Evi-1 is a gene, encoding a zinc finger protein, associated with a common viral integration site in murine leukemias. It is suggested that Evi-1 plays important roles in embryogenesis and transformation of myeloid cells. To elucidate mechanisms by which Evi-1 induces such biological effects, we analyzed the relationship between Evi-1 and AP-1 which could regulate cellular proliferation and differentiation. When Evi-1 was expressed, transactivation through a 12-O-tetradecanoylphorbol 13-acetate-responsive element was observed in NIH3T3 and P19 cells. Evi-1-transfected P19 cells showed some differentiated phenotypes and increased expression of endogenous c-Jun and c-Fos. These results indicate that Evi-1 raises AP-1 activity. Evi-1 caused stimulation of the c-fos promoter transactivation, which seems to be a main mechanism of AP-1 activation, through at least two portions of the promoter. Evi-1 has the first zinc finger domain at the N-terminal and the second zinc finger domain near the C-terminus. We constructed deletion mutants of Evi-1 and investigated the functions of these domains. It was shown that the second zinc finger domain is essential for the activation of AP-1 and transactivation of the c-fos promoter.

Evi-1 (ecotropic viral integration site 1) was first identified as a common locus of retroviral integration in myeloid tumors found in AKXD mice (1). Cloning of the cDNA corresponding to this locus and subsequent studies revealed that the gene encodes a nuclear-localized DNA-binding protein, which has 10 Cys_His-type zinc fingers (2-4). Evi-1 is not normally expressed in any lineage of hematopoietic cells. In some murine leukemias, however, inappropriate expression of Evi-1 is caused by retroviral insertions (5, 6). Ectopic expression of Evi-1 in murine 32D1 cell lines results in a block of granulocytic or erythroid differentiation (7, 8). Recently, it was shown that Evi-1 gene is transcriptionally activated in human acute myelogenous leukemias by translocations and inversions involving chromosome 3q26, which is the Evi-1 gene locus (9-11). These studies suggest that inappropriate expression of Evi-1 disturbs normal cellular proliferation and differentiation in hematopoiesis, probably resulting in or at least contributing to leukemic transformation of the cells.

Recently, the distribution of Evi-1 expression was reported in adult and embryonic mouse tissues (12). It was demonstrated that Evi-1 is expressed at high levels in several embryonic tissues, but at low levels in most adult tissues. Regions that exhibit high-level expression in the embryo include the urinary system, bronchial epithelium of the lung, developing limbs and some regions of the nasal cavity and heart. The restricted pattern of Evi-1 expression in embryonic tissues suggests that Evi-1 plays an important role in cellular proliferation and differentiation in mouse development. However, little is known about the mechanisms by which Evi-1 induces such biological effects.

The transcription factor AP-1 (Fos/Jun heterodimer or Jun/ Jun homodimer) represents a prototype of regulatory protein that converts extracellular signals into changes in the gene expression program (13). AP-1 is activated by growth stimuli, including growth factors, phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA), and various transforming oncogene products. In addition, AP-1 functions as a positive or negative regulator in a variety of cellular differentiation processes. AP-1 activity is found to change in the differentiation processes of embryonal carcinoma cell lines (14, 15) and myoblasts (16). It is probable that unclarified genes transcriptionally regulated by Evi-1 trigger certain signal pathways resulting in the change in AP-1 activity because Evi-1 plays an important role in cellular proliferation and differentiation. To elucidate mechanisms by which Evi-1 induces these biological effects in embryogenesis and transformation of myeloid cells, we investigated the relationship between Evi-1 and AP-1 in this study. In addition, we analyzed functions of domain structures found in the Evi-1 protein. Evi-1 has seven zinc fingers at the N-terminus (first zinc finger domain), three zinc fingers near the C-terminus (second zinc finger domain), and an acidic amino acid-rich region (acidic domain) (2). The consensus nucleotide sequence that each zinc finger domain recognizes has already been reported (7, 18, 19). However, biological functions of each zinc finger domain remain obscure. The acidic domain is a putative transcriptional activation domain in Evi-1, which has not yet been demonstrated. We analyzed the functions of these domains by estimating AP-1 activity using various Evi-1 deletion mutants.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture—**NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum as described.

1. The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; TRE, TPA-responsive element; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; EC, embryonal carcinoma; SIR, serum-inducible region.
previously (20). P19 cells were maintained on gelatin-coated dishes in DMEM (high glucose formulation) supplemented 10% fetal calf serum as described previously (21).

Plasmid Constructions—The cDNA of human Evi-1 was derived from AML1/Evi-1 fusion cDNA obtained from the SKH7 cell line, in which the truncated AML1 gene was revealed to fuse to the N terminal region of Evi-1 (22). The sequence of this Evi-1 cDNA was confirmed to be essentially identical to the cDNA which Morishita et al. (3) obtained. For construction of Evi-1 expression plasmids, an EcoRI site was introduced by the site-directed mutagenesis method as described previously (21). P19 p(wild TRE) X 3-tk-Luc, p(mut.TRE) X 3-tk-Luc, p(mut.TRE) X 3-tk-Luc and p(mut.TRE) X 3-tk-Luc, respectively, were transfected into NIH3T3 cells or P19 cells by the calcium-phosphate precipitation method. Deletion mutants of Evi-1 cDNA were created in the Evi-1 cDNA by the site-directed mutagenesis method as described above. The HindIII-Hind111 DNA fragment of the FC4 plasmid, in which two nucleotides were replaced at -308 in the c-fos promoter, was inserted into the Bam-HI site of pBLCAT2 (26). Site-directed mutagenesis was confirmed by estimating luciferase activity in NIH3T3 cells transfected with p(wild TRE)x3-tk-Luc, p(mut.TRE)x3-tk-Luc and p(mut.TRE)x3-tk-Luc DNA plasmid and stimulated by TPA. For construction of pFOS(-222)Luc and p(mut.TRE)x3-tk-Luc reporter plasmid, in which three tandemly repeated TPA-responsive elements (TRE) were inserted immediately upstream of the herpes simplex virus thymidine kinase promoter, was amplified by the polymerase chain reaction method and inserted into the BamHI site of pUC13. as described previously (27). oligonucleotides (5'GATCGTGAGTCAGCGCGGTGAGTCAR
CGCGCTGTCTCCCCGCGCTGTCTCCCCGCGCTGTCTCCC-3') were annealed and inserted into the BamHI site of pBLCAT2 (26). For construction of a p(wild TRE)x3-tk-Luc reporter plasmid, the cDNA was inserted into the Smal site of pUC000Luc, respectively. Site-directed mutagenesis was performed to make Sacl sites at -364 or -298 base pairs from transcription initiation of the human c-fos promoter and each Sall-HindIII DNA fragment was blunted by Klenow fragment and inserted into the Smal site of pUC000Luc, respectively. Site-directed mutagenesis was performed by the polymerase chain reaction method, to give pFOS(-222)Luc and pFOS(-88)Luc, respectively. The first antibodies used in this study were anti-c-Fos antibody (34), anti-c-Jun antibody (35) and anti-SSEA-1 (36) (mouse monoclonal IgM, AH-6) and anti-Hsp47 (37) (rabbit polyclonal IgG). The second antibodies were fluorescein-conjugated goat antibodies against mouse IgM and rat IgG (TACO, Inc.).

RESULTS

Evi-1 Activates AP-1 Depending on Second Zinc Finger Domain

Table 1

| Cell line | Reporter | Cotransfected expression plasmid |
|-----------|----------|---------------------------------|
| NIH3T3    | p(wild TRE)x3-tk-Luc | pME-Evi-1, pME-Evi-R |
|           | p(mut.TRE)x3-tk-Luc | 5.1 ± 0.3, 1.2 ± 0.1 |
| P19       | p(wild TRE)x3-tk-Luc | 5.3 ± 0.5, 1.2 ± 0.1 |
|           | p(mut.TRE)x3-tk-Luc | 1.2 ± 0.3, 1.4 ± 0.1 |

Data were analyzed by a standard deviation of three independent experiments (25). The values (means ± standard deviations) were analyzed by a standard deviation of three independent experiments (25).

Preparation of Anti-Evi-1 Serum—To obtain protein for immunization, a DNA fragment from ~88 to ~790 base pairs from the start site of translation of human Evi-1 cDNA was amplified by the polymerase chain reaction method and inserted into the BamHI and XhoI sites of pMAL-c2 vector (New England Biolabs) in the same frame as male blue gene of Escherichia coli. This construct was expressed in E. coli DH5α cells in the presence of isopropyl-β-D-thiogalactoside. The product (fusion protein of maltose-binding protein and partial Evi-1) was purified using an amylose resin column, according to manufacturer’s protocol (New England Biolabs) and used to immunize a rabbit.

Western Blots—Cells were lysed on ice by the lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.05% SDS, 1% deoxycholate, 1% Triton X-100, 10 units/ml aprotinin and 2 μg phenylmethylsulfonyl fluoride). Protein concentrations were quantified using the Bradford method, and cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene difluoride filters (Millipore), then reacted with anti-c-Jun antibody (OPA 131 purchased from Medac Molecular Biology) (34), anti-c-Fos antibody (PC05 purchased from Oncogene Science) (35), anti-a-tubulin (DM1A, gift from Dr. N. Hirokawa (University of Tokyo)) or anti-Evi-1 serum (described above). The blots were visualized by Protoblot system (Promega).

Immunoafluorescent Staining—P19 cells (1.5 × 10⁴) were transfected by the calcium-phosphate precipitation method, with pSV2neo (50 ng) and p(wild TRE)x3-tk-Luc (3.5 μg) DNA plasmid and pME18S DNA, then selected by G418 (200 μg/ml) for 6 days. Procedures for the fixation of cells and incubation with the first and second antibodies have been described previously (36). The first antibodies used in this study were anti-SSEA-1 (36) (mouse monoclonal IgM, AH-6) and anti-Hsp47 (37) (rat monoclonal IgG, TC4). The second antibodies were fluorescein-conjugated goat antibodies against mouse IgM and rat IgG (TACO, Inc.).

Cell line Reporter Cotransfected expression plasmid
| NIH3T3 | p(wild TRE)x3-tk-Luc | pME-Evi-1, pME-Evi-R |
|--------|---------------------|---------------------|
|        | p(mut.TRE)x3-tk-Luc | 5.1 ± 0.3, 1.2 ± 0.1 |
| P19    | p(wild TRE)x3-tk-Luc | 5.3 ± 0.5, 1.2 ± 0.1 |
|        | p(mut.TRE)x3-tk-Luc | 1.2 ± 0.3, 1.4 ± 0.1 |

Data were analyzed by a standard deviation of three independent experiments (25). The values (means ± standard deviations) were analyzed by a standard deviation of three independent experiments (25).
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Expression of c-Jun and c-Fos in P19 Cells Transfected with pME18S (left) or pME-Evi-1 (right). P19 cells were transfected as described in the legend of Fig. 1. Each cell lysate (50 μg) was subjected to SDS-PAGE, blotted to a filter, and probed with anti-c-Jun, anti-c-Fos, or anti-α-tubulin antibody. Expression of α-tubulin serves as an internal control for the amount of protein loaded in each lane.

The c-fos Promoter Is Transactivated in the Presence of Evi-1—Transcriptional activation of the c-fos gene is one of the main mechanisms by which various growth factors and oncogenes raise AP-1 activity (42). We examined whether the c-fos promoter is transactivated in the presence of Evi-1. The human c-fos promoter sequence containing nucleotide base pairs between -403 and +43, relative to the start site of transcription, was positioned downstream of the firefly luciferase cDNA. This reporter plasmid, designated pFOS(-403)Luc, was co-transfected with the Evi-1-expression vector (pME-Evi-1) or a control plasmid (pME-Evi-R) into NIH3T3 and P19 cells. The reporter was transfected 26.5-fold when Evi-1 was co-transfected into NIH3T3 cells (Table II). Similarly, weak but significant transactivation of the c-fos promoter was observed in P19 cells when Evi-1 was cotransfected, which is consistent with elevated expression of c-Fos protein shown in Fig. 2.

Many reports indicate that AP-1 positively regulates c-Jun transcription through the AP-1 recognition site in the c-Jun promoter. On the other hand, AP-1 negatively regulates c-fos transcription through the serum response element in the c-fos promoter (13, 43–45). There is an AP-1 recognition site at the position of around -295 in the c-fos promoter (46), but this site does not contribute at all to the c-fos promoter transactivation in response to the serum stimulation or overexpressed c-fos (44). To clarify that c-fos promoter transactivation in the presence of Evi-1 is not the result of a rise in AP-1 activity induced by Evi-1, but is a mechanism for it, because the c-fos promoter is reported to be negatively regulated by activated AP-1 (13, 45) and in fact, the AP-1 site in the c-fos promoter does not contribute to up-regulation of the c-fos promoter by Evi-1, and because increased c-fos-expression leads to a rise in AP-1 activity (47).

When primary cultures were selected for G418 resistance 5–7 days after cotransfection of pME-Evi-1 and pSV2neo, surviving cells formed colonies and 85–94% of them showed morphological differences from control P19 cells which were transfected with pME18S and pSV2neo. Evi-1-transfected P19 cells showed differentiated phenotypes, characterized by a flattened and enlarged morphology (Fig. 1A). These changes were indistinguishable from the morphological changes seen in P19 cells treated with retinoic acid or transfected with c-Jun (38, 39). To confirm the differentiation of P19 cells induced by Evi-1, stem-cell and differentiation marker proteins were examined by the indirect immunofluorescence method (Fig. 1B). The stage-specific embryonic antigen SSEA-1, known as a stem cell marker in EC cells, was detected in P19 cells without Evi-1 transfection, but not in Evi-1-transfected cells. In contrast to SSEA-1, the heat shock protein Hsp47 (37), known as a differentiation marker, was detected in Evi-1-transfected P19 cells, but not in the absence of Evi-1 transfection. In addition, we observed elevated expression of endogenous c-Jun and c-Fos in Evi-1-transfected P19 cells, compared with pME18S-transfected P19 cells (Fig. 2). The increased expression of endogenous c-Jun is also reported in EC cells transfected with exogenous c-jun (39) or activated c-H-ras (36) and treated with retinoic acid (14). It has also been reported that expression of endogenous c-Fos is increased along with EC cell differentiation induced by retinoic acid or dimethyl sulfoxide (40, 41).

Fig. 1. A, the typical morphology of P19 cells transfected with a control or Evi-1-expression plasmid. P19 cells (1.5 × 10^6) were transfected with pSV2neo (50 ng) and either pME-Evi-1 (3.2 μg) (right) or equivalent-molar pME18S (left). Phase-contrast photomicrographs were taken after 6 days of selection by G418 (200 μg/ml). B, immunofluorescent staining of P19 cells transfected with pSV2neo and either pME18S (a) or pME-Evi-1 (b). Cells were stained with anti-SSEA-1 (1) or anti-Hsp47 (2) monoclonal antibody.

Evi-1 Induces Differentiation and Increases c-Fos and c-Jun Expression in P19 Embryonal Carcinoma Cells—To explore biological responses to the AP-1 activity increased by Evi-1, we used the P19 embryonal carcinoma (EC) cell line, because ectopic expression of c-jun or c-fos in EC cells leads to cellular differentiation (14, 15). We investigated alteration of AP-1 activity and cellular differentiation in Evi-1-transfected P19 cells. The AP-1-dependent reporter gene was transactivated by Evi-1 in P19 cells, although the fold induction was lower than in NIH3T3 cells (Table I). This activation is dependent on the AP-1-recognition sequence, since no significant transactivation in the presence of Evi-1 was observed with the mutated AP-1 recognition sequence.
Evi-1 Activates AP-1 Depending on Second Zinc Finger Domain

### Table II

| Cell line | Expression plasmid |
|-----------|-------------------|
|           | pME-Evi-1         | pME-Evi-R        |
| NIH3T3    | 26.5 ± 3.0        | 1.3 ± 0.4        |
| P19       | 5.6 ± 0.6         | 1.0 ± 0.1        |

**Evi-1 Contributes to Transactivation of the c-fos Promoter through At Least Two Portions of the Promoter** — To identify the sites in the c-fos promoter where Evi-1 contributes to transactivation, we prepared various 5’ deletion constructs of the c-fos promoter fused with luciferase cDNA, transfected them with pME-Evi-1 or control pME18S into NIH3T3 cells and measured luciferase activity (Fig. 3). The similar levels of induction by Evi-1 were observed with deletions to nucleotides -403, -334, and -298. The deletion to -222 diminished the fold induction significantly from 29-27 to 4-7. Deletions to -222, -142, and -100 showed similar levels of fold inductions. The deletion to -88 decreased the fold induction significantly from 4-7 to about 1. These data indicate that there are at least two portions in the c-fos promoter where Evi-1 contributes to its transactivation: one site (possibly two or more separate sites) is between nucleotides -298 and -222 and another is between nucleotides -100 and -88. We compared the nucleotide sequences of these two portions of the c-fos promoter with Evi-1 binding consensus sequences (7, 18, 19). We did not find sequences similar to the binding consensus for the first zinc finger domain of Evi-1 but did find the sequence CTCACTCCTG around -235, which resembles the consensus CTCACTCTG (one base mismatch) for the second zinc finger domain. We performed further experiments including the gel shift analysis to investigate direct binding of Evi-1 to this site, but did not demonstrate such a phenomenon (data not shown).

The Second Zinc Finger Domain of Evi-1 Is Essential for Raising AP-1 Activity and Transactivation of the c-fos Promoter — To investigate the role of the two zinc finger domains and acidic domain, various deletion mutants of Evi-1 were constructed and transfected into NIH3T3 cells (Fig. 4A). The expression of the mutants was examined by Western blot analysis using anti-Evi-1 serum. Each mutant protein was expressed sufficiently in the anticipated size (Fig. 4B).

These mutants showed various levels of AP-1 activation (Fig. 5A). ΔZF1 showed an equivalent level of activation to that of the wild Evi-1. ΔZF2-4 and ΔZF5-7 presented 43 and 18% activation of AP-1, respectively, compared with wild Evi-1. Surprisingly, deletion of zinc fingers 6–10 completely abolished the activation of AP-1 (Fig. 5A, ΔZF8-10). These results show that the latter part of the first zinc finger domain is necessary for full activation of AP-1, and that the second zinc finger domain is essential for raising AP-1 activity. Deletion of the acidic domain (ΔC-end) only diminished to a slight extent the activation of AP-1 by Evi-1. The acidic domain is probably unnecessary for the activation of AP-1. When pFOS(-403)Luc was used as a reporter, the pattern of responses to these Evi-1 deletion mutants was almost the same as that of p(wild TRE)x3-tk-Luc reporter (Fig. 5B). These results confirm the importance of the second zinc finger domain and also provide evidence that transactivation of the c-fos promoter is the main mechanism for raising AP-1 activity.

**Discussion**

To elucidate mechanisms by which Evi-1 induces biological effects in cellular proliferation and differentiation, we analyzed the relationship between Evi-1 and AP-1 activity. When Evi-1 was expressed, transactivation through a TPA-responsive element was observed in NIH3T3 and P19 cells. In addition, elevated c-Jun and c-Fos expression and some differentiated phenotypes were found in Evi-1-transfected P19 cells. These phenotypes resembled those observed along with activation of AP-1 in P19 cells treated with retinoic acid or transfected with c-jun (39, 40). These results indicate that Evi-1 raises AP-1 activity. Moreover, the c-fos promoter was transactivated in the presence of Evi-1, which might be one of the mechanisms for the activation of AP-1 by Evi-1.

Evi-1 is transcriptionally activated in some human and murine leukemias and suggested to play important roles in transformation of myeloid cells. It is possible that Evi-1 can transform myeloid cells by continuously stimulating the c-fos transcription and activating AP-1, because there have been many observations that overexpressed c-Fos and c-Jun, component proteins of AP-1 complex, have transforming activity upon various cells (13). Moreover, Evi-1 should play an important role in embryogenesis because it is expressed in restricted tissues in mouse development (12). Most of these tissues, including developing endocardial cushions in the heart and developing limbs, are in which cell proliferation is especially enhanced. In these developing tissues, enhanced AP-1 activity by Evi-1 may induce enhanced cellular proliferation. In fact, it seems that stimulated expression of c-fos (48-50) is accompanied by enhanced expression of Evi-1 (12) in some developing tissues, as seen in truncus swelling of the heart and some regions of the nasal cavity.

A change in AP-1 activity is a crucial step for some cellular differentiation processes as well as proliferation. Studies so far suggest that AP-1 positively regulates the differentiation of EC cells such as F9 and P19 (14, 15). Evi-1-transfected P19 cells showed differentiated phenotypes in our study. Elevated expression of c-Jun and c-Fos can be observed along with EC cell differentiation (40, 41). We also observed elevated c-Jun and c-Fos expression and transactivation of the c-fos promoter in Evi-1-expressed P19 cells. It is reported that, in EC cells, c-fos expression is a crucial step for AP-1 elevation and development of their differentiated phenotypes (51). Transactivation of the c-fos promoter may be an indispensable event for these changes generated by Evi-1.

We showed high levels of TRE and c-fos promoter transactivation in NIH3T3 cells. In P19 cells, however, both transactivation levels were lower than in NIH3T3 cells. These observations are consistent with our presumption that transactivation of the c-fos promoter is a main mechanism for raising AP-1 activity. We suppose such discrepancies are due to that other transcription factors, which influence the c-fos promoter transactivation and AP-1 activity in cooperation with Evi-1, do not work so well in P19 cells as in NIH3T3 cells. Several reports, in fact, indicate a low level of AP-1 activity and c-fos expression in EC cells (40, 52).

Our results show that expression of Evi-1 leads to a rise in AP-1 activity and transactivation of the c-fos promoter. Our data suggest that the latter is the main mechanism of the former. How does Evi-1 regulate the transcription of c-fos. Analyses using 5’ deletion constructs of the c-fos promoter revealed that at least two portions of the promoter are important for transactivation when Evi-1 was expressed. One part between nucleotides -298 and -222 includes a sequence similar to the consensus sequence for binding of the Evi-1 second zinc finger domain. However, direct binding by Evi-1 to this portion
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Depending on Second Zinc Finger Domain Fold Induction by Evi-1

| pFOS(-403)Luc | luciferase |
| pFOS(-334)Luc | luciferase |
| pFOS(-298)Luc | luciferase |
| pFOS(-222)Luc | luciferase |
| pFOS(-142)Luc | luciferase |
| pFOS(-100)Luc | luciferase |
| pFOS(-88)Luc | luciferase |
| pFOS(mAP1)Luc | luciferase |

Exp. 1 | Exp. 2
---|---
25.0 | 24.6
23.8 | 22.5
27.4 | 27.0
4.7 | 7.1
4.6 | 5.3
4.3 | 5.7
1.3 | 1.1
23.5 | 20.8

Fig. 3. The 5′ deletion constructs of the c-fos promoter and their fold induction in transactivation when Evi-1 was expressed. Locations of regulatory elements reported previously in the c-fos promoter are shown by boxes; a sis-inducible element (SIE) (46), a serum response element (SRE), the AP-1 recognition site (API), a serum-inducible region with repeat elements (SIR) (53), the direct repeat (DR) (54), and a cyclic AMP-response element (CRE). Various 5′ deletion constructs of the c-fos promoter were fused with luciferase cDNA. Each 5-μg reporter plasmid was transfected with pME-Evi-1 (3.2 μg) or equivalent-molar pME18S into NIH3T3 cells (3 × 10⁵ cells), and the luciferase activities were measured. Fold induction represents the luciferase activity when pME-Evi-1 was cotransfected with each reporter plasmid, relative to the activity in cotransfection of pME18S with the corresponding reporter. The data of two independent experiments are shown.

was not observed in our studies so far. This part also includes a serum-inducible region with repeat elements (SIR), on which c-fos transcription induction by nerve growth factor or serum depends (53). It is possible that c-fos transcription induction by Evi-1 partly takes place through SIR, even if Evi-1 does not bind directly on the c-fos promoter. Another part responsible for the stimulated c-fos promoter transactivation by Evi-1 is between nucleotides -100 and -88. Deletion to -88 removes half of the tandemly repeated octanucleotide sequence (direct repeat, DR) (54). It is reported that c-fos transcription induction by Raf kinase partly depends on DR (55). It is likely that c-fos transcription induction by Evi-1 partly takes place at DR, possibly by stimulating the Raf signal transduction pathway. In addition to the transactivation of the c-fos promoter, there are possible mechanisms through which Evi-1 affects AP-1 activity. The main components of AP-1 complex in mammalian cells are c-Fos and c-Jun, and several molecules belonging to the jun/fos family were recently identified. It should be investigated whether Evi-1 also takes part in transcriptional control of c-jun and other members of the jun/fos family. We assume that the c-jun promoter can be transactivated in the presence of Evi-1 because c-jun transcription is positively regulated by AP-1 through the AP-1 binding site in the promoter (13, 43). We should also analyze the relationship between Evi-1 and post-translational control of AP-1 activity, such as phosphorylation of c-Jun.

Several DNA-binding proteins are reported to contain two widely separated zinc finger motifs, for example, hunchback...
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A. transactivation of AP-1 recognition site-containing promoter by cotransfection of each Evi-1 mutant. NIH3T3 cells (9 x 10^5 cells) were transfected with each mutant construct inserted in pME18S (3.2 μg of pME-Evi-1 indicated as wild Evi-1 or an equivalent-mole of each mutant Evi-1 construct) or equivalent-molar pME18S (indicated as vector). The reporter plasmid (5 μg), p(wild TRE)x3-tk-Luc, was cotransfected with each expression plasmid. Bars show luciferase activities in percentages relative to the activity of wild Evi-1 transfectants. B. transactivation of the c-fos upstream sequence in cotransfection of each Evi-1 mutant. NIH3T3 cells were transfected as described in Fig. 5A except that the reporter was pFOS(-403)Luc (5 μg).

That Evi-1 manifests its roles in embryogenesis and transformation of myeloid cells, at least partly, owing to the AP-1 activation. Further studies would clarify those relationships. It has also been revealed that the second zinc finger domain of Evi-1 is essential for activating AP-1 and stimulating the c-fos promoter transactivation. The mechanism should be further investigated, by which the second zinc finger domain induces such effects.

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Evi-1 Activates AP-1 Depending on Second Zinc Finger Domain

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T Tanaka, J Nishida, K Mitani, S Ogawa, Y Yazaki and H Hirai

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