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Regulation of c-*fos* Is Affected by Electromagnetic Fields

Sharmila Rao and Ann S. Henderson

Institute for Biomolecular Structure and Function, Biological Sciences, Hunter College-CUNY, The Graduate Division of the City University of New York, New York, New York 10021

Abstract The goal of the present study was to determine if regulatory regions of the c-*fos* gene were responsive to electromagnetic field exposure. The research design used transfected cells to increase the sensitivity of assays designed to identify changes following exposure. HeLa cells were transiently transfected with plasmids containing upstream regulating regions of c-*fos* up to −700 base pairs, coupled with the prokaryotic reporter gene CAT. Cells were exposed to an environmentally relevant EMF of 60 Hz at 60 mG_{rms}. CAT expression above control levels in transfected cells (region +42 to −700 bp) was observed following 5 min exposure to the electromagnetic field, with a peak at 20 min. The expression was at basal levels following 40 min exposure. Deletion analysis of upstream DNA narrowed the responsive region to 138 base pairs from −363 to −225, which contains the SRE/AP-1 sites.

Key words: c-*fos*, TPA, CAT expression, electric and magnetic fields

This study was designed to develop an endpoint for measurement in cells exposed to electromagnetic fields (EMFs), which was sensitive, unambiguous, and could be correlated with what is known about inductive pathways in the cell. The intensive research done on the c-*fos* gene makes it an excellent model for studying effects of EMF exposure. The Fos protein is an early nuclear transcription factor [Doucet et al., 1990]. The c-*fos* gene has been subjected to extensive analysis of its regulation [Prywes et al., 1988]. Specific upstream regulatory regions show sensitivity to many inducing factors [Greenberg and Ziff, 1984]; induced c-*fos* expression is rapid and transient. It was previously shown that steady state levels of transcripts coding for c-*fos* are transiently increased when human HL-60 cells are exposed for short periods to EMFs [Phillips et al., 1992].

This approach also provided a means of testing possible mechanisms for EMF-cell interaction. There are many proposed routes by which EMF exposures could theoretically affect cells. One of the most persuasive is that some event at the cell surface is translated by coupling to the cell's signaling pathways [Luben, 1991; Adey, 1993; Liburdy, 1994]. Several studies suggest that EMFs could use Ca^{+2} phospholipid-dependent PKC induction in a manner similar to 12-O-tetradecanoylphorbol-13-acetate (TPA) or other mitogenic agents [reviewed in Wallaczek, 1992; Goodman et al., 1993; Liburdy, 1994]. A direct role involving calcium flux is one of the most consistent findings in EMF research. This is a ubiquitous response to many stimuli initiating at the cell membrane. Evidence for changes in Ca^{+2} flux in EMF exposures, however, has been derived from data obtained from many laboratories [see Adey et al., 1982; Blackman et al., 1989; Wallaczek and Liburdy, 1990; Liburdy, 1992; Karabakhtsian et al., 1994]. TPA activates Ca^{+2} and phospholipid-dependent protein kinase C via the diacyl glycerol binding site [reviewed in Nishizuka, 1986]. TPA also causes an increase in the activity of the c-*fos* gene; a 20- to 50-fold increase in c-*fos* mRNA transcription in HeLa cells occurs within 30 min of induction [Fisch et al., 1987].

The results show that regulation per se of fos gene transcription can be affected by EMF exposure and give credence to proposed mechanisms of EMF activity that invokes interactive signal transduction mechanisms. The response of c-*fos* upstream regulatory regions to EMF exposure in the present experiments was time dependent, similar to the timed expression elicited by mito-

Abbreviations used: CAT, chloramphenicol transferase; elf, extremely low frequency; EMF, electric and magnetic fields; Hz, Hertz; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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Address reprint requests to Ann S. Henderson, Department of Biological Sciences, Hunter College of the City University of New York, 695 Park Avenue, New York, NY 10021.

genic and other inducing factors. This is supported by deletion analysis showing that the response of cells to EMF exposure is mediated by the SRE/AP-1 site.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human fibroblast cell line HeLa was used for transfections. The cells were maintained in Dulbecco Modified Medium (DMEM), containing 10% fetal calf serum (Sigma, St. Louis, MO) and 1% antibiotic-antimycotic (Gibco BRL, Grand Island, NY). HeLa cells were obtained from Dr. R. Dalla-Favera, Columbia University. Cell concentration was determined using a hemocytometer.

Conditions of EMF Exposures

HeLa cells were exposed to EMFs at 60 Hz (60 mG_{rms}). The calculated corresponding induced electric field was 11 uV/meter [Bassen et al., 1992]. Except for EMF exposure, control and exposed cells were subjected to identical environments within a double door incubator. The magnetic fields in the growth incubator have been measured over a period of 5 years at 2 mG. Control and exposed cells were derived from the same "parent flask" to ensure that the transfection efficiency was the same for both. The flasks were exposed simultaneously to either no field or the EMF at a concentration of 8×10^5 cells per ml.

Details of the placement of cells, physical separation of experimental and control flasks, and the composition and construction of the mu metal container shielding the exposure signal are described in Goodman et al. [1992]. The magnetic field in the mu metal containers in the absence of an applied field was 1.2 mG. Control cells were in a different compartment of the same incubator as the experimental cells to reduce thermal differences that could arise by using separate incubators. The signal generator was placed outside the incubation. All experiments were carried out at 37°C.

Early experiments used a continuous sinusoidal 60 Hz field generated by a pair of Helmholtz coils designed by Electro-Biology Inc., Parsippany, NJ [Goodman et al., 1992]. These included the determination of time required to maximize protein synthesis, initial studies of the time dependent response of the promoter region −700 to +42, and portions of the deletion analysis. All samples were coded using an alphanumeric code. All other experiments used a Double Blind Helmholtz Coil Exposure System designed by Electric Research and Management, Inc., State College, PA. This system produces identical magnetic field exposure conditions to that used previously, but adds the capability of conducting simultaneous sham/exposure in a dual device under double blind conditions to eliminate experimenter bias. Exposures were for different times depending on the experimental objective.

Plasmid and Cell Transfection

Plasmid pFC-700, containing the upstream regulatory region of the c-*fos* gene (from −700 to +42) was obtained from Dr. David Foster, Hunter College-CUNY. The promoter region was subcloned into a PUC-19 vector containing CAT. Five constructs containing different regions of the c-*fos* promoter complex were obtained from Dr. R. Prywes, Columbia University. The constructs, p350-tk CAT, p250-tk CAT, and ttk-CAT, were obtained from Dr. Harel-Bellan (Institut Gustave Roussy) [Trouche et al., 1991].

Plasmids were introduced by the lipofectin method as described (Gibco lipofectin reagent, Gibco BRL). Ten micrograms of DNA was used per 50 μl of lipofectin. HeLa cells were at a concentration of 8×10^5 cells/ml. After 12–16 h of incubation, the media was changed to DMEM with 10% FCS. The cells were harvested after 24 h and assayed for CAT activity.

CAT Assays

The percentage of chloramphenicol converted to acetylated ^{14}C chloramphenicol was measured by thin layered chromatography [Gorman et al., 1982]. The alternate of phase extraction was used to confirm results for some experiments [Seed and Sheen, 1988]. Northern blots of CAT mRNA were also used in some experiments. Conditions were essentially as described in Ausabel et al. [1994], using an annealing temperature of 40°C in 50% formamide.

Protein Extraction, Quantitation

Proteins were extracted using the freeze-thaw method [Gorman et al., 1982]. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad, Richmond, CA). Standard curves were obtained using Sigma software.

TPA Induction

Transfected HeLa cells (8×10^5 cells/ml) were exposed to TPA (162 nM) as a positive control. The appropriate TPA concentration was determined by doing time and concentration experiments using protocols as described in Trouche et al. [1991].

RESULTS

CAT Expression Is Increased in Human Cells Exposed to 60 Hz Sinusoidal EMFs

HeLa cells, transfected with a c-*fos* promoter-CAT construct (−700 to +42), were exposed for 5, 10, 15, 20, 25, 30, or 40 min to EMF. Exposure to EMF was followed by a 1 h incubation at 37°C, the peak in CAT protein following EMF exposure. The experimental determination of "lagtime" is given in Figure 1. An increase in CAT protein was observed 5 min following EMF exposure (Fig. 2). A peak increase of about 25% was seen after 20 min of exposure, but protein levels fell rapidly to basal levels with 40 min of EMF exposure. These results are consistent with determinations of increased c-*fos* steady state transcript levels (data not shown). The level of steady state CAT mRNA was also analyzed to ensure that the increases seen in expression of CAT protein were due to the effect of EMFs on the regulation of the gene rather than a downstream effect. CAT RNA from transiently transfected cells was analyzed using β2-microglobulin as an internal control (Fig. 3). Steady state levels of CAT RNA were comparable to those observed for CAT protein.

To verify that the fields were the cause of the increase seen in CAT protein in exposed cells, we conducted a series of "sham-sham" experiments. The experimental design for these experiments was comparable to the 20 min exposures except that the cells were isolated from EMFs within a mu metal box. No increase in CAT expression was observed (Fig. 4). The construct pFC-700 has been previously shown to be responsive to TPA [Prywes et al., 1988], providing a "positive control." Transfected cells were exposed to TPA (162 nM) for different times (Fig. 5). The CAT construct responded to TPA in a time-dependent manner. A response of 50% above basal levels was observed after 6 h.

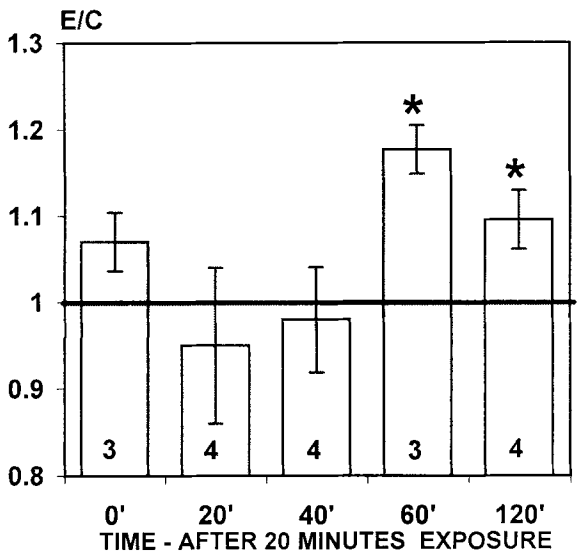

Fig. 1. CAT protein is increased by EMF exposure; determination of "lagtime" for CAT protein expression following EMF exposure. Protein from HeLa cells, transfected with pFC 700, was extracted at different times (0, 20, 40, 60, and 120 min) following 20 min exposures to an EMF of 60 Hz at 60 mG. CAT protein was determined by the chromatographic method. Peak expression was observed 60 min after exposure was ended. This was used as the "lagtime" in subsequent experiments which measured CAT activity. The numbers in each column are the number of times each experiment was repeated. E/C: ratios of experimental to control value (±SE of the mean). All values were determined by both phosphoimaging (PSI Image QuanTMb) and direct scintillation counts of cut spots, but both gave comparable results. Data for the graph here, and that in Figures 2–7 was obtained by phosphoimaging. Background is determined and automatically subtracted by the computer program. Significant deviation from one (*t*-test; $P \leq 0.05$) is shown by an asterisk (*). Other points are not significantly different from one.

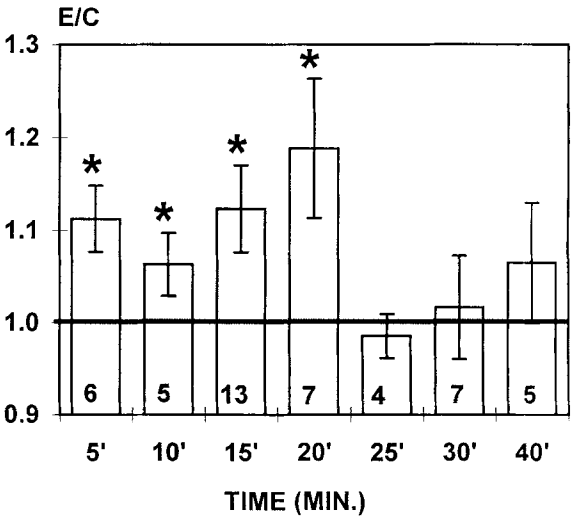

Fig. 2. Effect of time of EMF exposure on CAT protein. Methods are as given in Figure 1. HeLa cells, transfected with pFC 700, were exposed to EMF for different lengths of time between 5 and 40 min, and the cells harvested for CAT assays 1 h after exposure. Mean values at 5, 10, 15, and 20 min are significantly different from one ($P \leq 0.05$) and from each other ($P \leq 0.02$).

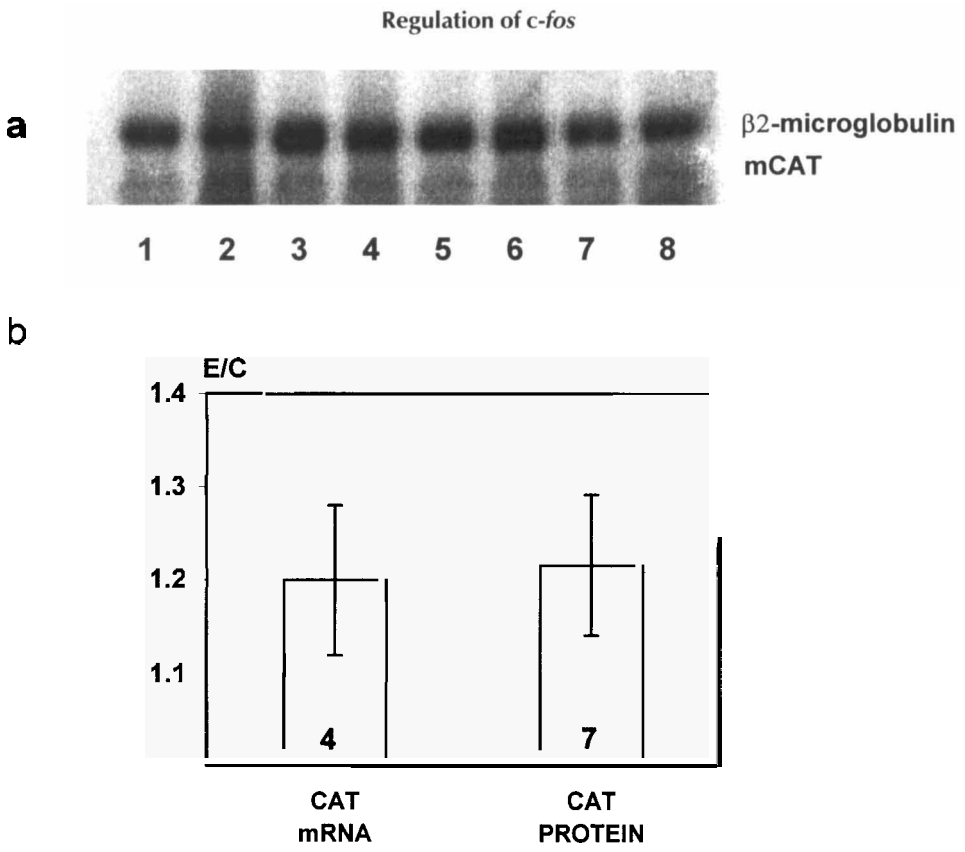

Fig. 3. Analysis of CAT mRNA by Northern blots. **a:** Northern blot hybridization of mRNA from transfected HeLa cells against ^{32}P-labeled ptri-CAT (RNA probe from Ambion, Austin, TX). RNA was extracted from HeLa cells immediately after exposure to a field of 60 Hz (60 mG) for 20 min. The odd-numbered lanes contained RNA from unexposed cells; the even lanes, RNA from exposed cells. The membrane was exposed overnight at −70°C. **b:** Comparison of CAT mRNA and CAT expression. Quantitation of the Northern blot was by phosphoimaging (PSI Image QuanTMb). E/C values for CAT mRNA were corrected to those of β2-microglobulin.

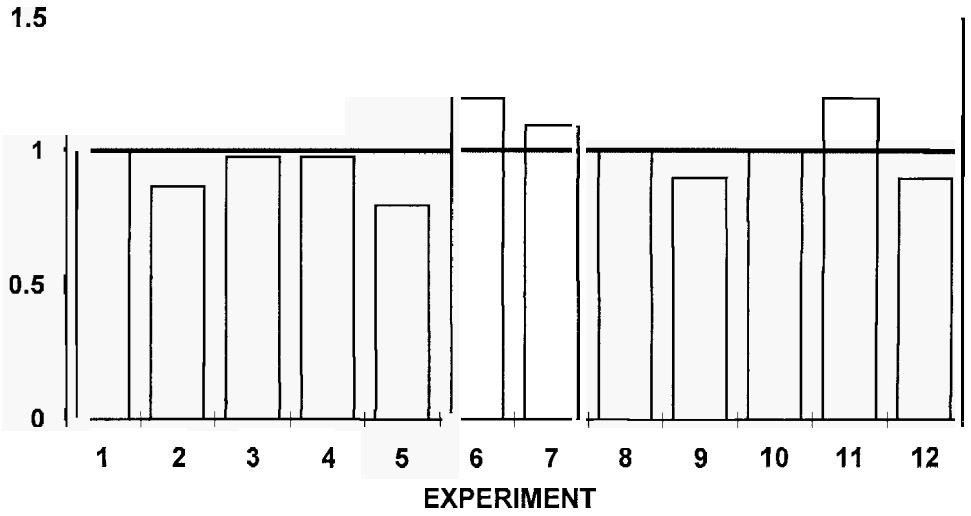

Fig. 4. Sham-Sham experiments. Transfected cells were treated under the same conditions as those used for 20-min exposures (see Fig. 1), but no field was applied. Each experiment point was derived *from three sets of flasks.*

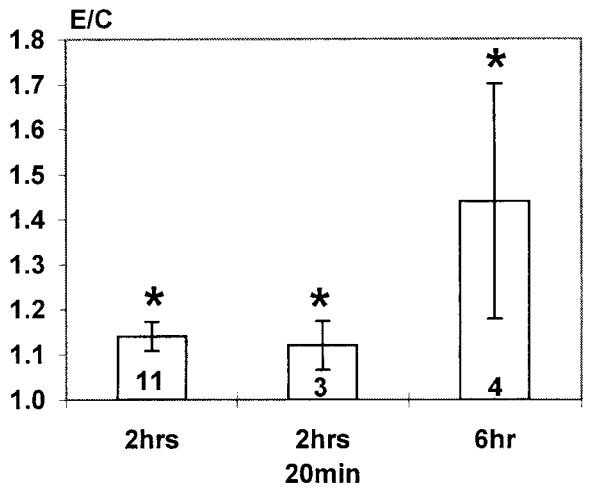

Fig. 5. Transfected HeLa cells are responsive to TPA. TPA was used as a positive control under the following conditions: 48 h following transfection of the cells with the pFC-700 construct, the cells were exposed to TPA (162 nM). Following incubation in the presence of TPA, protein was extracted and examined for CAT activity. To ensure that the solvent for TPA, DMSO, was not causing the effect, DMSO was also added to unexposed cells. The E/C ratio is given for CAT activity.

Deletion analysis was used to narrow the upstream region necessary to realize an effect of EMF exposure. The deletion constructs are illustrated in Figure 6. The region from −700 to −225, containing the SRE and AP1 binding sites, produced CAT expression similar to the complete upstream regulatory region. These results show that the region −225 to −53, containing the cAMP, DRS, and CRE, do not have an imperative function in the response to EMFs. Transfection of the construct pfc363, which lacked regions −700 to −363, also resulted in no change of expression of CAT in exposed cells, although the SRE and AP1 regions were present. This showed that either regions from −700 to −363 (only) are responsive to EMFs or the inclusion of the region containing bases −225 to −53 quenches the response. To determine which of the scenarios was correct, we first analyzed the construct p350, which contained basepairs −363 to −700 directly attached to the TAATA box. There was no change in CAT expression following exposure to EMFs, proving that this region alone was insufficient to elicit a response to EMF exposure. The construct p250 was then analyzed to determine if the SRE/AP1 site could respond to the field if the downstream regions were not present. This construct contains bases from −362 to −225, including the SRE and AP-1 sites directly attached to the TAATA box. CAT expression was affected by EMF exposure, giving similar results to those observed when the original construct (−700 to +42 bp) was used.

We conclude that the region −362 to −225 plays an important role in the expression of c-*fos* in cells exposed to EMFs. It can also be inferred that regions upstream of the SRE/AP-1 site, while not responsive to EMF exposure, appear to function in negating an inhibitory effect of the downstream regions.

The constructs, p350 and p250, have the c-*fos* promoter region attached to a thymidine kinase promoter. Transient transfection with a construct containing only the basic promoter region of herpes thymidine kinase (without the enhancer) showed no increase in CAT activity in exposed cells (Fig. 7). This ensured that the response of the p350 and p250 constructs to EMF was due to the upstream regulatory region of the c-*fos* promoter per se.

A final experiment showed that the SRE site alone (construct pFC53 E), was inadequate (see Fig. 6). Taken together, the results show that the AP-1 regions have an influence on the expression of the c-*fos* promoter after exposure to EMFs, which is mediated by the AP-1 site, or the AP-1 and SRE sites in concert.

DISCUSSION

The impetus for the present research was the possibility that EMF exposure is a health risk. Virtually every study on the effects of EMF exposure, however, is subject to controversy. The debate that has arisen from EMF exposure studies results in part from a lack of a proven mechanism [see Bennett, 1994], but also from variability in both population and molecular data [Saffer and Thurston, 1995; Lacy-Hulbert et al., 1995]. The most compelling studies relating EMF exposure to cancers of various types are epidemiological. The strength of the correlation varies widely, however, depending on the study [see Wertheimer and Leeper, 1979; McDowell, 1986; Feychting and Ahlbom, 1993]. High risk factors have been reported for childhood leukemia and adult breast cancer (male and female) on the basis of epidemiological data [Demers et al., 1991]. A consistent proposal is that EMF exposure cannot initiate tumor development, but could promote malignant processes once initiated. This is supported by experimental studies which show that exposure to EMFs acceler-

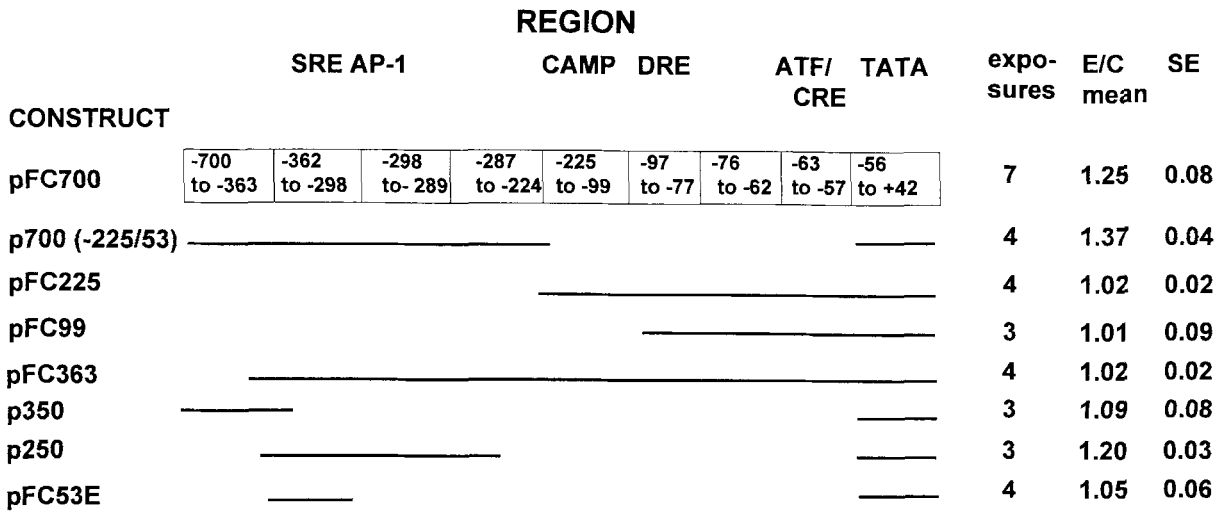

Fig. 6. Summary of deletion analysis. Transfected HeLa cells were exposed to an EMF for 20 min; other conditions are as given in Figure 1. Constructs were −700 (pFC700), −363(pFC363), −225(pFC225), and −99(pFC-99), all extending to +42. The construct, pfc 53E, contained 27 base pairs of the oligonucleotide TF4, spanning the human c-*fos* SRE (−319 to 297) cloned at position −53. The construct, pFC 700-225, contains basepairs spanning from −700 to −225. Constructs p350-ttkCAT and p250-ttkCAT contain the regions from −700 to −362 and −362 to −225, respectively.

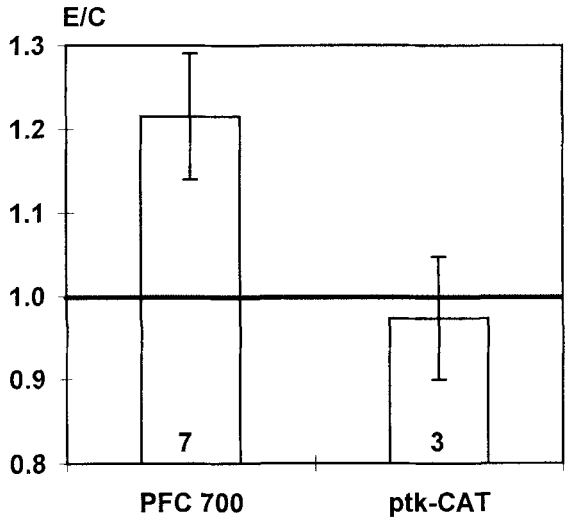

Fig. 7. Comparison of CAT activity in cells transfected with the construct pFC700 and ptkCAT. The cells were exposed for 20 min. Other conditions are as given in Figure 1. The thymidine kinase promoter alone is not responsive to EMFs.

ates tumorigenesis in animals exposed to carcinogens [Stuchly et al., 1991; McLean et al., 1991].

A direct association between EMF exposure and malignancy requires experimental substantiation that a cell function(s) altered during malignant progression or transformation can also be altered by exposure. How cells could translate "information" from EMF exposure to a neoplastic condition as a part of a multi-step progression is puzzling. Yet, the possibility of health risk provides a strong motivation to provide possible mechanisms by which EMFs could affect cell function using mechanisms consistent with tumor promotion and/or progression. Based on the present results and research from other laboratories, our working model is to consider EMF exposure as a tumor promoter akin in some fashion to TPA, but with much lowered efficacy. This assumes that a cell surface phenomenon initiated by EMF exposure activates second messengers either directly or indirectly. Theoretically this could include modification (loss or gain of function) in signal transduction systems where amplification and transmission of signals normally act coordinately in regulation of cell activity. The present experiments build on earlier studies that showed that the presence of increased c-*myc* transcripts following EMF exposure involves some facet of regulation of the c-*myc* gene [Lin et al., 1994].

Portions of our research findings are consistent with this working model. The most persuasive finding is that there is an involvement of the SRE/AP-1 site following EMF exposure. The AP-1 site is directly involved in the response to TPA. Exposure to TPA results in the translocation of conventional PKC isoforms from the cytosolic to the membrane fractions [Blumberg, 1988]. Translocation of PKC results in transcriptional activation via AP-1, leading to early expression and regulation of some genes,

including fos [Hirai et al., 1994; McCafferey et al., 1987].

There are still many questions to be answered, including the relationship between time and dose of TPA vs. EMF exposure. Further, there is no a priori means of knowing the effect of what is a very transient response on the part of EMF-exposed cells relative to the potential for participation in malignant progression. Proof of a general mechanism for EMF effects on cells, or as a health risk for the population, will require addressing these problems as well as further investigations as to feasible means for interaction of EMFs at the cell membrane level.

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