Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 290-297

COOPERATIVE INTERACTION BETWEEN ETS AND AP-1 TRANSCRIPTION FACTORS REGULATES INDUCTION OF GLUTATHIONE S-TRANSFERASE Ya GENE EXPRESSION

Svetlana Bergelson and Violet Daniel*

Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Received February 21, 1994

SUMMARY: Induction of mouse glutathione S-transferase (GST) Ya gene expression by a variety of chemical agents is mediated by a regulatory element, EpRE, composed of an Ets and two adjacent AP-1-like binding sites. In this report we present evidence that the basal and inducible activity of EpRE is mediated by AP-1 transcription factor and that the cooperative interaction between AP-1 and an Ets protein contributes to enhance the EpRE inducibility. We also show that EpRE, similar to a single AP-1 site, when ligated to GST Ya gene promoter, is transactivated by c-Fos/c-Jun or c-Fos/Jun-B heterodimer and that c-Jun/c-Jun homodimer is an activator of an AP-1 site only in the context of collagenase gene promoter.

The recent study of a mouse glutathione S-transferase (GST) Ya gene has shown that a variety of chemical agents such as planar aromatic hydrocarbons, diphenols, PMA, phenobarbital, H_2O_2, electrophilic compounds, arsenite, arsenate and heavy metals all operate through a single *cis*-regulatory element to induce the expression of this gene (1-5). The regulatory element, defined as EpRE, was found to be composed of two adjacent AP-1-like sequences that bind and are transactivated by Fos/Jun heterodimeric complex (AP-1 transcription factor) (2,5). Similarly, the highly homologous ARE regulatory element described in rat GST Ya gene (6) was also found to be activated by the AP-1 complex (5). Recently it was observed that the two adjacent AP-1-like sites of EpRE and ARE are preceded by a putative Ets-binding site which may contribute to enhance the inducibility of these regulatory elements (7). The protein products of c-*ets* proto-oncogenes are a family of transcription factors known to cooperate with other regulatory proteins for transcriptional activation of a variety of cellular and viral gene promoters (8-11).

*To whom correspondence should be addressed. Fax: 972-8-344118.

Abbreviations: GST, glutathione S-transferase; PMA, phorbol-12 myristate 13-acetate; CAT, chloramphenicol acetyl transferase.

0006-291X/94 $5.00

In the present study we have examined the interactions of EpRE regulatory sites with transcription factors and their contribution to the enhancing activity conferred by EpRE to the GST Ya gene promoter.

MATERIALS AND METHODS

Oligonucleotides and plasmid constructs: 41 bpEpRE enhancer of mouse GST Ya gene (2) (see Fig. 1); 5'-ctag AGCAGGAAG (Ets), the Ets-binding site from polyoma virus enhancer PEA3 (8); 5'-ctag ATGACATTGCT and 5'-gatc GTGACAAAGCA the first (A) and second (B) AP-1-like binding sites of EPRE; 5'-ctag ATGACATTGCTGATCGTGACAAAGCA (ΔEpRE) was obtained by ligation of the two AP-1-like binding sites of EpRE; 5'-gatc AGCTTGGAAATGACATTGCTAATGG (Ets-A) contains the putative Ets-binding site and the first AP-1-like motif of EpRE; 5'-ctag TGAGTCAGC is an AP-1 site from collagenase gene and 5'-ctag AGCTTGGAAATGAGTCAGC (Ets-AP-1) contains the putative Ets-binding site from EpRE upstream the AP-1 site. The oligonucleotides were ligated into the -187 site of GST Ya gene promoter driving the expression of *cat* gene (12). To serve as probes for protein factor binding in electrophoretic mobility shift assays, the double stranded EpRE, Ets-AP-1 oligonucleotides as well as the AP-1 consensus sequence, cloned into the Bam HI site of pGEM-1 (Promega) and excised as a 43 bp oligonucleotide by *Xba* I and *Eco* R1, were labeled at the 3'-recessed ends by Klenow DNA polymerase. Oligonucleotides representing the chemical-inducible enhancers ARE of rat GST Ya (6) and rat quinone reductase (13) genes were synthesized as described (5). -73 Col-cat plasmid construct of the AP-1 containing collagenase promoter (14) was obtained from Y. Shaul (Weizmann Institute).

Cell culture, transfection and CAT assays were carried out as previously described (12).

Electrophoretic mobility shift assay and immunoblotting: HepG2 cells were grown on 100 mm plates and after 3 h exposure to 100 nM PMA were used to prepare nuclear extracts (15). Mobility shift assays were carried out as described (5)

A combination of gel-shift assay and immunoblot transfer,"Shift-Western blotting" technique (16) was used for the identification of the c-Jun protein component in the gel retarded complex of nuclear extracts with AP-1 and EpRE oligonucleotides. Primary c-Jun antibody (Oncogene Science) was applied at a dilution of 1:300 and enhanced chemoluminescent protein detection was done as described by Amersham using anti-rabbit peroxidase-conjugated antibodies from Amersham.

RESULTS

The inducible regulatory element of GST Ya gene

Fig. 1 shows the structure of the EpRE enhancer which was found to mediate the inducible expression of the mouse GST Ya gene by a variety of chemical agents (1-5). This regulatory element is composed of an Ets and two adjacent AP-1-like binding sites. The sequence of the Ets-binding site appears to be related to the polyoma virus enhancer PEA3 (AGCAGGAAG) known to bind the Ets-1 and Ets-2 protein products of the *ets* proto-oncogene family (8). The inducible regulatory element of rat GST Ya gene, termed ARE, has an identical structure with the EpRE from which it differs by a single nucleotide change A→G (TGGCATTGC) in the first AP-1-like site (6).

Binding of nuclear transcription factors to EpRE

Nuclear extracts from HepG2 cells exposed for 3h to PMA were analyzed by electrophoretic mobility shift assay for EpRE binding activity using as probe a ^{32}P-labeled EpRE

Fig. 1. Structure of EpRE enhancer from mouse GST Ya gene. Bold characters indicate core Ets and AP-1-like binding sites.

oligonucleotide. The PMA-induced nuclear extract was also analyzed for AP-1 and Ets-AP-1 binding activity using as probes ^{32}P-labeled oligonucleotides representing consensus sequence AP-1 or Ets-AP-1 containing the Ets site of EpRE. The results indicate that, the AP-1 complex binds to EpRE with much lower affinity than to a single AP-1 or Ets-AP-1 site (Fig. 2A lanes 1

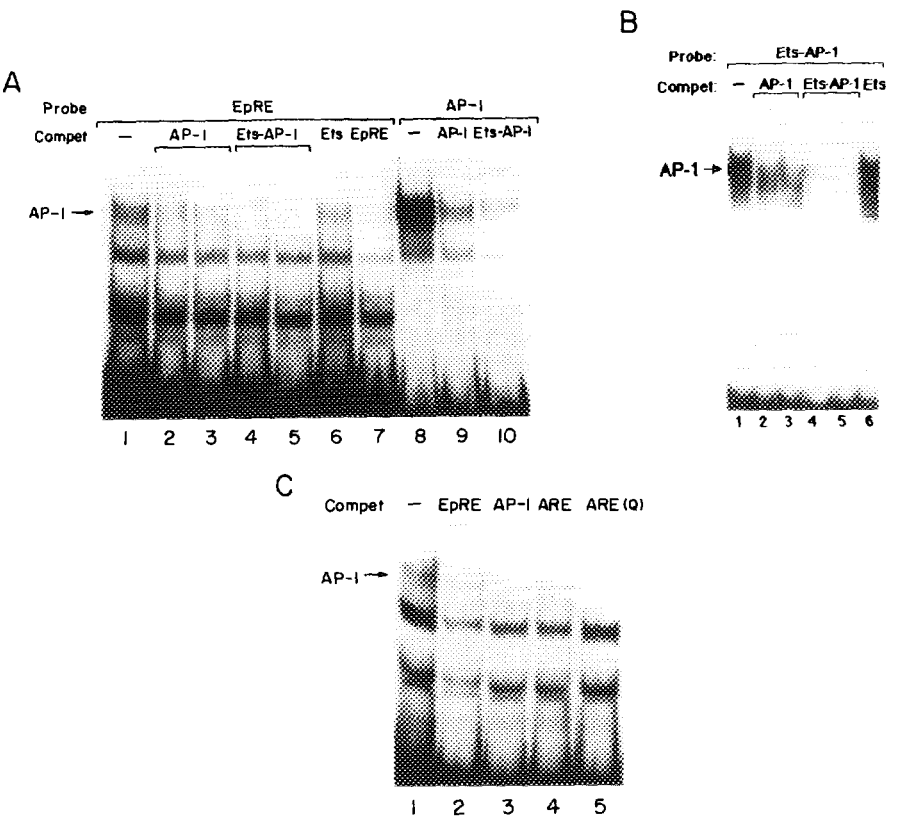

Fig. 2. Gel retardation assay of EpRE, AP-1 and Ets-AP-1 regulatory sites with nuclear proteins from 3h PMA-treated HepG2 cells. (**A**) The ^{32}P-labeled 41 bp EpRE oligonucleotide probe was incubated with nuclear extract in the absence (lane 1) or in the presence of 100 and 200-fold molar concentration of unlabeled AP-1 (lanes 2 and 3) and Ets-AP-1 (lanes 4 and 5) or 500-fold unlabeled Ets (lane 6) oligonucleotides. The binding of ^{32}P-labeled AP-1 oligonucleotide probe interacting with nuclear extract (lane 7) was competed by 100-fold unlabeled AP-1 (lane 8) or Ets-AP-1 (lane 9) oligonucletides. (**B**) The ^{32}P-labeled Ets-AP-1 oligonucleotide probe interacting with nuclear extract (lane 1) was incubated with 50- and 100-fold molar excess of unlabeled AP-1 (lanes 2 and 3), Ets-AP-1 (lanes 4 and 5) or 500-fold Ets (lane 6) oligonucleotides. (**C**) The ^{32}P-labeled EpRE probe interacting with nuclear extract (lane 1) was competed by unlabeled 100-fold molar concentration oligonucleotides of EpRE (lane 2), AP-1 (lane 3) and ARE from rat GST Ya (ARE) or rat quinone reductase (ARE(Q)) genes (lanes 4 and 5, respectively).

and 8 and Fig. 2B lane 1). This binding was competed by a 100 and 200 fold molar excess of unlabeled AP-1 (lanes 2 and 3) or Ets-AP-1 (lanes 4 and 5) oligonucleotide. The unlabeled Ets-AP-1 was consistently observed to be somewhat more efficient than the AP-1 oligonucleotide in competing the formation of the AP-1 complex band with EpRE. Unlabeled Ets oligonucleotide alone however, even in a 500-fold molar excess did not compete the formation of the AP-1 band (Fig. 2A, lane 6). The functional role of the Ets-binding site from EpRE is illustrated better by the experiments with a ^{32}P-labeled Ets AP-1 probe presented in Fig. 2B. The formation of the AP-1 complex band with the Ets-AP-1 probe (lane 1) is competed by an excess of 50- and 100-fold unlabeled AP-1 oligonucleotide (lanes 2 and 3 respectively). A 50- and 100- fold molar excess unlabeled Ets-AP-1 however appears to be a more efficient competitor for AP-1 complex band formation than unlabeled AP-1 oligonucleotide (lanes 4 and 5) and a 500-fold molar excess unlabeled Ets oligonucleotide had no effect (lane 6). The interaction of AP-1 complex with EpRE was found to be also competed by a 100-fold excess of oligonucleotides representing regulatory elements composed of two AP-1-like sites, ARE, of rat GST Ya and rat quinone reductase genes (Fig. 2C lanes 4 and 5 respectively).

Identification of c-Jun in the nuclear protein complex with EpRE

To identify the c-Jun component in the retarded nuclear protein-EpRE complex we have used the "Shift-Western blotting" technique (16). The protein-DNA complexes formed by ^{32}P-labeled AP-1 or EpRE oligonucleotides with nuclear extracts from PMA-induced HepG2 cells and separated by polyacrylamide gel electrophoresis, were transferred onto stacked nitrocellulose and anion-exchange membranes. The proteins bound to nitrocellulose were identified by immunoblotting using anti-c-Jun antibody while the ^{32}P-labeled oligonucleotides bound to the anion-exchange membrane were detected by autoradiography. Fig. 3 indicates that the retarded AP-1 complexes formed with the AP-1 and EpRE binding sites both contain c-Jun protein.

Transactivation of EpRE by AP-1 complex

Using undifferentiated F9 embryonal carcinoma cells which lack endogenous AP-1 activity, we have previously shown that the transactivation of EpRE enhancer specifically requires the Fos/Jun heterodimeric complex (2). In contradistinction with these findings an AP-

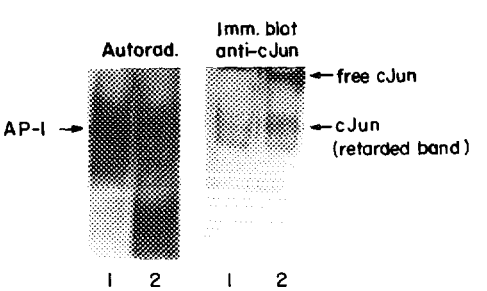

Fig. 3. Identification of c-Jun in the nuclear protein complex with EpRE. 50 and 100 µg nuclear extract protein from PMA-induced HepG2 cells were incubated with ^{32}P-labeled AP-1 or EpRE oligonucleotide, respectively. Complexes were separated by gel electrophoresis (5), transferred to nitrocellulose and DE-81 filters and analysed with anti-c-Jun antibody or autoradiography respectively as described (16).

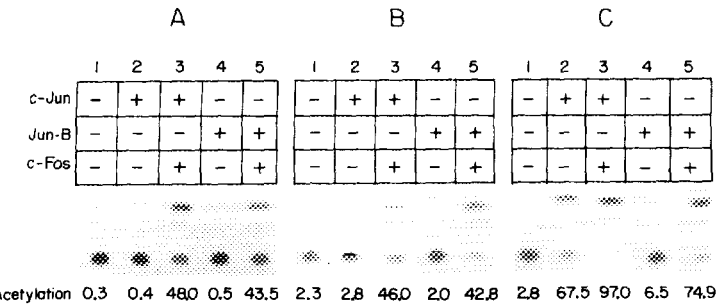

Fig. 4. Transactivation of EpRE by Fos/Jun complex. 5 μg plasmid DNA containing EpRE Ya cat, AP-1col Ya cat or -73Col cat gene constructs were cotransfected in F9 cells with 5 μg RSV-gal, internal control, in the absence or presence of 5 μg each of RSV-c-Jun, RSV-c-Fos or RSV-Jun B expression vector DNA as indicated. CAT activities were measured 24h after transfection and were normalized to the β-galactosidase activity.

1 site in the -73 Col-cat gene construct containing the human collagenase promoter was reported to require only c-Jun for transactivation in F9 cells (14). From this, and similar experiments with AP-1 sequences ligated to c-*jun* and thymidine kinase promoters (14,17) it was concluded that the AP-1 binding site is generally transactivated by c-Jun homodimer. To understand the discrepancy with the EpRE data we have ligated a collagenase AP-1 sequence upstream the GST Ya-minimal promoter driving *cat* gene expression (-187Ya cat)to form the AP-1 col Ya cat and compared its transactivation by Jun and Fos proteins in F9 cells with that of -73 Col cat and the EpRE Ya cat. Fig. 4 shows that, similar to EpRE Ya cat, the AP-1 col Ya cat requires the heterodimeric Fos/Jun complex for transactivation (panels A and B) and only when present in the collagenase promoter the AP-1 site was efficiently transactivated by c-Jun (panel C). Transactivation by Jun-B appears in all cases to depend upon the cooperative interaction with c-Fos (Fig. 4).

Role of Ets and AP-1-like motifs in the basal and inducible activity of EpRE

To study the contribution of the putative Ets-binding site alone or in association with the AP-1-like binding sites to the basal and inducible activity of EpRE, oligonucleotides representing these sequences were ligated into minimal promoter -187Ya cat and the plasmid constructs were transfected for expression into HepG2 cells. To determine the inducibility of the constructs the transfected cells were exposed to β-naphthoflavone. Fig. 5 shows that the Ets-binding site alone does not confer any activity to the -187Ya cat minimal gene promoter. In addition, the presence of Ets upstream the first AP-1-like motif (A) of EpRE did not affect the low basal activity and lack of inducibility of this site. In association with the two adjacent AP-1-like motifs of EpRE, however, the contribution of the Ets site to the inducible activity of EpRE becomes evident. Fig. 5 indicates that the inducibility of the ΔEpRE, containing only the two AP-1-like binding sites, is lower than that of EpRE which includes the Ets-binding sequence. The basal activities of ΔEpRE and EpRE are however similar.

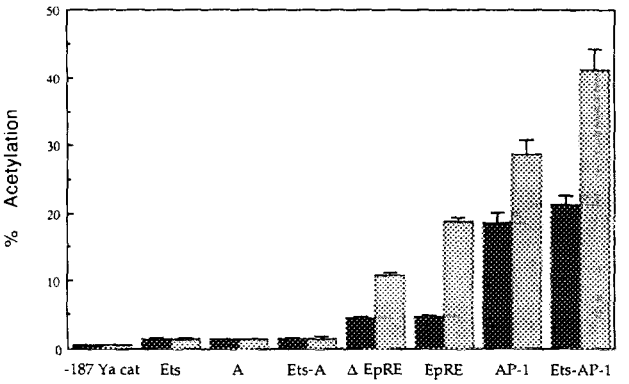

Fig. 5. Basal and inducible activities of Ets and AP-1-like binding sites of EpRE. 5 μg plasmid DNA constructs of oligonucleotides representing the Ets-binding site (Ets), the first AP-1-like site (A), the Ets and first AP-1-like site (Ets-A), the two adjacent AP-1-like binding sites (ΔEpRE), the 41 bp EpRE, an AP-1 consensus sequence (AP-1) or the Ets upstream the AP-1 sequence (Ets-AP-1) ligated into minimal promoter -187 Ya cat were transfected together with 5 μg RSV-gal and 10 μg pGEM-1 carrier DNA into HepG2 cells. After transfection cells were untreated (black columns) or exposed for 16 h to 50 μM β-naphthoflavone (grey columns). All CAT activities were normalized to β-galactosidase activity and calculated from three separate transfection experiments.

To further examine the functional activity of the Ets site of EpRE, we have synthesized an oligonucleotide, Ets-AP-1, which contains this Ets site upstream an AP-1 consensus sequence binding site and ligated it into the minimal promoter -187Ya cat construct. We observe that, compared with the AP-1 binding site alone, the Ets-AP-1 has an increased inducible activity (Fig. 5).

DISCUSSION

We have previously shown that EpRE binds *in vitro* synthesized Fos/Jun heterodimeric complex with a lower affinity than a single AP-1 site (2). Similarly, the AP-1 binding activity of nuclear extracts from PMA-induced cells presents a weaker interaction with EpRE than with an AP-1 or Ets AP-1 site (Fig. 2). The radioactive EpRE oligonucleotide probe interacting with the nuclear extracts forms three gel retarded bands. The competition experiments with unlabeled AP-1 and Ets-AP-1 oligonucleotide help identify the AP-1 complex with the slower migrating inducible band (4,5) and show that the two faster migrating bands are not related to this complex (Fig. 2A). Moreover, the interaction of EpRE with the AP-1 complex is competed by oligonucleotides representing the ARE regulatory elements of rat GST Ya and rat quinone reductase genes (Fig. 2C). These results bring additional support as to the involvement of AP-1 complex in the regulation of the inducible elements EpRE and ARE in the GST Ya and quinone reductase genes. Furthermore, by immunoblot assay with anti-c-Jun antibody we demonstrate the presence of c-Jun protein in the retarded band formed by AP-1 complex with EpRE (Fig. 3). The same experiment also shows that the faster migrating EpRE retarded band does not

contain c-Jun. In previous studies using gel supershift experiments with anti-c-Fos antibody, we have observed the presence of c-Fos protein in the retarded band of AP-1 complex with EpRE (4).

Transactivation experiments in F9 cells have shown that the EpRE enhancer ligated to -187Ya cat minimal promoter requires the heterodimeric Fos/Jun complex for activity (2). Since a single AP-1 site was shown to be transactivated by c-Jun alone forming the Jun/Jun homodimer (14,17), it would appear that the EpRE enhancer may have different features. In the present study we show that the transactivation of an AP-1 site by Jun/Jun homodimer or Fos/Jun heterodimer depends upon the promoter context in which the AP-1 site is located. Thus upon ligation of a collagenase gene-derived AP-1 site to the -187Ya cat minimal promoter the resulting AP-1-col Ya cat, similar to EpRE Ya cat, requires Fos/Jun heterodimeric complex for transactivation. The same AP-1 site however when present in the collagenase promoter, -73 Col cat, was transactivated by Jun/Jun homodimer and this activity was further increased by c-Fos (Fig. 4).

The finding of an Ets binding site adjacent to the two AP-1-like binding sites in the EpRE and ARE enhancers raises the question of the contribution of the Ets protein in the enhancer activity. The Ets binding site of GST Ya gene alone does not appear to mediate transcriptional activation when linked to -187Ya cat minimal promoter or to the first AP-1 like site which lacks basal or inducible activity (Fig. 5). An Ets protein seems however to interact synergistically with the Fos/Jun heterodimeric (AP-1) complex binding at the two adjacent AP-1-like sites of EpRE or to a consensus sequence AP-1 site to enhance inducibility by β-naphthoflavone (Fig. 5). This, together with the observation that an Ets oligonucleotide alone does not compete the binding of AP-1 complex to the EpRE site (Fig. 2A), may indicate that the Ets protein interacts primarily with the EpRE-bound AP-1 complex to activate transcription of GST Ya gene. The *ets*-1 and *ets*-2 genes are known to be induced by extracellular signals such as serum and activators of protein kinase C (11). It would be interesting to explore the effect of chemical agents on an induction of Ets protein activities.

ACKNOWLEDGMENTS

This work was supported in part by the Israel-U.S.A. Binational Science Foundation and the Leo and Julia Forchheimer Center for Molecular Genetics.

REFERENCES

1. Friling, R.S., Bensimon, A., Tichauer, Y. and Daniel, V. (1990) Proc. Natl. Acad. Sci USA 87, 6258-6262.
2. Friling, R.S., Bergelson, S. and Daniel, V. (1992) Proc. Natl. Acad. Sci. USA 89, 668-672.
3. Pinkus, R., Bergelson, S. and Daniel, V. (1993) Biochem. J. 290, 637-640.
4. Daniel, V., Bergelson, S. and Pinkus, R. (1993) Structure and Function of Glutathione S-transferases. Tew, K.D., Pickett, C.B., Mantle, T.J., Mannervik, B. and Hayes, J. (eds) CRC Press, Boca Raton, FL pp 129-136.

5. Bergelson, S., Pinkus, R. and Daniel, V. (1994) Oncogene (in press).
6. Rushmore, T.H. and Pickett, C.B. (1990) J. Biol. Chem. 265, 14648-14653.
7. Prestera, T., Holtzclaw, D.W., Zhang, Y. and Talalay, P. (1993) Proc. Natl. Acad. Sci. USA 90, 2965-2969.
8. Wasylyk, B., Wasylyk, C. Flores, P., Beque, A., Leprince, D. and Stehelin, D. (1990) Nature 346, 191-193.
9. Gutman, A. and Wasylyk, B. (1991) TIG 7, 49-54.
10. Macleod, K., Leprince, D. and Stehelin, D. (1992) TIBS 17, 251-256.
11. Janknecht, R. and Nordheim, A. (1993) Biochim. Biophys. Acta 1155, 346-356.
12. Daniel, V., Sharon, R. and Bensimon, A. (1989) DNA 8, 399-408.
13. Favreau, L.V. and Pickett, C.B. (1991) J. Biol. Chem. 266, 4556-4561.
14. Chiu, R., Angel, P. and Karin, M. (1989) Cell 59, 979-986.
15. Schreiber, E., Matthias, P., Mueller, M.M. and Schaffner, W. (1989) Nucleic Acid Res. 17, 6419.
16. Demczuk, S., Harbers, M. and Vennström, B. (1993) Proc. Natl. Acad. Sci USA 90, 2574-2578.
17. Okuda, A., Imagawa, M., Sakai, M. and Muramatsu, M. (1990) EMBO J. 9, 1131-1135.