Osteopontin is an arginine-glycine-aspartic acid-containing cell adhesion protein, which is frequently expressed in transformed cells and is thought to play a role in tumorigenesis. v-Src is a transforming viral oncogene product encoded by Rous sarcoma virus (RSV). We report that v-Src expression in HT1080 fibrosarcoma cells significantly stimulates mouse osteopontin promoter activity. We also determined the v-Src response element in the osteopontin promoter an inverted CCAAT box located at −53 to −49 from the transcription start site. Mutations of the CCAAT box disrupts protein-DNA interaction and diminishes both v-Src stimulation and basal promoter activity. A CCAAT box-containing fragment corresponding to −155 to −122 of RSV long terminal repeat competed with the −72 to −38 fragment of mouse osteopontin promoter for specific protein binding in the gel shift assay. A polyclonal antibody against CBF, a CCAAT box-binding factor, supershifted in gel shift assays the protein-DNA complex formed by nuclear extract of HT1080 with either the RSV CCAAT box fragment or with the osteopontin −72 to −38 fragment. Moreover, both osteopontin mRNA levels and enhancer activity of CCAAT box-containing −72 to −38 fragment were significantly elevated in v-sr-transfected NIH 3T3 cells relative to parental cells. These findings suggest that the elevated osteopontin expression in transformed cells could be, at least in part, to v-Src stimulation of the osteopontin promoter and that this effect is mediated by a CBF-like factor.

The Rous sarcoma virus (RSV)1 is a potent tumor inducer in host animals. A 60-kDa tyrosine kinase, v-Src, is essential for the transforming activity of RSV (1). Transformation by v-Src and/or transient expression of v-Src induces a number of genes, such as 9E3/CEF-4, collagenase, transforming growth factor β, c-fos, junB, and matrix metalloproteinase-9, proposed to play a role in tumor growth and metastasis (2–7).

Osteopontin is a multifunctional extracellular matrix protein thought to be involved in cell adhesion and signaling (8). Osteopontin expression is increased by cell transformation (9–14) and is stimulated in mouse epidermis by tumor promoters (15, 16). Osteopontin gene expression is also stimulated by growth factors, such as transforming growth factor β and epidermal growth factor (17, 18). Moreover, suppression of osteopontin expression by antisense RNA prevented malignant transformation, suggesting that osteopontin plays a critical role in tumorigenesis (19–21). Recently, it was reported that osteopontin expression is diminished in various tissues of c-src-deficient mice (22), suggesting that osteopontin expression is stimulated by c-Src. Osteopontin is one of the genes highly expressed in osteoclasts (23, 24), and c-src deficiency causes inactivation of osteoclasts and osteopetrosis (25, 26). Interestingly, epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate stimulation of osteopontin expression in c-src-deficient cells is similar to that in wild type cells, suggesting that c-Src stimulates osteopontin expression via a different pathway, which seems to be physiologically important (22).

In this report, we investigated the effect of v-Src on osteopontin promoter activity and tried to elucidate the mechanism of its stimulatory effects by identifying the transcription factor(s) involved.

MATERIALS AND METHODS

Cells and Reagents—The HT1080 human fibrosarcoma cell line, which was used in the previous study to investigate v-Src stimulation of matrix metalloproteinase-9 promoter (7), was obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (JRH Biochemicals, Lenexa, KS). NIH 3T3 cells transformed by v-src (27) and their parental NIH 3T3 cell line were kindly provided by Drs. N. E. Kohl and J. B. Gibbs (Merck Research Laboratories) and maintained in DMEM supplemented with 10% fetal bovine serum. Chemicals were obtained from Sigma. Oligonucleotides were obtained from Life Technologies, Inc.

Construction of Plasmids—Plasmid pEcoRIB containing RSV src region (28, 29) was obtained from ATCC. An EcoRI-HindIII fragment containing the v-src-coding region was excised from pEcoRIB and cloned into pcDNA3 vector (Invitrogen, San Diego, CA) under the control of cytomegalovirus promoter. Deletion mutants of osteopontin promoter were constructed, using polymerase chain reaction (PCR) with a PstI fragment containing the −910 to +90 region of the mouse osteopontin gene as a template (30), and cloned upstream of the firefly luciferase gene of the pXT2 vector (31), generating op-910, op-79, op-72, op-65, op-55, and op-37 which contain the −910, −79, −72, −65, −55, and −37 to +79 region of the mouse osteopontin promoter, respectively. Mutations were introduced by PCR using primers containing mutations as shown in Fig. 1, and sequences were confirmed by sequencing using a Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp.). A fragment containing −57 to +52 of herpes simplex virus (HSV) tk minimal promoter was prepared from pBLCAT2 vector (32) by PCR and inserted between the RgIII and HindIII sites of pGL2-Basic vector (Promega, Madison, WI), generating tk-LUC. Two double strand synthetic oligonucleotides corresponding to −72 to −56 and −72 to −38 of mouse osteopontin promoter were prepared and inserted upstream of HSV tk minimal promoter of tk-LUC plasmid in forward orientations, generating reporter plasmids as follows: RE3tk and RE3tk contain three and one copies of −72 to −38 fragments, respectively, upstream of tk minimal promoter. OP67tk contains one copy of the −72 to −56 fragment upstream of the tk minimal promoter.

Transfection and Luciferase Assays—Cells were transfected with
calcium phosphate-DNA coprecipitation method as described previously (33). HT1080 cells were plated onto six-well multiwell dishes (Costar, Cambridge, MA) at a density of 3 × 10^5 cells/well. Eighteen hours later, cells were transfected with reporter plasmids (2 μg/well) together with the v-Src expression plasmid or pcDNA3 plasmid (2 μg/well). To control for transfection efficiency and nonspecific effects of v-Src coexpression, the promoter activity of each promoter-luciferase reporter construct with or without v-Src coexpression was calculated and expressed relative to tk-LUC promoter activity, transfected into parallel separate cells with or without v-Src coexpression in the same experiments. HT1080 cells were harvested and used for luciferase assays 48 h after transfection. For NIH3T3 cells, medium was changed to serum-free DMEM 5 h post transfection, and a cell lysate was prepared 43 h later. Luciferase assays were performed as described previously (34).

Electrophoretic Mobility Gel Shift Assay—Preparation of nuclear extracts and gel shift assays were performed as described previously (34). Oligonucleotide competitors for AP-1, AP-2, CTF/NF-1, NF-κB, and Sp1 were obtained from Stratagene (La Jolla, CA). An oligonucleotide competitor for C/EBP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CBF-A antibody (35) was kindly provided by Drs. S. Marriott and B. de Crombrugghe (University of Texas). For the antibody shift experiments, 0.5–1 μl of anti-CBF antibody or rabbit preimmune serum was incubated with the binding reaction mixture for 15 min before the probes were added. The reaction mixture was loaded onto a 4–20% polyacrylamide precast gel (NOVEX, San Diego, CA).

Methylation Interference Assay—Methylation interference assay was performed as described previously (36). A double strand oligonucleotide corresponding to the −72 to −38 sequence of the osteopontin promoter was labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (Amersham Corp.) at either end. Labeled oligonucleotides were partially methylated by dimethyl sulfate, incubated with nuclear extract of HT1080 cells, and subjected to gel mobility shift assays. Retarded and free probes were purified from the gel and treated with piperidine. The interrupted residues were visualized by gel electrophoresis on an 8% sequence gel.

Northern Blot Analysis—When v-src-transformed NIH3T3 cells and parental NIH3T3 cells became semiconfluent, medium was changed to serum-free DMEM, and cultured for additional 48 h. Total RNA was prepared as described previously (37). Ten micrograms of total RNA was separated by agarose gel electrophoresis and blotted onto nylon membrane (Hybond-N, Amersham). The radioactive probe was prepared from mouse osteopontin cDNA and human β-actin gene (38), and Northern blotting was performed as described previously (39).

RESULTS AND DISCUSSION

v-Src Stimulates Mouse Osteopontin Promoter Activity via a Response Element Located between −65 and −38—Cotransfection of op-910 and op-79 with a v-Src expression plasmid into HT1080 cells increased the expression of both constructs by approximately 5–6-fold (Fig. 2). Deletion up to −66 had no effect on the v-Src stimulation (Fig. 2). This suggests that the distal AP-1-like sequence in this fragment does not play a major role in v-Src stimulation. Removal of the −65 to −56 segment significantly decreased both v-Src stimulation and basal promoter activity. It should be noted that constructs op-55 and op-37 lost v-Src stimulation but still retained significant promoter activity as high as that of the tk-minimal promoter used as a control. Next, heterologous promoter constructs were made, in which the −72 to −56 or −72 to −38 fragment of the osteopontin promoter was inserted upstream of the HSV tk minimal promoter of tk-LUC. As shown in Fig. 3, the −72 to −38 fragment increased basal activity and conferred v-Src response to a tk minimal promoter, whereas the −72 to −56 fragment moderately increased basal activity but did not confer v-Src response. These observations suggested that the v-Src response element (v-SrcRE) is located between −65 and −38.

Characterization of the Nuclear Factor Binding to v-SrcRE as a CBF-like Factor—To characterize the nuclear factor(s) which bind to v-SrcRE, gel shift assays were performed using the double strand oligonucleotide corresponding to −72 to −38 of the mouse osteopontin promoter (Fig. 4). One major retarded band was observed, and its intensity was decreased by the addition of increasing amounts of cold oligonucleotide (Fig. 4, lanes 1–4). A 200–400-fold excess of cold oligonucleotide which contains the known consensus sequence for one of the following, AP-1, AP-2, Sp-1, NF-κB, CTF/NF-1, or C/EBP, did not compete with the binding of the −72 to −38 fragment (data not shown). To localize the protein binding site, a methylation

Fig. 1. Nucleotide sequence of proximal region of the mouse osteopontin promoter. The nucleotide preceding the transcription start site was designated −1. Two AP-1-like sequences, an inverted CCAAT box, and a TATA box are boxed. Nucleotides mutated in the constructs, muAP-1, muGC, and muCCAAT, are shown. Dots represent unchanged nucleotides.

v-Src Stimulation of Osteopontin Promoter

v-Src Stimulation of Osteopontin Promoter Activity via a Response Element Located between −65 and −38—Cotransfection of op-910 and op-79 with a v-Src expression plasmid into HT1080 cells increased the expression of both constructs by approximately 5–6-fold (Fig. 2). Deletion up to −66 had no effect on the v-Src stimulation (Fig. 2). This suggests that the distal AP-1-like sequence in this fragment does not play a major role in v-Src stimulation. Removal of the −65 to −56 segment significantly decreased both v-Src stimulation and basal promoter activity. It should be noted that constructs op-55 and op-37 lost v-Src stimulation but still retained significant promoter activity as high as that of the tk-minimal promoter used as a control. Next, heterologous promoter constructs were made, in which the −72 to −56 or −72 to −38 fragment of the osteopontin promoter was inserted upstream of the HSV tk minimal promoter of tk-LUC. As shown in Fig. 3, the −72 to −38 fragment increased basal activity and conferred v-Src response to a tk minimal promoter, whereas the −72 to −56 fragment moderately increased basal activity but did not confer v-Src response. These observations suggested that the v-Src response element (v-SrcRE) is located between −65 and −38.

Characterization of the Nuclear Factor Binding to v-SrcRE as a CBF-like Factor—To characterize the nuclear factor(s) which bind to v-SrcRE, gel shift assays were performed using the double strand oligonucleotide corresponding to −72 to −38 of the mouse osteopontin promoter (Fig. 4). One major retarded band was observed, and its intensity was decreased by the addition of increasing amounts of cold oligonucleotide (Fig. 4, lanes 1–4). A 200–400-fold excess of cold oligonucleotide which contains the known consensus sequence for one of the following, AP-1, AP-2, Sp-1, NF-κB, CTF/NF-1, or C/EBP, did not compete with the binding of the −72 to −38 fragment (data not shown). To localize the protein binding site, a methylation

Fig. 2. Stimulation of mouse osteopontin promoter by v-Src. Reporter plasmids containing various 5′-deletion mutants of the mouse osteopontin promoter were cotransfected with v-Src expression plasmid (closed bar) or control pcDNA3 plasmid (open bar) into HT1080 cells. Forty-eight hours post transfection, luciferase activity was measured. Means and standard deviation of triplicate samples are shown.

Fig. 3. Effect of v-Src coexpression on heterologous promoter constructs. Each heterologous promoter construct together with the v-Src expression plasmid (closed bars) or with the pcDNA3 plasmid (open bars) were cotransfected into HT1080 cells. A large arrow represents one osteopontin −72 to −38 segment. A small arrow represents one osteopontin −72 to −56 segment. Means and standard deviations of triplicate samples are shown.
interference assay was performed (Fig. 5). Methylation of residues −57, −54, −53, −50, or −49 interfered with protein binding to the −72 to −38 fragment. This result indicates that the −57 to −49 segment, which contains an inverted CCAAT box, is important for binding. This was further confirmed by the fact that a fragment corresponding to the −72 to −38 sequence in which the CCAAT box was mutated (−57 CCT-GATTGG −49 to ACTGATTT) did not compete with the wild type −72 to −38 fragment for protein binding (Fig. 4, lane 8).

To confirm that the CCAAT box plays a role in v-Src stimulation of the osteopontin promoter, mutations were introduced into the op-72 and op-79 constructs, as shown in Fig. 1. Mutation of AP-1-like sequence of op-79 had no effect on v-Src stimulation (data not shown). That is consistent with the fact that op-65 which lacks the AP-1-like sequence still retained v-Src stimulation (Fig. 2). Mutation of the CCAAT-box of op-72 significantly diminished both basal promoter activity and v-Src stimulation, whereas mutation of the adjacent GC-rich sequence had little effect on v-Src stimulation (Fig. 6).

We next searched for the possible type of CCAAT-binding factor involved. Oligonucleotides corresponding to consensus sequences for CTF/NF-1 or C/EBP did not compete with the −72 to −38 fragment for specific protein binding (data not shown). However, there is another class of CCAAT box-binding factors, called NF-Y or CBF, originally identified as factors binding to the major histocompatibility complex class II and to the α2 chain of type I collagen promoters, respectively (40, 41). Biochemical characterization of the protein complex binding to the two CCAAT boxes of RSV LTR suggested that it is identical or closely related to CBF or NF-Y (42). A fragment corresponding to the −155 to −122 sequence of RSV LTR, containing the distal CCAAT box, partially competed with the −72 to −38 fragment of mouse osteopontin promoter in the gel shift assay (Fig. 4, lanes 5–7). A fragment corresponding to the −72 to −56 sequence of the osteopontin promoter did not compete by itself with the −72 to −38 fragment; however, the combination of the RSV CCAAT fragment and the osteopontin −72 to −56 fragment competed with the −72 to −38 fragment as effectively as the −72 to −38 fragment itself (data not shown). On the other hand, excess cold osteopontin fragment −72 to −38, as well as the RSV CCAAT fragment itself, displaced to the same extent binding activity to the RSV CCAAT fragment (Fig. 4, lanes 9–15). These results indicate that there are two factors binding to the osteopontin −72 to −38 fragment, and they migrate to similar positions in the gel shift assays. One seems to bind to the inverted CCAAT box, and the other to the −72 to −56 segment. The binding activity to the −72 to −56 fragment in HT1080 nuclear extract was also confirmed by gel shift assay (data not shown). Interestingly, in Fig. 5, methylation of residues −65, −63, −62, −60, or −59 increased the binding, suggesting that these residues may also affect the protein-DNA interaction. However, heterologous promoter analysis (Fig. 3) and mutation analysis (Fig. 6) strongly suggested that the inverted CCAAT box but not the −72 to −56 segment plays a critical role in v-Src stimulation of the osteopontin promoter. Therefore, we concentrated on the CCAAT box-binding factor.

To further characterize this CCAAT box-binding factor, we used anti-CBF-A antibody which can supershift the CBF-complex in gel shift assays (35). As shown in Fig. 7, the anti-CBF antibody supershifted both the complex formed with the osteopontin −72 to −38 fragment and with the RSV CCAAT fragment. These findings suggest that the factor binding to the osteopontin −72 to −38 fragment is either the CBF, which binds to the CCAAT box of RSV LTR, or is closely related to it. Dutta et al. (43) reported that two CCAAT boxes in RSV LTR play an important role in serum-dependent transcription. They also found that v-Src stimulates binding activity to the CCAAT box by using a temperature-sensitive mutant of v-Src. Our

![Image](http://www.jbc.org/)

**Fig. 4.** Specific protein binding to the −72 to −38 fragment of mouse osteopontin promoter. Binding activity in HT1080 nuclear extract to the −72 to −38 fragment (OPSrcRE) or the −155 to −122 fragment of RSV LTR (RSV-CCAAT), was examined by gel shift assays. Ten micrograms of nuclear protein were incubated with the probe, with or without cold competitors. **Mut** represents the mutated OPSrcRE fragment, in which the following mutations were introduced into the CCAAT fragment: −57 C to A, −54 G to T, and −49 G to T. **B** and **F** indicate the positions of bound and free probes, respectively.

**Fig. 5.** Methylation interference assay. A, either the sense or antisense strands of the mouse osteopontin −72 to −38 fragment were end-labeled and used for the methylation interference assay. **F** and **B** represent free and bound probes, respectively. **Closed triangles** and **open triangles** indicate strong and weak inhibition of binding, respectively. **B**, nucleotide sequence of the mouse osteopontin −72 to −38 fragment. The inverted CCAAT box is boxed. **Closed and open arrowheads** indicate the position of strong and weak inhibition of binding, respectively.
findings suggest that a similar mechanism is involved in the stimulation of the osteopontin promoter by v-Src. This mechanism may play a role in the transformation of cells by RSV and the participation of osteopontin in this process.

**Osteopontin Expression Is Stimulated by Transformation with v-Src**—To examine if v-Src stimulation of the osteopontin promoter occurs in situ, we compared osteopontin mRNA levels in v-src-transformed NIH 3T3 and in parental cells. In v-src-transformed NIH 3T3, osteopontin mRNA levels were significantly higher than in the parental NIH 3T3 (Fig. 8). The relative promoter activity of op-72 to op-37 was also significantly higher in v-src-transformed cells than in the parental cells (Fig. 9). Moreover, the stimulation was not observed when the CCAAT box was mutated (Fig. 9). These results suggest that, upon transformation by v-src, osteopontin expression increases, and at least part of the stimulation is due to the increased promoter activity mediated by the v-srcRE that we identified. In a gel shift assay, we could not see any difference in the protein-DNA complex formed with the −72 to −38 fragment using nuclear extract prepared from v-src transformed and wild type NIH 3T3 cells (data not shown), suggesting that the effect may not be due to the abundance of CBF.

It has been reported that a dominant negative Ras mutant or a farnesyltransferase inhibitor, which interferes with Ras activity, blocked oncogenic action of v-Src, suggesting that at least part of v-Src action is mediated by Ras (27, 44). Recently, a ras-activated enhancer was identified in the mouse osteopontin promoter, which interacts with an ETS-related transcription factor (45). The response element, GGAGGCAGG, was located at −725 to −717, which is far upstream of the v-SrcRE identified here. However, that study also suggested that the −88 to +79 region contains regulatory elements which contribute to the increased expression in ras-transformed cells. Our findings are consistent with those results and suggest that several enhancer elements may contribute to the increased expression of osteopontin upon transformation.

The protooncogene of v-src, c-src, plays an important role in cell signaling (46). Gene knockout of c-src resulted in osteopetrosis in mice (25). In c-src-deficient mice, osteoclasts are inactive and do not resorb bone (26). Recently, it was reported that osteopontin expression was diminished in c-src-deficient cells (22). Our results complement these findings, although c-Src and v-Src may have different modes of action.
tin is highly expressed in osteoblasts and osteoclasts in bone tissue (23, 24). The mechanism by which c-src deficiency causes osteoclast inactivation has not been elucidated. A reduction in osteopontin expression in osteoblasts and/or osteoclasts of c-src-deficient mice could play a role in osteoclast inactivation. Thus, stimulation of the osteopontin promoter by Src may have a function not only in tumor cells but also in the physiological function of normal tissues.

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Stimulation of Mouse Osteopontin Promoter by v-Src Is Mediated by a CCAAT Box-binding Factor
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