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Reciprocal Modulation Between Sp1 and Egr-1

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Abstract Many ubiquitously expressed genes, including oncogenes, lack a proximal TATA or CAAT box but have a region of G + C-rich sequences that appears to replace the usual promoter initiation site. The zinc-finger protein Sp1 is one of the prevalent activators of these genes. The Egr-1 zinc-finger protein has a similar binding site and if the two sites occur in the same region, a variety of activation or inhibitory responses may be obtained. We show that competition between the two factors for overlapping sites on growth-promoting genes could explain why the overexpression of Egr-1 suppresses transformed growth in a number of cell types [Huang et al. (1995): Cancer Res 55:5054–5062; Huang et al. (1997): Int J Cancer]. We demonstrate here that Egr-1 and Sp1 can bind to the same G + C-rich sites and that Egr-1 can displace Sp1 and hence inhibit its activity. We measured the responses of synthetic consensus binding sites and natural promoter sequences linked to a reporter gene and showed that Egr-1 inhibited the activation of transcription by Sp1 on overlapping Sp1/Egr-1 sites. In contrast, Sp1 activity could be augmented by Egr-1 at nonoverlapping sites in the *Egr-1* gene promoter, in transient reporter gene studies in *Drosophila* SL2 cells. In addition, over-expression of exogenous Sp1 in mammalian cells, also leads to increased Egr-1 protein expression, which further inhibits Sp1 transactivation of numerous genes. Therefore, we can account for some of the complex responses of G + C-rich enhancer/promoters by a form of "facilitated inhibition" of Sp1 by Egr-1 at overlapping sites. J. Cell. Biochem. 66:489–499, 1997.

Key words: competition; gel retardation; synthetic DNA binding site; reporter gene activation; immunoblotting; overexpression

An important group of zinc-finger transcription factors typically transmodulate their responsive genes by binding to G + C-rich 5′ regions of these genes. Growth-associated G + C-rich promoters include epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, PDGF, IL-2, MDR1, c-*ras*, c-*fos*, WT-1, and Egr-1. The examples are not limited to growth-related genes and include adenosine deaminase (ADA), phenylethanolamine-N-methyl transferase (PNMT), Hox1.4, thrombospondin, and tissue factor (TF) [Ackerman et al., 1991; Cui et al., 1996; Ebert and Wong, 1995; Shingu and Bornstein, 1994]. Genes that lack TATA and CAAT boxes invariably have a G + C-rich region within about 50 bp of the start of transcription site that may act in the place of the TATA box. Several of these genes have been shown to be activated by Sp1. Examples include EGF receptor [Kageyama et al., 1988]; transforming growth factor-α (TGF-α) [Chen et al., 1992, 1994]; TGF-β1 [Geiser et al., 1991], IGF-IR [Werner et al., 1993a], and WT1 [Hofmann et al., 1993; Molnar et al., 1994].

Sp1 is the most studied of the factors that bind to the regulatory G + C-rich regions. Increasingly, the products of the *Egr-1* family [Sukhatme, 1990] Egr-1 (Zif268, Krox-24, NGFIA, TIS8), Egr-2, Egr-3, Egr-4 and WT1 (Wilm's tumor suppressor gene) [Rauscher et al., 1990] are being recognized and reported as both inhibiting and stimulating transcription of gene promoters at their G + C-rich elements. Sp1 is usually an activating factor but gives complex responses when in the presence of Egr-1 or WT1. Similarly, Egr-1 can either transactivate target promoter sequences or inhibit; the

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same is true for WT1, although it is usually an inhibitor. These reports in the literature provide a possible mechanism for the ability of Egr-1 to suppress transformed growth [Huang et al., 1995]. We have shown recently that Egr-1 overexpression represses transformed growth in HT1080 cells by the stimulation of TGF-β expression [Liu et al., 1996], but this does not explain why the isolated DNA-binding zinc-finger domain also has growth suppressive properties. This effect might be produced by competition between Egr-1 and other factors that bind to similar DNA motifs. Competition between Sp1 and Egr-1 was first reported by Ackerman and coworkers [Ackerman et al., 1991] for binding and activation of the ADA gene promoter. Several reports since then have indicated that there is a complex relationship between Egr-1 and Sp1 that needs further study.

We show here that Sp1 is a strong activator of consensus Sp1 binding sites in SL2 cells, a cell line derived from *Drosophila* that expresses no endogenous Sp proteins. Moreover, Egr-1 expression augments the Sp1 activation of non-overlapping Sp1 + Egr-1 sites, but inhibits Sp1 activity when the sites are overlapping by competing with Sp1 for the binding site. In addition, we find that Sp1 is a strong inducer of *Egr-1* in mammalian cells and suggest a mechanism in which Sp1 facilitates the inhibition of its own transactivating potential by induction of Egr-1. This "facilitated inhibition" of Sp1 transactivation activity by Egr-1 could be a common mechanism for the regulation of a wide range of growth-related genes.

EXPERIMENTAL PROCEDURES

Cells and Culture

NIH 3T3 cells were maintained in DMEM containing 5% calf serum. Insect cells, Sf9 infected with a baculovirus construct and that produce Egr-1 [Ragona et al., 1991], were cultured in serum-free medium, Sf900 (Gibco, Gaithersburg, MD) at room temperature. *Drosophila* Schneider cells, SL-2 were obtained from Dr. John Thomas (Salk Institute, San Diego) and cultured in Schneider's *Drosophila* medium (Gibco, Gaithersburg, MD) with 12.5% fetal bovine serum (FBS).

Preparation of Protein Extracts

Egr-1 protein was extracted from nuclear preparations of the Sf9 cells as described [Ragona et al., 1991]. Extracts contained 1–2 µg/µl Egr-1; total protein was measured using the BioRad (Hercules, CA) protein assay kit. Sp1 was obtained as a commercial product from Promega (Madison, WI) and contained 25–30 ng/µl.

Plasmids

Plasmids were constructed with standard recombinant DNA manipulation techniques as described [Sambrook et al., 1989]. Expression vectors active in *Drosophila* cells were constructed as follows. For pPacEgr-1, a *Bgl*II fragment (nucleotide 306–1960 derived from CMV-neoEgr-1 [Huang et al., 1995] was blunted and subcloned into pPacU + NdeI vector after digestion with *Xho*I and blunting. The *Drosophila* promoter plasmids were kindly provided by Dr. R. Tjian [Courey and Tjian, 1988] and contain the *Drosophila* actin 5C promoter to drive expression. The 100 bp fragment (nt −555 to −655) of the Egr-1 promoter was derived by Ava I digestion of the 268CATAMC plasmid kindly provided by Dr. D. Nathans [Christy and Nathans, 1989]. This fragment was inserted into pBLCAT-2. Other chloramphenicol acetyltransferase (CAT) constructs were prepared by insertion of corresponding oligonucleotides (listed below) into the blunted BamH1 site of pBLCAT-2. All plasmids were subjected to DNA sequence analysis to determine the orientation and copy number. pXKCAT containing 250 bp from the human keratin 18 promoter and its "empty" version dP1-CAT, were kindly provided by Dr. Robert Oshima of this Institute.

Probes and Electrophoretic Migration Shift Assays

The sequence **GCGGGGGCG** is an Egr-1 binding site; **GGGCGGG** contains an Sp1 site. The following synthetic double-stranded oligodeoxynucleotides were synthesized in the DNA laboratory of the Burnham Institute: A probe CGTA**GCGTGGGCGGGG**CTGTG is a sequence from the ADA promoter that contains overlapping Egr-1 and Sp1 binding sites, named (ES). In addition, this probe was synthesized in two mutated versions: CGTAGAATGGT**GGGCGGGG**CTGTG is the same as (ES) except that the Egr-1 site is mutated (mutES); EmutS is CGTA**GCGTGGGCG**AAGCTGTG with the Sp1 site mutated. A probe that contains non-overlapping Sp1 and Egr-1 binding sites was prepared by restriction enzyme digestion (*Ava*I) of the

Egr-1 960-bp promoter plasmid. The relevant sequence N_7**GGGCGGG**N_{44}**CGCCCCCGC**N_{34} (100-bp Egr-1 promoter, or S-E) contains both sites separated by 44 bp. A promoter sequence of 287 bp from the human keratin 18 gene, XK-CAT proximal promoter, contains two overlapping binding sites for Egr-1 and Sp1. Two other Sp1 sites also occur in the center section of N_{91}**GGGCGCGGGGGTGGGG**N_{78}**GGGGC-GGGGGCGGGG**N_{87}.

Gel-retardation assays were carried out as described [Huang and Adamson, 1993; Huang et al., 1994a]. Oligonucleotides were labeled using γ[^{32}P]-ATP and T4 kinase. The labeled 100-bp DNA fragment was prepared using [^{32}P]-dCTP and T4 polymerase. In brief, 1 ng [^{32}P]-labeled oligonucleotide was incubated with the Egr-1 extracted from nuclei of infected Sf9 cells, or with recombinant Sp1 for 30 min at room temperature. Egr-1 and Sp1 were added together to the DNA probe for competition studies, and reached equilibrium in less than the 30-min incubation time. The amount of Sp1 in recombinant extracts was 25–30 ng/μl, while 50–100 times this amount of Egr-1 was needed for competition. The same finding was reported by others [Cui et al., 1996].

Chloramphenicol Acetyl Transferase Assays to Determine Transactivation of Binding Sites

Chloramphenicol acetyl transferase (CAT) assays were performed essentially as described [Huang et al., 1996]. SL-2 cells (1.5×10^6) were seeded into 60-mm tissue culture dishes 16 h before transfection. Total plasmid DNA of 8 μg including 2 μg Copia β-galactosidase plasmid, and expression vector DNA or carrier pUC8 DNA as indicated in the figures, was transfected into SL-2 cells using the calcium phosphate procedure. The cells were harvested after 40 h and lysed; β-galactosidase activity was determined in order to equalize the aliquot volumes that gave equal expression. The CAT activities of lysates were measured by thin layer chromatography and normalized. All assays were conducted with equal amounts of DNA with the empty vector making up the difference. Signals were visualized and quantified using a phosphorimager system (BioRad Laboratories, Hercules, CA).

Stable Transfection

pSVSp1-F and pSVSp1-FX are Sp1-containing expression vectors driven by SV40 promoter and were obtained from Dr. J. Saffer. The FX vector is modified to create a frameshift so that Sp1 is not expressed. The vectors were cotransfected into NIH3T3 cells with 2 μg SVneo to provide G418 resistance to clones. After selection with 400 μg/ml G418, clones were isolated using cloning rings.

Western Blotting

Immunoblotting was performed as described [Huang et al., 1996]. Equal amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were electrophoretically transferred to polyvinylidinedifluoride membranes (Immobilon, Millipore Corporation, Bedford, MA). Specific proteins were detected by anti-Egr-1 antibody [Huang et al., 1994b] or anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to the enhanced chemiluminescence system (ECL, Amersham, Little Chalfont, UK).

RESULTS

Competition at Overlapping Sites

In many promoters, the Sp1 and Egr-1 sites are overlapping, and a synthetic oligonucleotide containing the motif found in the ADA gene promoter was tested for its ability to bind Sp1 and Egr-1 both separately and together. This motif is named ES and is shown at the top of Figure 1B. When increasing amounts of Egr-1 were added with constant Sp1, Sp1 was competed from its binding site and Egr-1 was preferentially bound (Fig. 1A, lanes 5–7). The same oligodeoxynucleotide was used in a reporter gene and coexpressed with vectors for Egr-1 or Sp1 in *Drosophila* SL2 cells, which do not have endogenous Sp1. The reporter gene was transactivated strongly only by Sp1, and very weakly if at all by Egr-1 (Fig. 1B). Egr-1 expression was active, as shown with a reporter construct containing multiple Egr-1 binding sites, such as in Figure 3. The lack of effect of Egr-1 on a single binding site in a promoter-reporter construct was a consistent finding for transfections in NIH 3T3 and other cells (data not shown). When the two transcription factors were expressed together in SL2 cells, there was a clear inhibitory dose-dependent effect of Egr-1 on the transactivating activity of Sp1, reducing the 13.5-fold activation by Sp1 to 3-fold (Fig. 1C). The amounts of protein needed for competition on the binding site and for activation of the

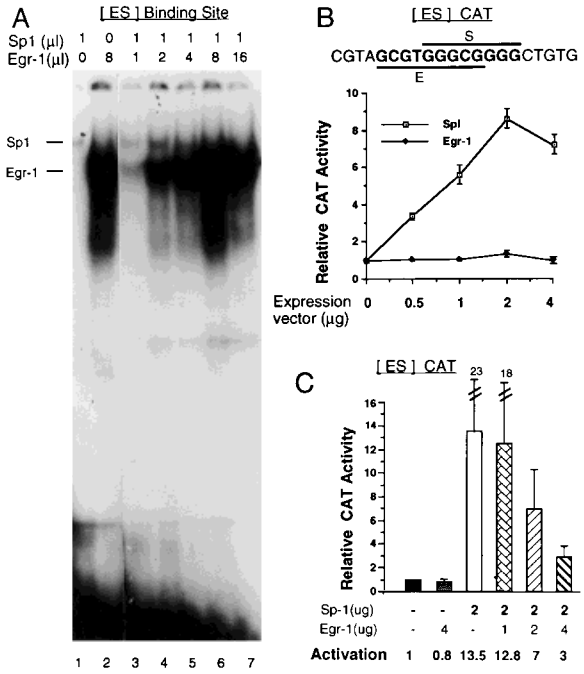

Fig. 1. Effect of Sp1 and Egr-1 on overlapping sites. **A:** Electrophoretic mobility shift assays (EMSA) to show competition for Sp1 binding to overlapping Sp1 and Egr-1 DNA binding sites in the ADA gene promoter by increasing levels of Egr-1. **B:** Transient transfection in *Drosophila* SL2 cells shows that only Sp1 activates overlapping Sp1/Egr-1 oligonucleotide-reporter gene. **C,** increasing levels of Egr-1 expressed in the presence of Sp1 and the Sp1/Egr-1 CAT reporter gene in SL2 cells, inhibit the activity of Sp1.

reporter cannot be compared because the active fraction likely differs from the level of protein present. In any case, we are not told the exact protein concentration of the commercial Sp1 preparation. The competition of Egr-1 for Sp1 binding and transactivation supports the previous findings reported by Ackerman et al. [1991], who showed that Egr-1 displaced Sp1 and decreased transactivation of the *ADA* gene.

Transactivation From Mutated Overlapping Sites

As a further test, we attempted to eliminate competition by mutating each of the binding sites in turn in the overlapping ES oligonucleotide-CAT reporter construct. First, using a mutated Egr-1 site (mutES), Sp1 activated CAT activity by 28-fold. Indeed, expression of Egr-1 augmented this activity modestly to 43-fold (Fig. 2A). As expected, Egr-1 did not inhibit Sp1 activity when the Egr-1 site was imperfect.

When the Sp1 site in the ES overlapping sites was mutated to a sequence that only contained the minimal core Sp1 site (EmutS), the transac-

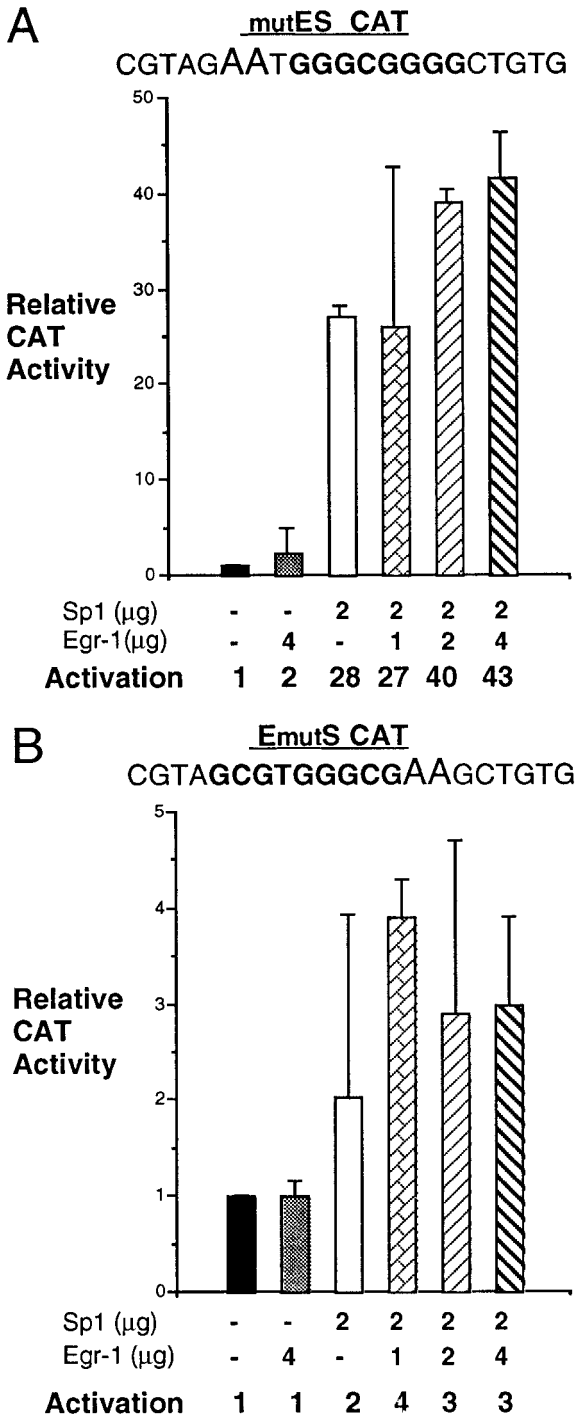

Fig. 2. Transactivating activity of Sp1 and Egr-1 on mutant (ES) binding site reporter genes transfected into SL2 cells. **A:** When the Egr-1 site in an overlapping site sequence was mutated (EmutS), Sp1 activated strongly while Egr-1 had a residual weak effect. The combination of the two factors indicated that Sp1 activity was augmented by the presence of Egr-1. **B:** When the Sp1 site in the reporter gene was mutated (ESmut), Sp1 alone activated 2-fold, and Egr-1 has a modest augmentation effect.

tivating activity of Sp1 was much lower (only 2-fold induced). While the addition of Egr-1 was able to augment this activity somewhat, but this may be within the range, rather than dose dependent (Fig. 2B). Here, Egr-1 did not inhibit Sp1 activity even though the Egr-1 site was intact. These results show that competitive inhibition of ES-CAT activity by Egr-1 (Fig. 1C) does not occur on mutated sites and therefore the effect is sequence specific.

Egr-1 Inhibition of Sp1 Transactivation by the Human K18 Gene Promoter

The keratin 18 gene is upregulated by Sp1 in tumorigenic SW613-S human colon carcinoma cells but not in the non-tumorigenic version [Gunther et al., 1995]. We observed that the Sp1 site overlapped an Egr-1 site in this promoter, and therefore tested for competitive inhibition. A 287 bp fragment of the K18 gene 5′ sequence in the K18 promoter contains two distinct overlapping Sp1 and Egr-1 sites (see the Methods section) and was used to make a reporter construct. The experiment was performed essentially as in Figure 1, with transient expression of Sp1 and/or Egr-1, as indicated in Figure 3. The results revealed that in this context Egr-1 added alone, had a modest transactivating effect (3.6-fold activation) on the K18 promoter-CAT construct, in comparison with 10-fold activation caused by Sp1. However, together the combination had a poorer transactivating effect than Sp1 alone. This result indicates clearly that Egr-1 inhibited the Sp1 transactivation of K18 promoter. We have not tested the expression of Egr-1 in the nontumorigenic SW613-S human colon carcinoma cell line, but if Egr-1 is expressed as predicted by other examples of non-tumorigenic cells quoted here, it could explain the lower K18 expression. The level of expression of K18 would not, in this case, be expected to affect the transforming activity of the cell line.

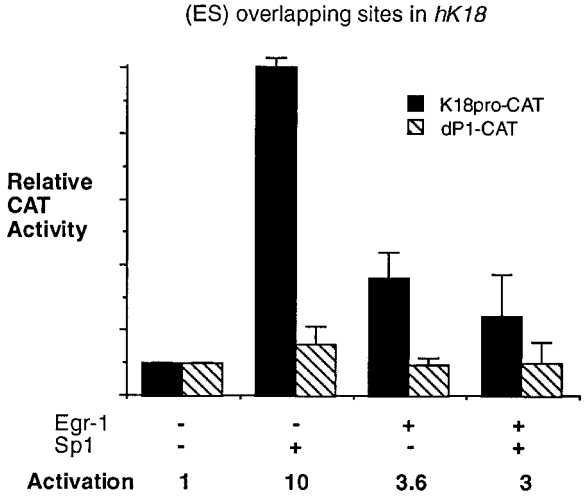

Fig. 3. Effect of Sp1 and Egr-1 on the transactivation of the human K18 gene promoter. The promoter contains overlapping Sp1 and Egr-1 binding sites and Sp1 strongly activates the reporter construct, while Egr-1 activates moderately. The combination, however, is inhibitory illustrating competition of the site by high levels of Egr-1.

Sp1 and Egr-1 Binding to Single DNA Sites

In order to interpret all possible effects between Egr-1 and Sp1, we also looked at single Egr-1 or Sp1 binding sites in gel-retardation assays and found that Sp1 binds to the Egr-1 site and Egr-1 binds to the Sp1 site (Fig. 4A,B, lanes 1 and 2). When the Sp1 protein is increased while Egr-1 is constant, both Egr-1 and Sp1 bind more efficiently to the Egr-1 site. This is specific because the addition of bovine serum albumin (BSA) does not have this effect (Fig. 4A, lane 7). A similar result occurred at the Sp1 binding site, where increasing Egr-1 levels bound dose dependently, but Sp1 also bound increasingly better in the presence of Egr-1 (Fig. 4B). We conclude that the two proteins have the potential to interact [Lin and Leonard, 1997] in some way that increases their abilities to bind to single nonoverlapping sites. This may considerably modulate the activities of both transcription factors when both are expressed together in a cell.

Transactivation From Nonoverlapping Egr-1 and Sp1 Sites

The promoter of the Egr-1 gene itself contains a fragment (base numbers −655 to −555) with one Sp1 site and one Egr-1 site that are separated by 44 bp. We examined competition for binding and for activation of a reporter construct using this natural 100-bp DNA fragment of the mouse Egr-1 gene. When the DNA probe was mixed with either protein factor alone, single complexes of characteristic migration rates were detected (Fig. 5A, lanes 1 and 2). Both factors bound separately to 100-bp fragments when present together (Fig. 5A, lane 3) because no "heterodimer" was seen. Both Sp1 and Egr-1 bound specifically because only Egr-1 was inhibited from binding with an Egr-1-specific antibody that largely removed it from

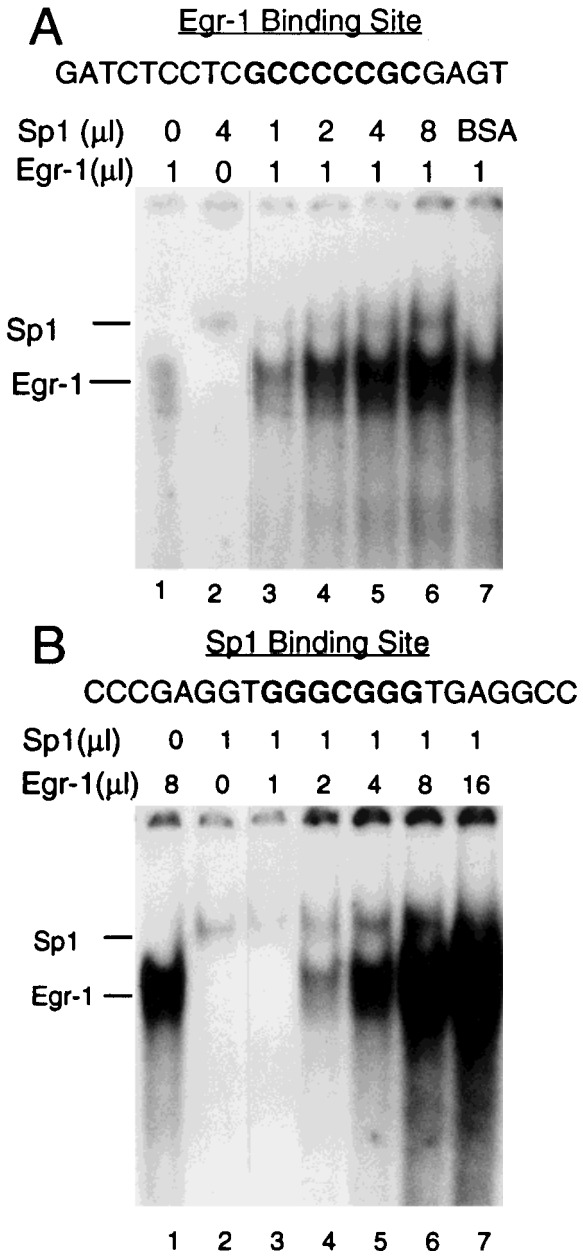

Fig. 4. Gel retardation assays to show Sp1 and Egr-1 binding to single DNA binding sites. **A:** The Egr-1 binding site is shown at the top and a constant amount of Egr-1 was added and allowed to bind in the presence of increasing levels of Sp1. **B:** Using the Sp1 binding site and constant amounts of Sp1, increasing amounts of Egr-1 were added and analyzed for retardation of the labeled oligonucleotide by the two proteins.

the lower retarded band in Figure 5A (lane 4) while preimmune serum (PI) had no effect (Fig. 5A, lane 5). Interestingly, the expression of Egr-1 had little activity on the 100-bp oligoCAT construct (Fig 5B), while Sp1 activated strongly, again demonstrating that Egr-1 has little effect on a single Egr-1 binding site. Studies of the transactivating activity of Sp1 in the presence of increasing levels of Egr-1 showed that Egr-1 greatly enhanced Sp1 transactivation from a basal 10-fold to an average of 50-fold (Fig. 5C). Only the direction of the effect was measurable because the effective levels of Egr-1 and Sp1 were not known in these transient assays. However, the increase in transactivating activity of Sp1 by the addition of Egr-1 was more than additive (e.g., Fig. 5C, lanes 2, 3, and 6). When the two factors bound at sufficiently separated sites, they did not interfere and Egr-1 facilitated the effect of the Sp1 in transactivation. This result indicates that Egr-1 stimulates the activation of its own transcription and that Sp1 might be an activator of the Egr-1 gene and this was tested next.

Sp1 Stimulates Egr-1 Gene and Egr-1 Protein Synthesis

In an attempt to determine whether the expression of Sp1 could activate the Egr-1 gene in vivo, we first tested this by transient coexpression of Sp1 in *Drosophila* SL2 cells with a reporter consisting of the 950-bp Egr-1 promoter/enhancer ligated to the CAT gene. The reporter was shown to be activated dose-dependently by Sp1 expression in transient assays in SL2 cells (Fig. 6A). We next turned to NIH 3T3 cells to test if Sp1 could up-regulate Egr-1 levels when Sp1 is expressed constitutively in a normal mammalian cell type. Cotransfection of the Sp1 cDNA driven by the CMV promoter, together with the pSVneo vector to allow selection for cells expressing both Sp1 and neo, led to the isolation of numerous clones. Two of these clones that expressed moderate (Sp1-11) and high levels (Sp1-25) of Sp1 (Fig. 6B) were selected. The levels of Egr-1 and Sp1 were analyzed by Western blotting using anti-Sp1 and anti-Egr-1 antisera, and normalized by reprobing with anti-β-actin antibody. The Egr-1 promoter contains 6 possible Egr-like and 24 putative Sp1 sites overlapping most of these sites. The net effect of expression of Sp1 expression on the endogenous Egr-1 gene was the dose dependent stimulation of Egr-1 expression, because Egr-1 expression was highest in clone Sp1-25 moderate in Sp1-11 and least in parental NIH3T3 cells (Fig. 6B, lanes 3, 2 and 1, respectively).

The correspondence of Egr-1 expression with Sp1, indicated that constitutive Sp1 expression leads to clearly, if modestly increased, constitu-

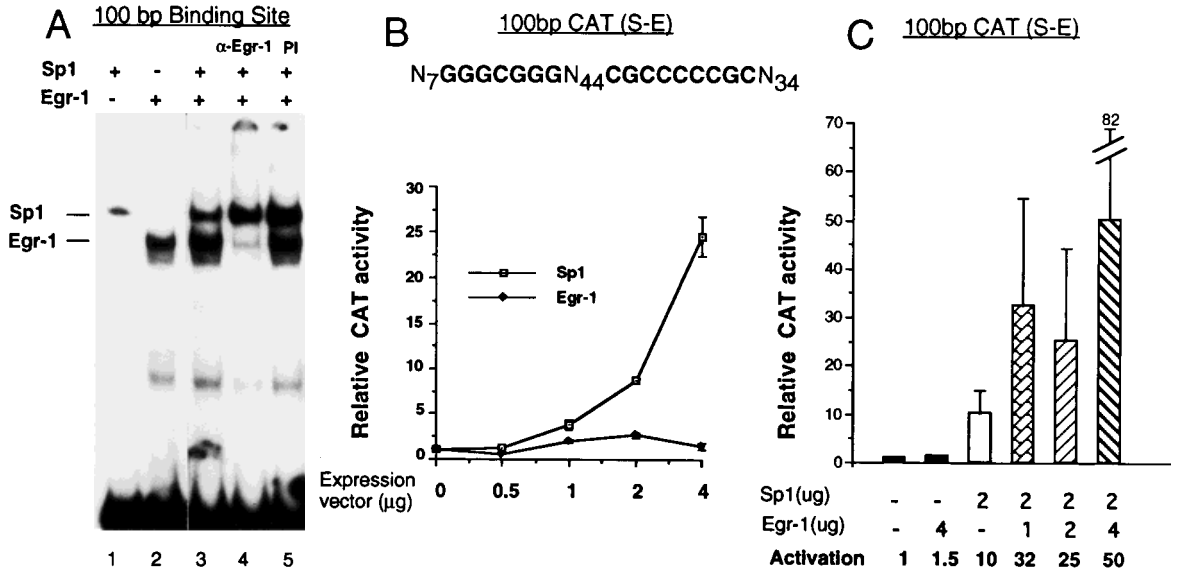

Fig. 5. Effect of Sp1 and Egr-1 on nonoverlapping Sp1 and Egr-1 binding sites (100-bp S-E domain). **A:** Sp1 and Egr-1E proteins bound to the 100-bp oligonucleotide sequence in EMSA. The proteins bind independently of each other (**lane 3**) when added together. Rabbit antibody to Egr-1 inhibited Egr-1 binding only, while proimmune rabbit serum had no effect. **B:** Transient expression of Sp1 activates the 100-bp CAT construct up to 25-fold, while very little transactivating activity was effected by Egr-1 alone on the same reporter gene. **C:** Simultaneous transient activity of Sp1 and Egr-1 demonstrated the augmentation of Sp1 activation by Egr-1 when their binding sites were nonoverlapping.

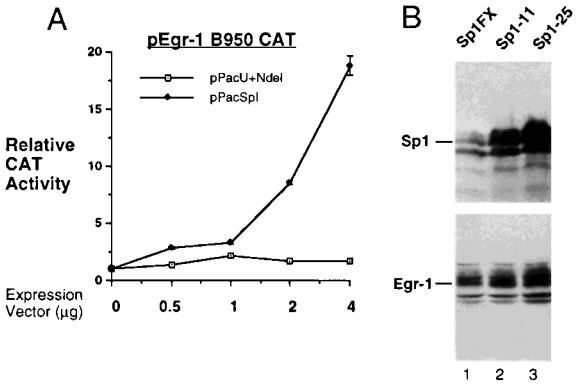

Fig. 6. The effect of Sp1 on the activity of the Egr-1 gene. **A:** *Drosophila* SL2 cells were transfected with the 950-bp Egr-1 promoter-CAT construct as a target for Sp1 activation provided by an expression vector. Increasing expression of Sp1 activated the promoter (pPacSp1), but not a control CAT construct. **B:** Immunoblotting to show the levels of transcription factors expressed by three NIH 3T3 clones. NIH 3T3 cells were transfected with pCMV-Sp1 and constitutively expressing clones were selected. Sp1-11 and Sp1-25 were two of the clones expressing moderate and low levels of Sp1, respectively. The resulting clones also expressed increasing levels of Egr-1.

tive Egr-1 expression. The results depicted in Figure 6A indicated that Sp1 achieved this result, at least in part, by transactivating the Egr-1 gene promoter. Thus, Sp1 increases the amount of Egr-1, which then becomes a potent competitor of Sp1 for binding to overlapping sites.

DISCUSSION

The results of our analyses on G + C-rich binding sites allow us to make two major conclusions. First, Sp1 as a major activator of genes with G + C-rich promoters, can be competitively inhibited by Egr-1 binding to overlapping Egr-1 and Sp1 sites (Figs. 1, 3). Second, Sp1 is an activator of the Egr-1 gene. Moreover, Egr-1 is autostimulatory for its own production when activating nonoverlapping sites in a 100-bp fragment of the Egr-1 promoter (Fig. 5). These two features combine to give the effect of "facilitated inhibition" of Egr-1 on Sp1 transactivation of overlapping Sp1/Egr-1 sites. Figure 7 is a model depicting this effect. The induction of Egr-1 would therefore enable the efficient reduction of Sp1 activation of numerous genes. We envision a mechanism of a form of inhibition (on overlapping sites) that is based on the physical displacement of Sp1 on DNA by Egr-1. This displacement of Sp1 combined with the ability of Sp1 to induce Egr-1 expression provides a simple explanation of facilitated inhibition. Several individual findings of this study led to these conclusions. First, the binding of Egr-1 to a single Egr-1 site is insufficient to activate

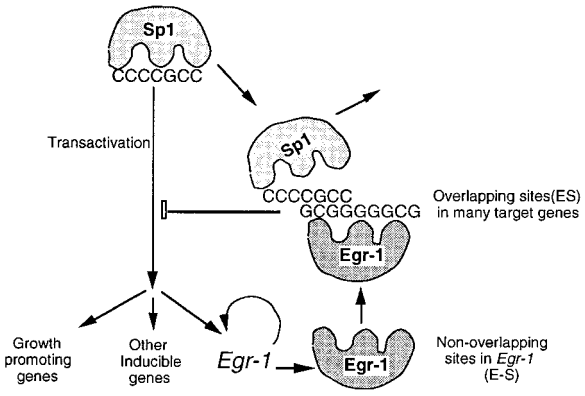

Fig. 7. Model for facilitated inhibition of Sp1 activity.

transcription, whereas Sp1 binding to a single Sp1 site is sufficient (Figs. 1A, 5B). Second, Egr-1 expression inhibits the transactivating activity of Sp1 on overlapping Sp1/Egr-1 site in a dose dependent manner (Fig. 1C). Third, the inhibition of Sp1 transactivation by Egr-1 is DNA sequence specific (Fig. 2). Fourth, Egr-1 augments the transactivation of Sp1 when Egr-1 and Sp1 sites are present in a nonoverlapping configuration, as in the Egr-1 promoter itself (Fig. 5). 5) Sp1 stimulates the expression of the Egr-1 promoter and leads to sustained Egr-1 and Sp1 expression (Fig. 6).

Genes whose activity is not associated with growth are also among the G + C-rich promoter types, such as the ADA gene in which competition between Sp1 and Egr-1 was strongly indicated [Ackerman et al., 1991]. In this gene the Egr-1 and Sp1 sites are overlapping, and it was this example that we modeled for the studies illustrated in Figure 1. The results agreed with the observations of Ackerman et al. [1991] that Egr-1 repressed activation by Sp1. We extended their observations by showing that the effect of Egr-1 was dose dependent. We also demonstrated repression of Sp1 activation of the human K18 promoter by Egr-1 on overlapping binding sites.

If a large number of Sp1 target genes are transforming oncogenes, growth factors, and receptors, our observations [Huang et al., 1994b, 1995] of the suppressive effect of Egr-1 on tumorigenicity would (at least in part) be explained. Sp1 is ubiquitously expressed in many tissues and is associated with the promotion of cell cycle genes [Karlseder et al., 1996; Molnar et al., 1994]. Sp1 is responsible for the basal activity of oncogenes such as *ret, rel, myc,* and *ras* [Itoh et al., 1992; Jordano and Perucho, 1988; Majello et al., 1995; Sif et al., 1993; Sif and Gilmore, 1994]. However, the regulation of Sp1 activity is clearly complex, and other genes besides Egr-1 are involved. For instance, *IGF-IR* and *EGFR* and TATA-less and CAAT-less growth factor receptor genes, that Sp1 up-regulates and WT1 represses [Werner et al., 1993a,b]. A cytokine gene, *CSF-1,* has a similar profile of activation by Sp1 and repression by WT1. In this case, the sites were shown to be overlapping at −273 to −265; we presume that this is so for the EGFR and IGF-IR genes as well. The effect of Egr-1 was not reported in these studies, but Egr-1 would almost certainly have an effect because the binding site is the same as for WT-1 [Nardelli et al., 1991; Rauscher et al., 1990]. In addition, Egr-1 is more broadly expressed in tissues compared to WT-1.

The *PDGF-A* gene is particularly rich in G+C regions, both in the 5′ sequences of the promoter and in the first intron. In this gene, a new binding sequence for Egr-1 and WT1 was first recognized: TCCTCCTCCTCC, a sequence that binds WT1 and Egr-1 much more strongly than the consensus and that also activates the gene more strongly [Wang et al., 1992]. In addition, this sequence binds Sp1 with greater affinity than GC-rich sites [Cohen et al., 1997], hence there is a potential for competition between all three zinc finger proteins for the same binding site in the *PDGF-A* promoter. There are at least three Sp1 sites with adjacent or overlapping Egr-1/WT1 sites and Sp1 stimulates transcription of the *PDGF-A* gene, while WT1 represses. Repression by WT-1 is brought about by mechanisms other than competition for Sp1 and is thought to act through its repressive domain [Madden et al., 1991, 1993]. In fact, the principal activator of the *WT1* gene is Sp1 [Cohen et al., 1997], indicating the possibility of interactive modulatory effects of all three zinc-finger transcription factors on each other and on target genes. It is not surprising, then, that the effect of Egr-1 differs according to the cell: in NIH 3T3 cells, Egr-1 represses the *PDGF-A* gene slightly but consistently, while in 293 cells, Egr-1 stimulates while WT1 represses. The promoter region −73 to −46 in the *PDGF-A* gene was shown to have three overlapping Egr-1 sites and yet Egr-1 had no transactivating effect. Instead, Sp1 bound in three positions on the same DNA [Kaetzel et al., 1994], illustrating the complexity of possible responses.

Other examples illustrate that competition between Sp1 and Egr-1 binding for overlapping sites is not universal. The human *IL2* gene provides another type of Sp1 regulation where overlapping Egr-1 and Sp1 sites occur, but do not lead to inhibition by Egr-1, probably because several adjacent sites are involved [Skerka et al., 1995]. A similar but more complex interaction occurs to regulate the serum responsive thrombospondin gene [Shingu and Bornstein, 1994]. Similarly for tissue factor (*TF,* a gene involved in the blood coagulation cascade) that has three overlapping Egr-1 and Sp1 elements with differing affinities at the three sites, such that Sp1 binds preferentially at site 1 and 3, while Egr-1 binds at site 2. The basal activity of *TF* is regulated by Sp1 in the absence of Egr-1. When *TF* is strongly up-regulated by TPA in HeLa cells, Egr-1 binding at site 2 is the inducible transactivating factor that accounts for this response [Cui et al., 1996]. We can now attribute this result to activation augmented by Egr-1 by the combined effect of two Sp1 and one Egr-1 sites adjacent but not overlapping.

We examined the promoter of *Egr-1* itself because it has an Sp1 site separated from an Egr-1 site. We showed that the two transcription factors together enhanced transactivation of the *Egr-1* gene in a more than additive fashion (Fig. 5). The mechanism for this effect is unknown at present because we can find no evidence that the two proteins can bind or interact directly with each other [Lin and Leonard, 1997]. The simplest explanation is that each factor activates from its own binding site, and the result is greater than additive by a mutually protective effect. Results from gel shift experiments show that Egr-1 can bind to Sp1 sites without activating, and Sp1 can bind to Egr-1 sites (Fig. 4), thus complicating the interpretation. Another possibility is a conformational effect on the DNA by the presence of the two zinc-finger proteins. The formation of stem-loop structures in G + C-rich promoters has been hypothesized [Ackerman et al., 1993] and this could bring the two proteins together in a way that facilitates either stability or activity, or both. One of the consequences of Sp1 binding stably to its DNA element is its phosphorylation and this can affect its activity [Leggatt et al., 1995] and stability [Jackson et al., 1990].

The effect of Sp1 on numerous target genes is growth stimulatory, while Egr-1 is growth inhibitory when constitutively expressed in transformed cells. The NIH 3T3 cells with high levels of both Egr-1 and Sp1 expression (Fig. 6B) did not differ in growth rates (data not shown). This example illustrates how one might have overexpression of a factor known to be a strong and general gene activator without a net change in cell behavior. We hypothesize that Egr-1 acts as a general dampener of rapid response and signaling genes such as *fos, jun, ras* and *myc,* at least partly through its inhibitory activity on Sp1 in a range of cell types. In HT1080 human fibrosarcoma cells and osteosarcoma cells [Huang et al., 1995] and in breast cancer cells [Huang et al., 1997], exogenous Egr-1 can inhibit transformation and tumorigenicity. One of the mechanisms for this effect, we believe, is competition between Sp1 and Egr-1 for the G + C rich enhancer/promoter elements of the predominantly growth associated genes. We have shown that at high levels of Egr-1 expression, Egr-1 can displace Sp1 from its activating element, often substituting an inhibitory or neutral effect on gene transcription. A large part of the repressive effect of Egr-1 can be mimicked by the overexpression of the zinc-finger domain alone (C domain), suggesting that at least one mechanism for the activity of Egr-1 and the C domain is that it need only bind to its DNA sequence. We show here that competition for a G + C-rich binding site can account for these observations. The facility with which Egr-1 inhibits growth will depend on the level of its expression, its state of phosphorylation [Huang and Adamson, 1994] and the cell type. We have shown that many types of tumor cell lines have little or no Egr-1 expression [Huang et al., 1995] and this presents another step in progressive growth deregulation leading to neoplasia. Lack of regulation of growth in the absence of Egr-1 leaves Sp1 without its natural competitor for the control of transcription of genes important for sending signals for cell proliferation.

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