcoma-specific chimeric genes also transactivate the MMP genes, contributing to the tumor's invasiveness and propensity for metastasis. To verify this hypothesis, we investigated the expression of MMPs in eight Ewing sarcoma cell lines. Surprisingly, MMP-1 and MMP-3 were not expressed at all in any of the cell lines. MMP-9 was expressed in four out of the eight cell lines, and MMP-2 and MT1-MMP in all of the cell lines. Ewing sarcoma-specific chimeric genes have been shown to transactivate the promoter of the MMP-1 gene by the reporter assay, and bind to the putative recognition sites in the MMP regulatory elements by the gel shift assay. However, an in vivo formaldehyde cross-linking study revealed that the chimeric protein did not bind to the predicted ETS recognition sites in the regulatory elements of the MMPs. These results indicate that the absence of the MMP expression in the tumor cells is at least in part due to the loss of accessibility of the ETS recognition sites in the regulatory elements of the MMP genes. Therefore, we should be careful before theorizing simply that a putative binding site is essential for the transcription of critical genes, since the binding of this fusion protein was found to be modulated in tumor cells in this study.

Keywords: Ewing sarcoma; ETS; E1AF; RNA binding protein

Ewing sarcoma, one of the most common malignant tumors of children and young adults, is an aggressive osteolytic tumor with a marked propensity for dissemination [1] and has neurogenic potential [2]. Recently, many human sarcomas have been reported to contain characteristic chromosomal translocations, which generate specific chimeric genes [3–5]. Ewing sarcoma is characterized by a specific chromosomal translocation, t(11; 22) [6,7], through which the EWS-FLI-1 chimeric gene is generated [8]. FLI-1, a component of the chimeric gene, is a member of the ETS family of transcription factors. Other members of the ETS family of genes, ERG, ETV1, and E1AF, are also fused to EWS through t(21; 22), t(7; 22), and t(17; 22), respectively [9–12], in Ewing sarcoma.

Ewing sarcoma-specific chimeric genes contain members of the ETS family on their 3′ side and are considered to play important roles in oncogenesis and malignancy. The target genes for these chimeric genes remain unclear. But, matrix metalloproteinase (MMP)-3

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(stromelysin-1) has been suggested to be a direct target of EWS-FLI-1 by representational difference analysis [13]. The promoters of human MMP-1 (collagenase-1) and MMP-3 have been reported to be transactivated by the c-ETS-1 and c-ETS-2 genes [14,15]. In addition, ERG transactivates MMP-1 [16], and E1AF transactivates MMP-1, MMP-3, and MMP-9 (gelatinase B) [17].

Based on these findings, we speculate that Ewing sarcoma-specific chimeric genes can transactivate MMP-1, MMP-3, and MMP-9 through their binding to putative recognition sites in their regulatory elements, and contribute to the invasiveness and propensity for metastasis of Ewing sarcoma. Although there are a few reports on the expression patterns of the MMP genes in osteosarcoma [18] and chondrosarcoma [19], MMP gene expression in Ewing sarcoma has not yet been investigated. In this report, we investigated the transcriptional activation of the MMP-1, MMP-3, and MMP-9 promoters by Ewing sarcoma-specific fusion products, and the expression patterns of the MMPs, including MMP-1, MMP-2 (gelatinase A), MMP-3, MMP-9, TIMP-1, TIMP-2, and MT1-MMP, in Ewing sarcoma cell lines and primary Ewing sarcoma tumors. Surprisingly, Ewing sarcoma cells did not express MMP-1 and -3. However, they did express MMP-2 and -9 and MT1-MMP. Lack of MMP-1 and -3 expression seems to result from the loss of accessibility of the ETS recognition sites in the regulatory elements of the MMPs to the Ewing sarcoma-specific chimera.

Materials and methods

Tumors and cell lines. Five surgical specimens of Ewing sarcoma/peripheral primitive neuroectodermal tumor (PNET) were obtained from Japanese patients. The patient characteristics are summarized in Table 1B. The tumors were diagnosed as Ewing sarcoma or PNET based on the clinical and pathological findings. Two new Ewing sarcoma cell lines, K-EW2 and K-EW3, were established from the tumor specimens of two patients (Table 1A). The tumor specimens were minced and cultured in dishes of Dulbecco's modified minimum essential medium (GIBCO) in the presence of 10% fetal bovine serum (GIBCO). Both lines grew stably for more than 30 passages. In addition to these two newly established cell lines, six previously established Ewing sarcoma cell lines (NCR-EW2 [20], NCR-EW3 [20], SK-ES1, SCCH-196 [21], W-ES [22], and K-EW1 [23]) are being maintained in our laboratory (Table 1A). SK-ES1 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MRC-5, which represents immortalized cell clones from transformed human fibroblasts [24], used as control cells, is also maintained in our laboratory. The cells were cultured at 37 °C in Dulbecco's modified minimum essential medium in the presence of 10% fetal bovine serum and 5% CO_2 in 25-cm^2 culture flasks. The cultured cells were then suspended in phosphate-buffered saline containing 0.05 M EDTA, without calcium and magnesium.

Reverse transcription and polymerase chain reaction (RT-PCR), and RNA blot analysis. Total RNAs from the tumors and cell lines were extracted by the guanidinium thiocyanate method, followed by centrifugation in cesium chloride solutions [25] or ISOGEN (Nippon Gene, Tokyo). The surgical specimens were minced with a small stick in a microcentrifuge tube. Complementary DNA was generated using the first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala). Approximately 1–5 μg of total RNA was transcribed. Polymerase chain reaction (PCR) was carried out in a 100 μl reaction mixture containing 1–7 μl of the cDNA template, 200 mM of deoxynucleotide triphosphates, 0.5 mM of each oligonucleotide primer, and 2.5 units of Taq polymerase in 10 mM Tris–HCl buffer (pH 8.8) containing 50 mM KCl, and 1.5 mM $MgCl_2$. The oligonucleotide primers used for the PCR were based on the sequences of ESBP-1 (EWS specific) and ESBP-2 (FLI-1 specific) [26]. PCR was performed in 35 cycles under the following protocol: denaturation step at 94 °C for 1 min, annealing at 65 °C for 1 min and elongation step at 72 °C for 1 min. The amplified products were analyzed on 1% agarose gel.

For the RNA blot analysis, 10 μg of total RNA was electrophoresed on 1.0% agarose gel, transferred to a nylon membrane and hybridized with a cDNA probe radiolabeled with [α-^{32}P]dCTP, by the random-primer method, at 65 °C for 14–16 h in a buffer containing 5× SSPE, and 5× Denhardt's solution. The blots were washed with 2× SSC containing 1% SDS at room temperature and at 65 °C. Final washings were done with 0.1× SSC containing 0.1% SDS at 65 °C. The blots were exposed to X-ray films using an intensifying screen.

Table 1
Description of Ewing/PNET cell lines and primary Ewing/PNET tumors in patients

Case	Cell line	Age/sex	Surgical samples	Origin	Diagnosis	Fusion transcript
(A) *Cell lines*						
1	NCR-EW2	13 yr/male	ST433	Pelvis (EO)	EWING	EWS exon7-FLI-1 exon5
2	SK-ES1	18 yr/male	–	unknown	EWING	EWS exon7-FLI-1 exon5
3	SCCH196	16 yr/female	ST854	Upper arm (EO)	EWING	EWS exon7-FLI-1 exon6
4	K-EW2	18 yr/male	KST1420	Fibula (O)	EWING	EWS exon7-FLI-1 exon6
5	K-EW3	13 yr/male	KST1446	Rib (O)	EWING	EWS exon7-FLI-1 exon6
6	K-EW1	15 yr/male	KST1166	Pelvic cavity (EO)	EWING	EWS exon7-ERG exon9
7	W-ES	14 yr/male	–	Chest wall (EO)	EWING	EWS exon7-ERG exon9
8	NCR-EW3	10 yr/male	ST480	Chest wall (EO)	EWING	EWS exon7-E1A-F
(B) *Primary tumors*						
		13 yr/male	ST433	Pelvis (EO)	EWING	EWS exon7-FLI-1 exon5
		30 yr/male	KST652	Thigh (EO)	PNET	EWS exon7-FLI-1 exon5
		24 yr/male	KST1461	Tibia (O)	EWING	EWS exon7-FLI-1 exon6
		15 yr/male	KST1166	Pelvic cavity (EO)	EWING	EWS exon7-ERG exon 9
		10 yr/male	ST480	Chest wall (EO)	EWING	EWS exon7-E1A-F

(O) Osseous; (EO) Extraosseous.

CAT assay. Chloramphenicol acetyltransferase (CAT) reporter genes driven by promoters of type I collagenase, stromelysin, and 92 kDa type IV collagenase genes were co-transfected into human HeLa cells containing a Ewing sarcoma-specific chimeric gene (EWS-FLI-1, EWS-ERG, and EWS-E1AF) expression vector and pACT-β gal plasmid as a reference by the lipofection method. After 48 h, cell extracts were prepared, and the CAT and β-galactosidase activities were assayed by a standard procedure [27,28].

Subcloning of the target genes of EWS-FLI-1 using the in vivo formaldehyde cross-linking technique, and hybridization analysis. The cell cultures of Ewing sarcoma cells (NCR-EW2), labeling and formaldehyde fixation, immunoprecipitation of in vivo-fixed chromatin fragments using an anti-human FLI-1 antibody (Santa Cruz Biotechnology, sc-356) or anti-human IgG antibody as a control, purification of the immunoprecipitated DNA, and linker-modified PCR amplification were performed according to the methods described by Orlando and Paro [29]. Subcloning of DNA was carried out using pGEM-T Vector Systems (Promega, Madison, WI, USA).

About 30 ng of amplified DNA probes immunoprecipitated by the anti-human FLI-1 antibody was labeled using an oligonucleotide random-primed DNA synthesis kit with [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol; Amersham). Slot-blot analyses were performed with a slot-type dot blotting apparatus (Bio-Rad) equipped with nylon filters. About 100 ng of template DNA was loaded onto a nylon membrane, and hybridized with labeled DNA probes at 65 °C for 14–16 h in a buffer containing 5× SSPE, and 5× Denhardt's solution. The blots were washed with 2× SSC containing 1% SDS at room temperature and at 65 °C. Final washings were done with 0.1× SSC containing 0.1% SDS at 65 °C. The blots were exposed to a phosphoimaging plate and image quantitation was performed using a phosphoimaging apparatus.

Gel mobility shift assay. Gel mobility shift assay was performed as described previously [30,31]. The annealed double-stranded oligonucleotide was labeled by the fill-in reaction of Sequenase version 2 (US Biochemicals), in the presence of radioactive dCTP on the AGCT extensions added to the oligonucleotide. The upper strand of the oligonucleotides (Col I-88 ets S: tcgaATCAAGAGGATGTTATAA) containing the ets site (underlined) of human collagenase (MMP-1) promoter was used. Nuclear proteins extracted from Ewing sarcoma cells (NCR-EW2) were prepared as described previously [30]. Nuclear protein–DNA binding reactions were performed in binding buffer containing 40,000 cpm of a ^{32}P-labeled probe, 15 μg of nuclear proteins, 10 μg of poly(dA-dT), for 10 min at 4 °C in the presence of an excess or absence of unlabeled intact or mutated oligonucleotide (100×, 250× and 500×). In one reaction, a specific antibody raised against the FLI-1 protein (Santa Cruz Biotechnology, sc-356) was used. The protein–DNA complexes were analyzed on a 5% polyacrylamide gel containing 0.4× TBE. The gel was dried and autoradiographed.

Results and discussion

Generation of new Ewing sarcoma cell lines

We established two new cell lines (K-EW2 and K-EW3) from the tumor specimens of Ewing sarcoma patients (Figs. 1A–D). Histology of the primary tumors showed small round cells with round to ovoid nuclei. Some tumor cells stained positive for the periodic acid–Schiff reaction, which were digested with diastase. These tumor cells were not arranged in any particular configuration, such as in the form of rosettes. K-EW2 cells grew to give a fibrocytic appearance, while K-EW3 cells grew as a polygonal cell sheet with the formation of cell aggregates at the center. In both of these cell lines, the EWS-FLI-1 chimeric gene was detected by RT-PCR (Fig. 1E).

Expression of the MMP and related genes in Ewing sarcoma cell lines and primary tumors

We investigated the expression of the MMP and related genes in Ewing sarcoma, since (a) specific fusion genes between EWS and ETS-related genes are present in Ewing sarcoma; (b) the MMP-1, -3, and -9 genes are activated by ETS-related oncogenes through binding to their regulatory elements; and (c) the MMP genes are candidate target genes for the specific fusion genes (Fig. 2 and Table 2A). Eight human tumor cell lines established from Ewing sarcoma cells, including the two new cell lines, K-EW2 and K-EW3, were examined for their expression of MMP-1, -2, -3, and -9, and MT1-MMP. MMP-1 and MMP-3 were not expressed in any of the Ewing sarcoma cell lines tested, while they were in MRC-5 used as the positive control (Fig. 2 and Table 2A). MMP-9 was expressed in four out of the eight cell lines (Fig. 2D). MMP-2, MT1-MMP, TIMP-1, and TIMP-2 were expressed in almost all the cell lines (Figs. 2C, E and F).

To eliminate the possibility of bias in the expression pattern of the MMP genes in Ewing sarcoma cell lines arising through the establishment of the cell lines from primary tumors, five human surgical specimens obtained from patients of Ewing sarcoma were examined for their expression of MMP-1, -2, -3, and -9, and MT1-MMP (Fig. 3 and Table 2B). MMP-1 and MMP-3 were not expressed in any of the Ewing sarcoma tumor specimens (Figs. 3A and B). MMP-2 and MT1-MMP were expressed in almost all, while MMP-9 was expressed in only a few of the specimens (Figs. 3C–E). This expression pattern of MMPs in the Ewing sarcoma tumor specimens was consistent with that in cell lines.

Transcriptional activation of the promoter of MMP-1 by Ewing sarcoma-specific chimeric genes

The promoters of MMP-1 and MMP-3 are known to be transactivated by ETS-related genes, including ERG and E1AF. However, our results show that MMP-1 and MMP-3 are not expressed in Ewing sarcoma cell lines and tumors. We hypothesize that this lack of expression of the MMP-1 and -3 genes is due to the loss of transcriptional activation of the promoters of MMP-1 and MMP-3 by the chimera genes. Therefore, we analyzed the transcriptional activation by the chimeric genes, i.e., EWS-FLI-1, EWS-ERG, and EWS-E1AF, of the promoters of MMP-1 and MMP-3. All the three chimeric genes tested clearly induced transcriptional activation on the MMP-1 promoter (−517/+63), although to

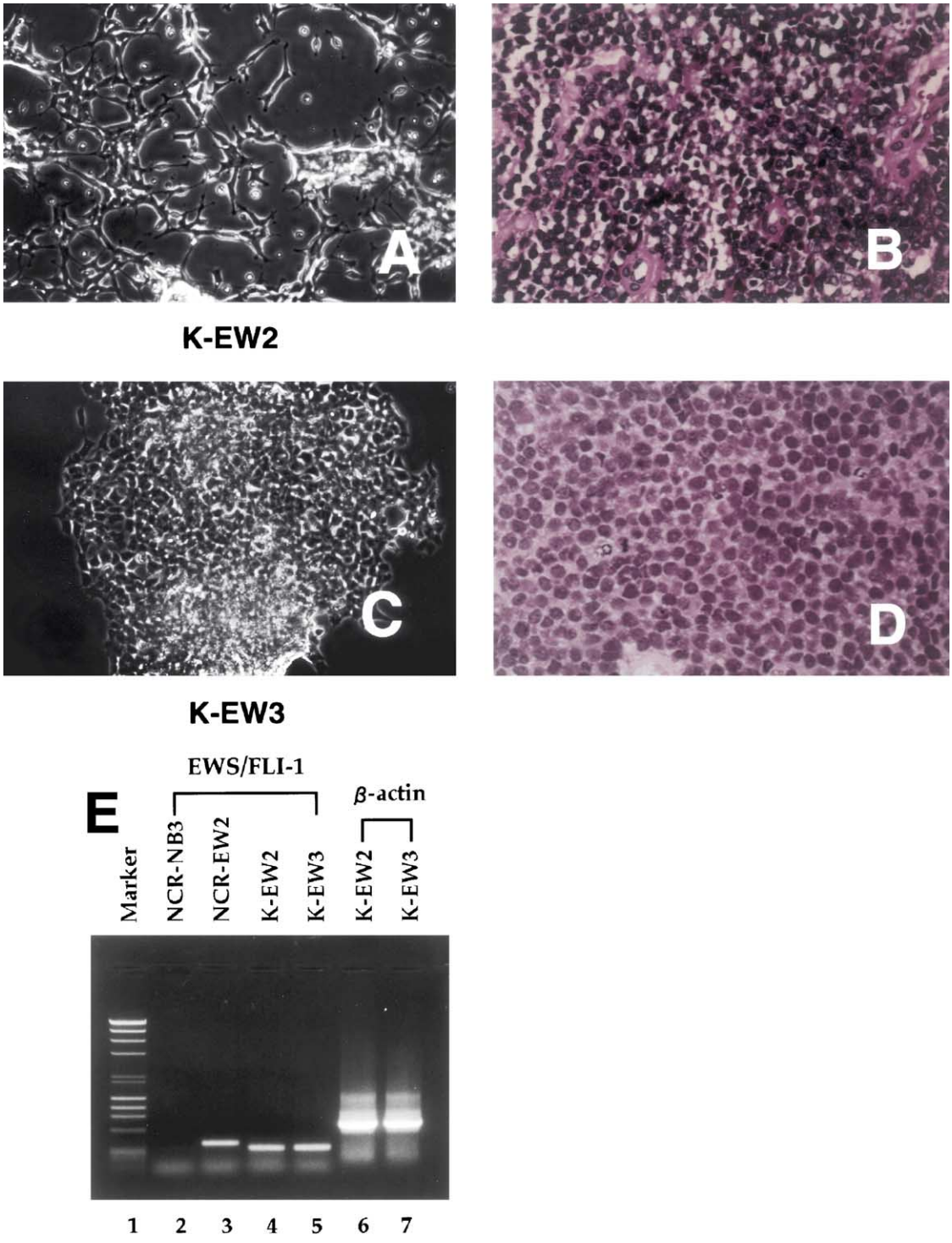

Fig. 1. Generation of two new Ewing sarcoma cell lines, K-EW2 and K-EW3, with the specific chimera gene, EWS-FLI1. (A) Phase-contrast photograph of K-EW2 cells in culture at a low cell density. The cells were fibrocytic in morphology (100×). (B) Histological appearance of the original tumor of K-EW2 cells. Small round tumor cells showing diffuse proliferative activity. Neither the Homer–Wright-type rosette formation nor that of any other specific structure was noted (HE, 100×). (C) Phase contrast photograph of K-EW3 cells. The cells grew as an attaching aggregate or a colony. (D) Histological appearance of the original tumor of K-EW3 (HE, 100×). (E) RT-PCR analysis of a cell line of Ewing sarcoma. Total RNAs were analyzed by RT-PCR using EU-1 (EWS specific) and EU-2 (FLI-1-specific)-sequence-based primers. The EWS-FLI-1 fusion gene was detected in both K-EW2 and K-EW3 cell lines (311 bp, lanes 4 and 5). The NCR-EW2 cell line, which contains EWS-FLI-1 fusion transcripts, was used as the positive control for the PCR reaction (377 bp, lane 3). NCR-NB3 cell line, established from a neuroblastoma, was used as the negative control (lane 2). β-actin gene is also detected in the K-EW2 and K-EW3 cell lines (868 bp, lanes 6 and 7).

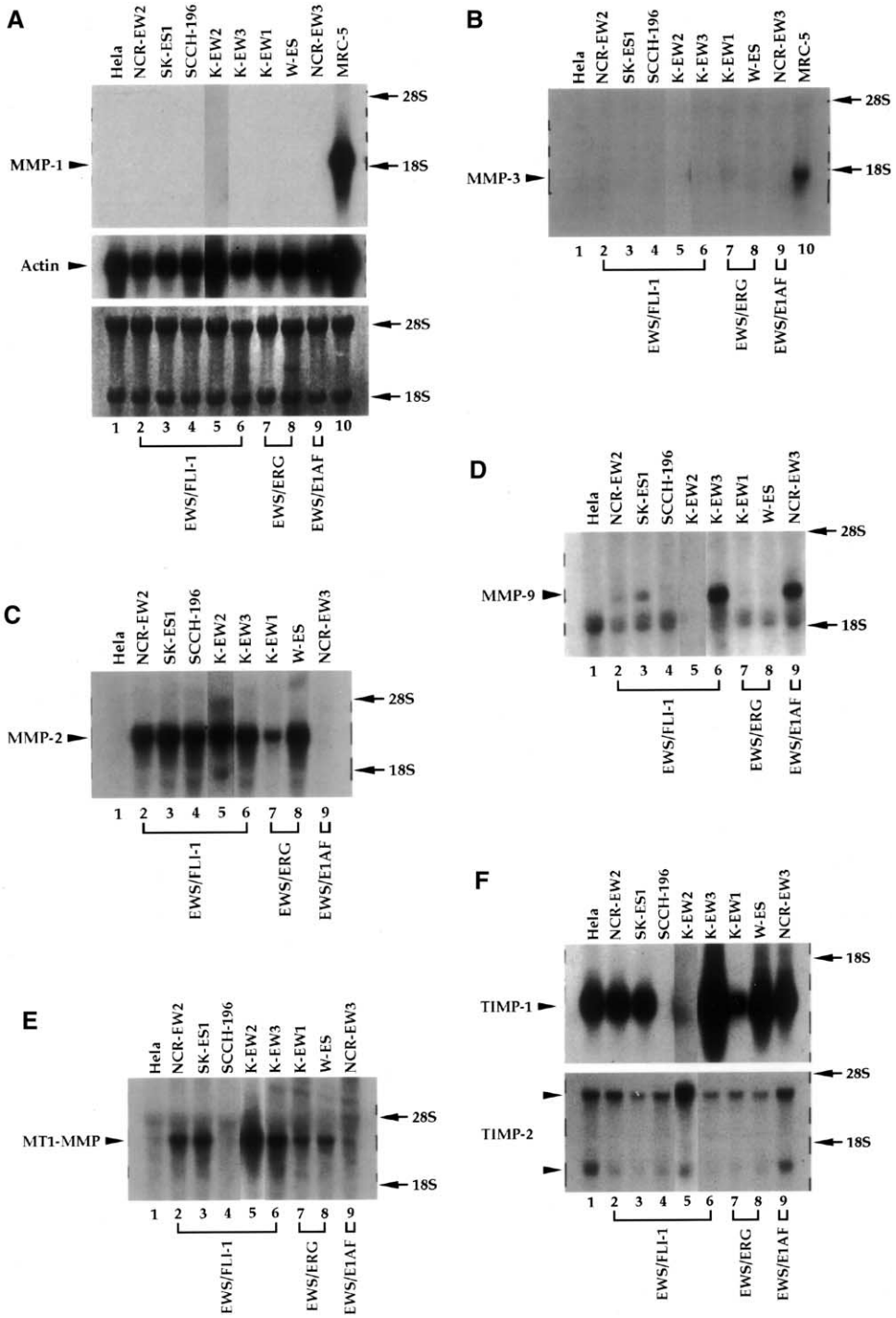

Fig. 2. Expression of MMPs and TIMPs in Ewing sarcoma cell lines. Northern blot analysis of MMP-1 (A), MMP-3 (B), MMP-2 (C), MMP-9 (D), MT1-MMP (E), and TIMP-1 and -2 (F) in Ewing sarcoma cells and control cells. Lanes 2–6: The cell lines established from Ewing sarcomas which have EWS-FLI-1, NCR-EW2, SK-ES1, SCCH196, K-EW2, and K-EW3. Lanes 7 and 8: K-EW1 and W-ES cells with EWS-ERG. Lane 9: NCR-EW3 with EWS-E1AF. HeLa cells (lane 1) and MRC-5 cells (lane 10) served as controls. The amount of total RNA was checked by the level of expression of the β-actin gene (A, middle part) and, 18S and 28S rRNA stained with methylene blue (A, lower part).

different levels (Fig. 4A). Thus, MMP-1 is not expressed in Ewing sarcoma cells and primary tumors, even though Ewing sarcoma-specific chimeric genes, including EWS-FLI-1 and EWS-ERG, can transactivate the promoter of MMP-1. In contrast, EWS-FLI-1 and EWS-ERG have a tendency to activate the MMP-3 promoter, although their activities are no more than those in the MMP-1 promoter (Fig. 4B).

Table 2
MMP and TIMP gene expressions in Ewing sarcoma/PNET cell lines

Type of MMPs and TIMPs	ets site	Gene expression								
		HeLa	EWS/FLI-1				EWS/ERG		EWS/E1AF NCR-EW3	MRC-5
			NCR-EW2	SK-ES1	SCCH196	K-EW2	K-EW3	K-EW1	W-ES		
(A) *PNET cell lines*											
MMP-1	+	–	–	–	–	–	–	–	–	–	+
MMP-2	–	–	+	+	+	+	+	+	+	–	
MMP-3	+	–	+	+	–	–	+	–	–	+	+
MMP-9	+	–	+	+	–	–	+	–	–	+	
MT1-MMP	+	–	+	+	–	+	+	+	+	+	
TIMP-1	+	+	+	+	–	–	+	+	+	+	
TIMP-2	–	+	+	+	+	+	+	+	+	+	

Type of MMPs and TIMPs	ets site	Gene expression					
		EWS/FLI-1			EWS/ERG	EWS/E1AF	MRC-5
		ST433	KST652	KST1461	KST1166	ST480	
(B) *PNET tumors*							
MMP-1	+	–	–	–	–	–	+
MMP-2	–	+	+	+	–	+	
MMP-3	+	–	–	–	–	–	+
MMP-9	+	+	–	–	–	–	
MT1-MMP	+	+	+	+	+	–	

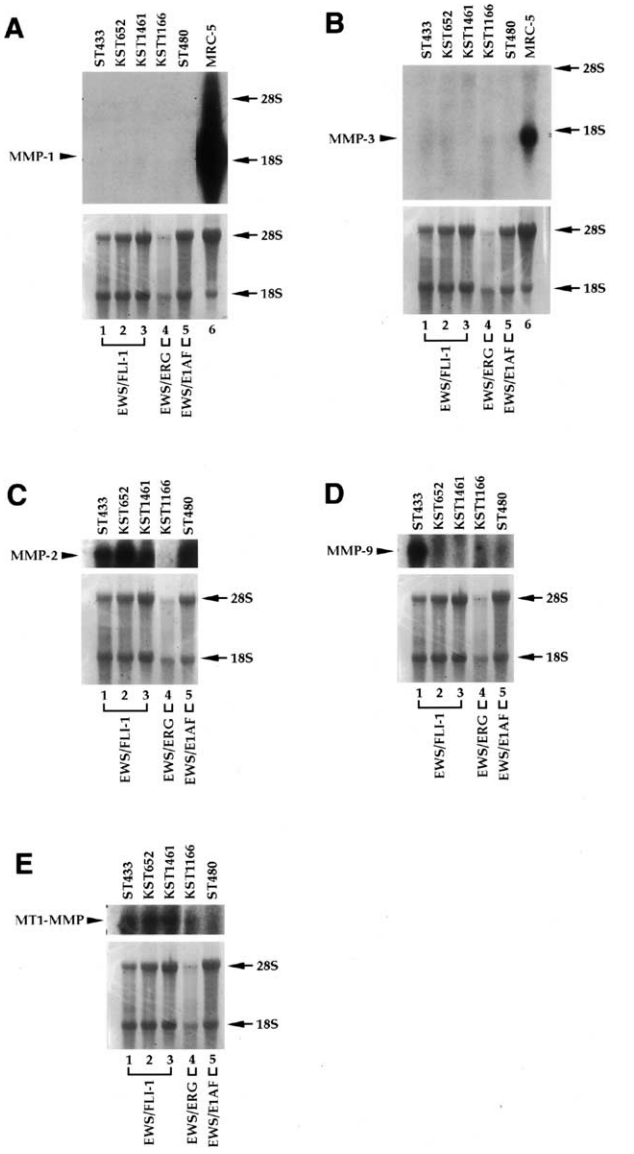

Fig. 3. Expression of MMPs in Ewing sarcoma specimens. Northern blot analysis of MMP-1 (A), MMP-3 (B), MMP-2 (C), MMP-9 (D) and MT1-MMP (E) in Ewing sarcoma specimens. Total RNA was isolated from ST433, KST652, and KST1461 (lanes 1–3) which have the EWS-FLI-1 fusion transcript; KST1166 (lanes 4) with the EWS-ERG chimeric gene; ST480 (lane 5) with the EWS-E1AF chimeric transcript. MRC-5 cells served as the control (A and B, lane 6). The amount of total RNA was checked by the level of expression of 18S and 28S rRNA stained with methylene blue (lower part).

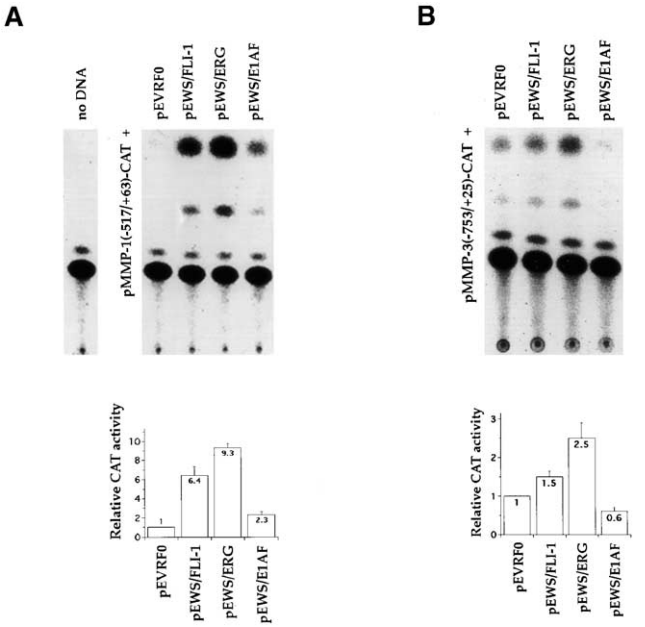

Fig. 4. Activation of MMP-1 and MMP-3 promoter activity by the EWS-FLI-1, EWS-ERG, and EWS-E1AF fusion proteins. Representative autoradiograms of CAT assays (upper part). By using the lipofection method, HeLa cells were co-transfected with 2 mg of the reporter plasmids (A: pMMP-1 (−517/+63); B: pMMP-3 (−753/+25)), 2 mg of internal control plasmids (actin-bgal) and 2 mg of empty vector (pEVRF0) or the expression vector of either EWS-FLI-1, EWS-ERG or EWS-E1AF driven by the cytomegalovirus (CMV) promoter. Cells were harvested 48 h after transfection, and the CAT activity was measured using a silica gel plate and visualized by autoradiography. After repeating the same experiments four and three times (A and B, respectively), the average and standard error of CAT activity corrected for variations in the internal control are indicated (lower part).

Loss of accessibility of the MMP regulatory element to the specific fusion protein in vivo

To elucidate the reason for the absence of MMP-1 expression despite the strong transcriptional activation of its promoter by the EWS-FLI-1 chimeric protein, we employed the in vivo formaldehyde cross-linking technique. With this technique, we investigated whether the EWS-FLI-1 chimeric protein binds to the ETS recognition sites in the regulatory elements of the MMP promoters in vivo. To determine whether the target sequences were precisely precipitated with the anti-FLI-1 antibody, we used Iti, Juyon, and Jugo, which have recently been isolated as target sequences (deposited in Genbank as AF177750, AF177751, and AF177752, unpublished observations). The signals of Iti, Juyon, and Jugo loaded on the nylon filter were enhanced reproducibly, indicating that the in vivo formaldehyde cross-linking and immunoprecipitation occurred precisely. In contrast, the signals of MMP-1, MMP-3, and MMP-9 promoter DNA were not enhanced when hybridized with the precipitated DNA, including the EWS-FLI-1 binding elements with the anti-FLI-1 antibody, as compared with that when hybridized with nonspecific DNA (Fig. 5A). Thus, the EWS-FLI-1 chimeric proteins did not bind to the promoters, including the predicted ETS recognition sites of MMP-1, MMP-3, and MMP-9, in Ewing sarcoma cells.

To determine whether the absence of in vivo binding of the Ewing sarcoma-specific chimeric proteins is due to the loss of the binding ability of the chimeric protein to the ETS recognition sites in the promoters, we performed the gel mobility shift assay. The signal of the

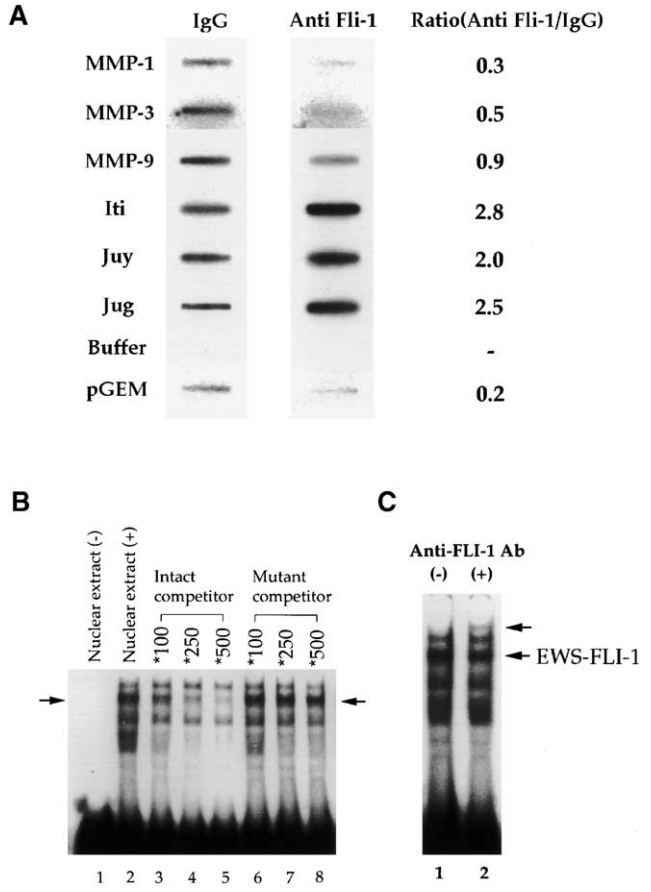

Fig. 5. Slot blot analysis of the DNA containing MMP-1, MMP-3, or MMP-9 regulatory elements and the gel shift assay. (A) DNA fragments containing the MMP-1 (−517/+63), MMP-3 (−9/−440), or MMP-9 (−670/+53) regulatory elements, were, respectively, applied to individual slots on a nylon filter. As positive controls, the DNA fragments (Iti, Juyon, and Jugo) cloned as including EWS-FLI-1 binding elements were applied onto individual slots on the same nylon filter. As negative controls, buffer only and pGEM vector only were applied onto the slots on the nylon filter. The loaded filter was hybridized with the DNA obtained by immunoprecipitation of cross-linked chromatin by anti-FLI-1 antibody and also with the anti-IgG antibody for comparison. The intensity of each of the signals enhanced by the FLI-1 antibody is calculated and shown on the right. Binding activity of a double-stranded oligonucleotide (Col I-88 ets) containing the ets site of the human collagenase (MMP-1) promoter detected by the gel shift assay. (B) Complexes were formed between the ^{32}P-labeled probe and nuclear proteins extracted from Ewing sarcoma cells (NCR-EW2). Lane 1: Probe alone; Lane 2: Probe and nuclear protein; Lanes 3–5: Competition with a 100-, 250-, or 500-fold molar excess of non-radioactive intact oligonucleotide; Lanes 6–8: Competition with a 100-, 250- or 500-fold molar excess of nonradioactive mutated oligonucleotide. (C) Lane 1: Probe and nuclear protein; Lane 2: Probe, nuclear protein, and specific antibody raised against the FLI-1 protein.

MMP-1 ET element was shifted with the addition of the nuclear extracts of Ewing sarcoma cells. This shifted signal was decreased by the intact competitor, but not by a mutant competitor (Fig. 5B). It was also super-shifted by the FLI-1 antibody (Fig. 5C). These results suggest that the EWS-FLI-1 chimeric protein can bind to the predicted ETS element in vitro, and that there are no inhibitors which prevent the chimeric protein from binding in the nuclear extracts of Ewing sarcoma cells.

Expression of metalloproteinase genes in Ewing sarcoma

In this study, we have demonstrated for the first time metalloproteinase gene expression in Ewing sarcoma and Ewing sarcoma cell lines, including two newly established lines. A series of metalloproteinase genes and tissue inhibitors of metalloproteinases have been purified or cloned. Extensive effort has been expended on investigating the expression profiles of such genes in a variety of carcinomas, since the expression patterns or levels are reported to be directly linked to the biological properties of the tumor, such as its propensity for metastasis and invasiveness. Most cancer cells produce higher levels of metalloproteinases, as compared with their normal counterparts. The expression profiles of MMPs have been analyzed in carcinomas of the breast, thyroid gland, endometrium, and oral cavity. However, quantitative expression analyses of the metalloproteinases have not yet been performed in sarcomas [18,19]. In this study, we show that Ewing sarcomas express MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2, but not MMP-1 or MMP-3. MMP-2 participates in the invasion by a carcinoma of the basement membrane, and is activated by MT1-MMP [32–34]. Activation and enhanced production of MMP-2 are detected in metastatic tumors of human thyroid carcinomas and human astrocytic tumors showing malignant transformation [35,36]. Similarly, the highly aggressive and invasive nature of Ewing sarcoma may be explained by the high level of expression of MMP-2 and MT1-MMP, which function cooperatively in the degradation of extracellular matrix.

Besides their pathological significance, the high expression levels of MMP-2, MT1-MMP, and TIMP in all the surgical specimens and cell lines of Ewing sarcoma examined evoked a possibility that EWS-FLI1 directly transactivates these genes. The regulation of expression of the MMP genes has been investigated at the mRNA level [37–42]. MT1-MMP is induced by v-src or erbB-2. MMP-2 and MMP-9 are regulated by integrins and E-cadherin, respectively. Hence, oncogenes and cell adhesion molecules are directly related to the expression levels of degradation enzymes of the extracellular matrix. Although the transcriptional regulation of such genes has not been elucidated yet, we cannot entirely deny the possibility that MMP-2, MT1-MMP, and TIMP are among the targets for the fusion protein.

Possible mechanism underlying the lack of MMP expression

The lack of expression of MMP-1 and -3 in Ewing sarcoma found by us was unexpected, since sarcomas originates from undifferentiated mesenchymal cells.

Most mesenchymal cells such as fibroblasts, synovial cells, and chondrocytes, which are of mesodermal origin, express MMP-1 and -3 at a high level. Furthermore, the MMP-1 and -3 genes are transactivated through the ETS recognition sites in their regulatory elements, and Ewing sarcoma has specific chimeric genes between those for EWS and ETS transcription factors. Therefore, the MMP-1 and -3 genes were expected to be expressed in Ewing sarcoma, which was not the case.

The chromatin immunoprecipitation method employed in this study provides information on the target genes of transcriptional factors in vivo. We employed this method for two purposes: (a) for in vivo binding of the fusion gene to their putative recognition sequences in the regulatory elements of MMP-1 and -3; (b) isolation of the target genes or sequences of the fusion gene. So far, MMP-3, cytochrome P450-F1, cytokeratin 15, EAT-2, and manic fringe have been suggested as target genes of the Ewing sarcoma-specific fusion protein [13,43,44]. However, as shown in this study, MMP-3 was not a target gene of the fusion protein of Ewing sarcoma in vivo. This is based on the evidence that MMP-3 was not expressed in any of the surgical specimens and cell lines of Ewing sarcoma, and EWS-FLI1 did not bind to the MMP-3 regulatory element in vivo. In addition to MMP-3, the transcriptional activation, which was detected by a transient transfection method, by the fusion genes of the MMP-1 regulatory element was not associated with the expression of the MMP-1 gene in vivo, either. Lack of binding of the fusion gene product in vivo to the regulatory elements of these two genes was rather surprising, since binding ability of the fusion protein to the ETS recognition sequence was observed in vitro. Therefore, we would like to emphasize here that the in vivo expression of the gene(s) and in vivo binding of the fusion protein to the recognition sites must be definitely investigated once the candidate gene(s) are isolated or identified.

Loss of accessibility of the recognition sequence to the fusion protein can be explained by two possible mechanisms, i.e., chromatin configuration and methylation (Fig. 6). Chromosomal packaging can prevent the access of the transcriptional machinery to nucleosomal DNA, leading to cell-type specific genetic regulation. MMP-1 and -3 expression may be influenced by the chromatin state of the gene, whereby tight chromosomal packaging may interfere with the fusion protein binding. Interestingly, MMP-1 and MMP-3 genes, both of which were not expressed in Ewing sarcoma, have been mapped to the same chromosomal locus, 11q22.3, while the MMP genes which were found to be expressed were mapped to the other loci, implying the possibility that the locus holding MMP-1 and MMP-3 is tightly packaged or silenced. The other possibility is that DNA methylation results in gene inactivation. Methylated DNA-binding proteins are responsible for transcriptional repression via an indirect mechanism, i.e., inhibition of DNA binding of transcriptional factors. Alternatively, DNA binding of ETS transcription factors is sensitive to DNA methylation [45]. Recently, these two mechanisms, i.e., chromatin state and methylation, have been molecularly shown to be tightly linked. Thus, we should be careful before theorizing simply that a putative recognition sequence is essential for the transcription of critical genes, because binding of the fusion protein was shown to be significantly affected in vivo in this study.

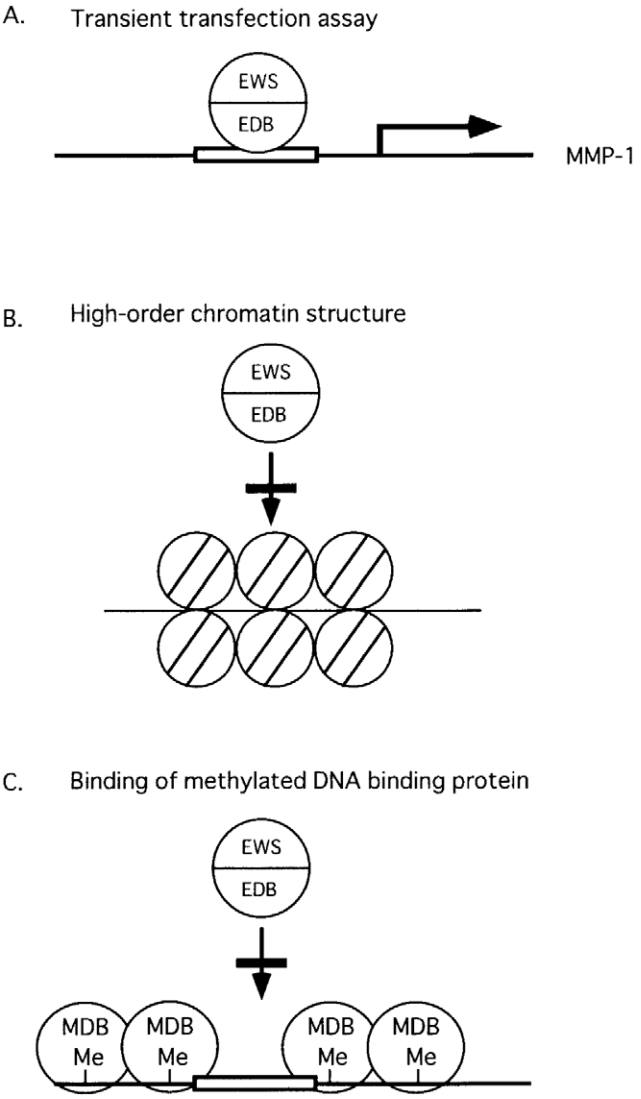

Fig. 6. Loss of accessibility of the MMP regulatory elements to the Ewing sarcoma-specific fusion protein. The chimeric protein between EWS and ETS was found to transactivate the MMPs through binding with the regulatory elements, by a transient transfection assay (A). Two possible mechanisms for this not being the case in vivo are postulated. (B) High-order chromatin structure of the regulatory elements inhibits DNA binding of the fusion protein. (C) Methylated DNA binding proteins prevent the fusion protein from binding to the recognition site.

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