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THE EFFECT OF EMF ON THE REGULATION OF THE C-FOS GENE

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

THE EFFECT OF EMF ON THE REGULATION OF THE *C-fos* GENE

by

Sharmila Rao

Advisor: Professor Ann S. Henderson

The present research was designed to test a proposed role for signal transduction in the response of cells to environmentally relevant electromagnetic fields (EMFs). Two approaches were taken. The first determined if defined upstream regulatory regions of the *c-fos* gene were critical; the second compared the effects of EMF exposure and the tumor promoting phorbal ester, TPA , on the regulation of *c-fos*. HeLa cells were transiently transfected with plasmids containing various portions of the *c-fos* promoter complex coupled with the prokaryotic reporter gene chloramphenicol acetyl transferase (CAT). Levels of CAT activity were used as an indirect marker relative to the response of the promoter to the fields. CAT expression, driven by the construct containing +42 to -700 base pairs, was above background levels following 5 minutes of EMF exposure, reached a maximum activity (~25%) by 20 minutes and returned to basal levels after 40 minutes of exposure. Deletion analysis of the upstream DNA showed that a 138 bp region from -363 to-225 (containing the SRE/AP1site) is

important in the response of cells to EMFs. This region is sensitive to several mitogenic factors, including TPA. Studies that compared the activities of TPA with EMF showed that they initiated similar pathways. This was supported by evidence that EMF affects on cells, like TPA, required both protein kinase C (PKC) and protein kinase A (PKA). Further, EMF exposure resulted in an increase in the uptake of ^{32}P by Fos protein by 50%. This research demonstrates that EMF affects the regulation of the *c-fos* gene and uses signaling pathways similar to that of TPA.

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INTRODUCTION

I. Background

Electromagnetic fields (EMFs) are produced when electric current flows through a conductor such as a power line. Although EMFs are usually associated with high voltage power lines and power stations, they are also produced by any electrically powered device, typical of those found in households or the work place. Appliances such as video display terminals, TV's, hair dryers and cellular phones emit EMFs (rev. in Goodman *et al.*, 1993). Human exposures are normally to extremely low frequency (elf) EMFs (defined as less than 200-300 Hz). Heightened public awareness has lead to the inclusion of exposure to elf EMFs as part of a growing series of environmental conditions related to "quality of life" in the industrial world. There is still, however, some resistance to accepting EMFs as a health hazard, perhaps since electricity is such as driving force in our lifestyle.

EMFs are also emitted continuously by the earth's magnetic field. This force is a direct current (DC) field and is usually constant in a given environment. The electric current generated for daily use is a fluctuating alternating current (AC) field. The fluctuation of the electric current corresponds with that of the magnetic signal, *i.e.*, fluctuations (frequencies) are measured as Hertz (Hz) values. Most electrical equipment in the USA runs at a frequency of 60 Hz.

The energy of EMF depends on the frequency (Figure 1). A very high

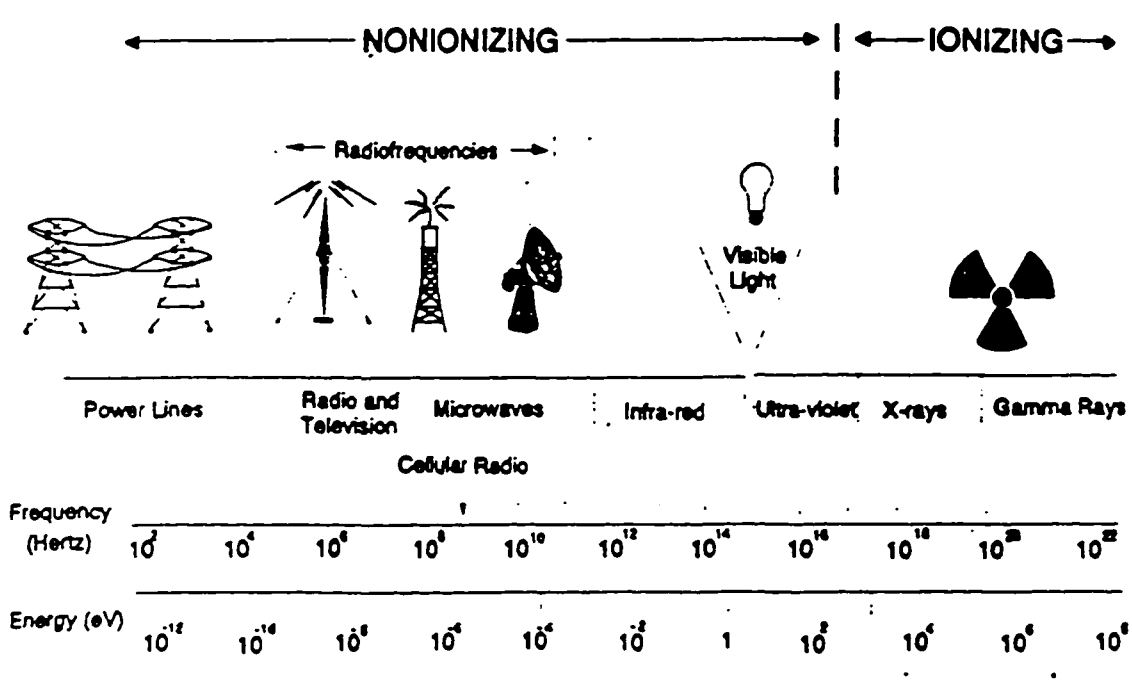


Figure 1. The electromagnetic spectrum.

Frequency is given in Hertz (Hz) values (one Hz equals one cycle per second). Corresponding energy values are also depicted. Elf EMF is on the lower end of the spectrum.

frequency range is generally termed "ionizing radiation", or radiation with sufficient energy to cause formation of ions. X -rays are a common example. Ionizing radiation is known to cause damage at the DNA and chromosomal level. Lower frequency waves, such as infrared, microwaves and radio waves, heat molecules. At the lowest ranges, or elf, the magnetic and electric fields are decoupled. Elf EMFs are non-thermal and therefore putative effects from elf EMF must be the results of nonlinear or non-equilibrium effects.

Elf EMF exposures are defined by magnetic field. The intensity of magnetic fields is measured in milligauss (mG) or Tesla (1 Tesla (T)=10^4 Gauss). Environmental intensities vary widely in magnitude, depending on location and proximity to an electrical device. In most homes, the magnetic field averages less than 2 mG (Bennet *et al.*, 1994). Everyday appliances, such as electric razors or hair dyers, can produce magnetic fields as high as 1000 mG at close range. Occupational exposures to EMFs are typically at 100 mG (or higher) levels.

Magnetic fields are not easily shielded like electrical fields. Mu metals and ferromagnetic materials shield the magnetic fields, whereas electric fields are shielded by trees, windows, walls and skin (Paris *et al.*, 1969). Magnetic and electric fields, however, weaken with increasing distance from the source.

EMF exposure and health. An early scientific link between EMF and cancer was proposed in 1979, on the basis of an epidemiological study conducted by Wertheimer and Leeper (1979). Their study focused on leukemia rates among

children who lived along unusual 60 Hz power line configurations that emitted higher magnetic fields (3 mG). This was a retroactive study using a sample size of 344 cases of children previously exposed to the magnetic field. Cancer rates of these children were compared to 344 control cases of children not exposed to the magnetic fields. The results indicated that the children in their study population showed a greater risk of leukemia; the odds ratio was greater than two (The odds ratio is defined by two independent binomial populations and is obtained by dividing the ratio of the odds in the first population or case exposure with the second or control population ratio). A problem associated with this study, however, was that the magnetic field was not measured directly. Instead, they estimated the magnetic field based on the wiring configuration, *i.e.*, the size and number of power line wires and the distance between the power line and the home. Savitz *et al.* (1988) replicated the Wertheimer and Leeper results. This study also investigated populations in Denver, but incorporated wire configuration, spot measurements and interviews about the use of appliances to estimate magnetic field exposures. This study used a sample size of 356 children exposed to a 2 mG field (278 control cases were analyzed) and reported an odds ratio of 1.9 for leukemia (95%confidence interval CI= 0.67-5.56) The effects of low level EMFs gained importance after a more recent Swedish study also directly correlated increased childhood leukemia with exposure to magnetic fields of intensity comparable to that used in previous studies (3 mG) (Feychting *et al.*,

1993). This corroborated other epidemiological studies, showing an odds ratio of 3.80 (CI= 1.4-9.3). It included a sample size of 572 children exposed to the magnetic field, including 142 diagnosed cancer case controls. They also showed a dose response, *i.e.*, closer proximity to the EMF source gave a higher odds ratio. In this case, the magnetic field was determined by the yearly average field values to obtain a more realistic estimate of the magnetic field at the time of cancer diagnosis. There have been many other epidemiological studies that have suggested a link between EMF and certain types of cancer, while other studies have found no such link (Figure 2). The risks in some cases were not reported due to the small sample sizes, small risk factors and uncertainties in actual exposure histories.

Other major epidemiological studies have been done on groups exposed to high voltage lines, such as electricians, power line workers and workers in related fields. Studies conducted by Demers *et al.* (1991) showed a six-fold greater incidence of certain cancers (one of which was breast cancer) in male electric workers, as compared with males in a more typical environment. This study used a sample size of 194 electricians, telephone linesmen and electric power workers (and 274 controls matched according to age). This study showed an odds ratio of 6.0, with a CI= 1.7-21. This was especially interesting since breast cancer in males is so rare. These results were further substantiated in a study by Matanoski *et al.* (1991), which also showed similar increases in breast

Study	Location	Leukemia	Other Cancer
	Child cancer		
Wertheimer and Leeper '79	Denver	OR=2.35*	All Cancer OR=2.22*
Fulton et al '80	Rhode Island	OR=1.09	Not studied
Tomenius '86	Sweden	OR=.30	CNS tumorsOR=3.70*
Savitz et al.'88	Denver	OR=1.54	All cancer OR=1.53*
Coleman et al. '89	U.K.	OR=1.50	Not studied
Myers et al. '90	U.K.	OR=1.14	All cancer OR=0.98
London et al.'91	Los Angeles	OR=2.15*	Not studied
Lowenthal et al. '91	Australia	O/E=2.00	
Feychting and Ahlbom '93	Sweden	OR=3.80*	All Cancer OR=1.30
Olsen et al.'93	Denmark	OR=1.50	All cancer OR=5.60*
Petridou '93	Greece	OR=1.19	Not studied
Verkasalo '93	Finland	SIR=1.60	All cancer SIR=1.50
	Adult cancer		
Wertheimer and Leeper '82	Denver	OR=1.00	All cancer OR=1.28*
McDowell '86	U.K.	SMR=143	Lung cancer SMR=215*
Severson et al. '88	Seattle	OR=0.80	Not studied
Coleman	U.K.	OR=0.90	Not studied
Youngson et al.'91	U.K.	OR=1.29	Not studied
Eriksson and Karlsson '92	Sweden	Not studied	Multiple Myeloma OR=0.94
Schreiber et al. '93	Neatherlands	No cases	All cancer SMR=85

Figure 2. Summary of Residential Power -Line Cancer Studies

This figure summarizes selected, and often cited results of the residential cancer studies.

Abbreviations and definitions: OR (Odds Ratio); SMR (Standardized Mortality Ratio, which is a weighed average of the ratios of the mortality rates of two populations at individual ages- an SMR of 100 means no increased or decreased risk; SIR (Standardized Incidence Ratio is the weighed average of the ratios of the incidence (cancer) of two populations at individual ages - an SIR of 1.00 mean no increased or decreased risk; CNS (central nervous system) O/E (Observed number of cases divided by the expected number of cases).

* The number is statistically significant.

cancer in male telephone company central office technicians with a standardized incidence ratio (a weighed average of the ratios of the incident/mortality rates of two populations at individual ages) of 6.5 (CI= 0.79-23.5,). Female breast cancer rates were also shown to increase significantly, but since other factors, such as family history etc. might be involved in this increase, the direct link to EMF is still unknown. Epidemiological studies, however, are not sufficient by themselves to prove a link between EMFs and cancer since many studies were carried out in urban or workplace environments, where multiple factors (confounders) could also predispose exposed individuals to cancer. In addition, the epidemiological determinations show large statistical variations. Although epidemiological studies initially raised concerns about potential health risks associated with exposure to EMFs, they were not sufficient to establish the relationship. The putative risks associated with EMF exposures are low. For example they are one tenth the risks associated with other environmental hazards such as smoking (Wald *et al.*, 1996)

Research at the basic level. Typically cancer arises from exposure of cells to chemical carcinogens, ionizing radiation or genetic factors. The result is a direct effect on DNA, causing breaks, deletions or mutations. Indirect effects may play a role in secondary multi-step processes leading to cancer by altering regulation of cell cycle controls. On the basis of several different types of studies, it has been proposed that EMF exposure cannot initiate tumor development, but

may rather promote the process once initiated; *i.e.*, EMF exposure could mimic characteristics of the "second or other-step" counterpart in the multiple independent events leading to neoplastic disease. This view is supported by studies that show that exposure to EMF accelerates tumorigenesis in animals exposed to carcinogens (Stuchly *et al.*, 1991; McLean *et al.*, 1991). The failure to show chromosomal disturbances, DNA crosslinks, changes in DNA repair, or sister-chromatid exchanges in cells exposed to 60 Hz fields, is more consistent with a role in tumor promotion, rather than in initiation (Nair *et al.*, 1989). These observations and the known characteristics of elf EMFs have given rise to the question of how these fields could initiate or cause changes in biologic events. Although most investigators agree that elf EMF interacts with the cells, the absence of a credible mechanism(s) of interaction decreases the credibility of the scientific evidence.

To tackle this, recent research has concentrated on identifying the mechanism by which EMFs could affect the cell. Researchers have approached this problem differently, investigating the effect of the field on several different systems and targets. Basically two types of studies have been done. The first is to expose animals or humans to fields to test the effect on body function, disease or behavior. The second type of study exposes single cells, or groups of cells under a variety of conditions to investigate tumorigenicity.

General animal studies. Magnetic fields affect a wide range of species,

ranging from bacteria to mammals. The changes observed vary from species to species and with the type of magnetic field. Animal studies have used various types of magnetic fields to measure effects on behavior and physiological processes, including rates of development and reproduction. For example, studies on *Paramacium tetraurelia* showed that a 72 Hz pulsed field [pulsed electromagnetic fields or PEMFs are a broad band signal, with frequency components ranging from ~DC to the low-mega Hertz region, based on Fourier analysis (Bassett *et al.*, 1994)] increased cell division by 85% (Diehl *et al.*, 1985). When an intense magnetic field of 0.126 Tesla (T) was applied, reduction and disorganization of movement was seen (Rosen and Rosen, 1990). It was hypothesized that the events occur because of an alternation in ion balance. Similarly, studies on the ameba, *Volvox aureatus,* and diatoms also showed that EMFs affected motility and shape through alteration of ionic flux (Palmer, 1963; Friend *et al.*, 1975).

Magnetic fields affect regeneration in the fiddler crab. Crabs placed in the south pole of a magnetic field of 10T regenerated and molted sooner than controls, while crabs at the north pole were delayed in development (Lee and Weiss, 1980). There are also a number of reports dealing with the developmental effects on amphibians exposed to a magnetic field. One of the best known effects of magnetic fields is on the regeneration of limbs. Abnormal limb regeneration has been reported in adults newts exposed to pulsed magnetic fields (Landesman and

Douglas, 1990).

Neurophysiology studies. Other research showed that EMFs affect growth and orientation of neurons. Studies on chick dorsal roots showed that the growing neurite migrated toward the cathode in a field of 70 millivolts/millimeter(mV/mm) (Erickson and Nutitelli, 1984). A similar observation was seen relative to neurite outgrowth of neuroblasts in the frog (Patel *et al.*, 1982; 1984). Neurites changed direction of growth toward the cathode. Young *et al.* (1994) showed that influx of Ca^{+2} ions, which are considered to amplify the signal generated by the weak fields into a measurable response, increased in cells exposed to a current from the normal value of 50 to 100nM to 0.5μM in a neuron.

Neuronal or neuroendocrine studies. Neuronal or neuroendocrine alternations induced by EMF stimulation have been demonstrated in the rat (Wilson *et al.*, 1980), hamsters (Yellon *et al.*, 1991), mouse (Groh *et al.*, 1990) and monkey (Seegal *et al.*, 1989). The major neuroendocrine system studied is the pineal gland, and in particular, the hormone, melatonin. Reiter *et al.* (1988) examined the effects of a 60Hz field dose on the rat pineal function. Exposure to the field showed approximately a 50% reduction in nocturnal peak melatonin. Melatonin has been shown to be protective against leukemia (Buswell, 1975), breast cancer (Blask, 1990), prostrate cancer (Philo and Berkowitz, 1988) and melanoma (Das Gupta and Terz, 1967). Melatonin suppression was also seen in

rats exposed to a 60 Hz electric field and 65 kV/m (kilovolts/meter) unperturbed magnetic field (Wilson *et al.*, 1980). Work by Welker and colleagues (1983) also showed that the reversal of application of a static magnetic field affects the serotonin N-acetyl transferase (SNAT) enzyme, a rate limiting enzyme for melatonin synthesis in a pineal gland, causing subsequent reduction in melatonin. A recent study by Liburdy *et al.* (1993) reported that a 12 mG 60 Hz magnetic field could block the *in vitro* growth inhibition of human MCF7 breast cancer cells by melatonin.

Cell and molecular studies. The first biosynthetic effects attributed to EMF were demonstrated by the successful treatment of non-union bone fractures in patients exposed to selected time-varying magnetic fields (Bassett *et al.*, 1974). On a cellular level the application of PEMFs has shown to cause modifications of cellular calcium and mineralization (Aaron *et al.*, 1989), synthesis of extracellular matrix (Murray et al., 1985) and new blood vessel formation (Yen-Patton *et al.*, 1988). One of the interesting characteristics of PEMFs has been the induction of cellular transcription, which relates directly to cellular regulation (Goodman *et al.*, 1983).

Many basic studies have been done to test whether EMF exposure promotes cancer. Research to date has shown that EMFs affect several different cellular functions that could be related to the promotion of carcinogenesis. For example, cells exposed to fields between 15 Hz and 4 kHz (2.3 mG to 5.6 mG)

(Liboff *et al.*, 1984) increased DNA synthesis from 30 to 70%. An increase in the growth-related enzyme, ornithine decarboxylase, was also observed (Byus *et al.*, 1988). Altered cell surface properties (Marron *et al.*, 1988), and effects on development (Delgado *et al.*, 1982) were some other events associated with EMF exposure.

A large body of research has been done on the effect of EMF on lymphocytes. Lymphocytes are an appropriate models for EMF study, since epidemiological data has indicated increases in various leukemias. Exposure to PEMFs (pulsed electromagnetic fields) triggered lymphocytes into the cell cycle, but was unable to further support the reactivation process (Grattorala *et al.*, 1985). These results suggested that EMFs exert a modulatory effect on lymphocyte reactivation. PEMFs have also been associated in the modulation of critical events such as Ca^{2+} influx and mobilization, surface receptors and redistribution of protein kinase C (PKC) activity in lymphocytes (Luben *et al.*, 1982;1994b).

Altered quantities of RNA after EMF exposure was first observed when increased transcription was seen in the salivary gland cells of dipterans (Goodman *et al.*, 1992a). The increase in steady state transcript levels was confirmed by Phillips *et al.* (1992) in CEM-CM3 human T-lymphoblastoid cells, where they showed that increased transcription was observed for genes encoding *c-myc*, *c-fos*, *c-jun* and PKC following EMF exposure. The increase in

c-*myc* was dependent on the time of exposure and on the strength of EMFs (Goodman *et al.*, 1992b). Cells exposed to EMFs retain transcript levels above control for up to 2 hours after EMFs were removed. This was observed when the cells were continuously exposed or exposed to EMFs for 20 minutes and then removed from EMFs (Goodman *et al.*, 1992b)

EMF and cellular mechanism(s). Membrane involvement was an early clue that signal transduction mechanisms could play a role in the response of the cell to EMFs (Adey 1994). Direct interaction of EMFs on the membrane was initially shown in studies conducted to investigate the mechanism of EMFs on bone healing. It had been hypothesized that EMF causes changes in bone activity by modulating the response of cells to growth factors, cell density nutrient levels and hormones, particularly the parathyroid hormone (PTH). PTH is involved in bone absorption and regulates bone growth. EMF exposure inhibits the cellular responses induced by this hormone (Raisz, 1988). Cain *et al.* (1987) demonstrated that the hormone receptor was the probable locus for this EMF effect. Associated studies showed that EMF changed the accessibility of some PTH receptor epitopes to monoclonal antibodies (Luben *et al.*, 1991). These studies suggested that EMFs induced a conformational change in the binding domains of the receptor that resulted in a decrease in signal transduction capacity (Luben and Cain, 1986). The transcript response of both c-*myc* and c-*fos* is dependent on the condition and presence of the plasma membrane. Addition of

colchicine, a microtubule disrupter completely abolished the response indicating that EMFs affect the cell at the membrane level (Broude *et al.*, 1994).

One of the most significant results of EMF research has been the demonstration of influx of Ca^{2+} into cells following EMF exposure. Direct measurements show an increase in intracellular calcium in cells stimulated to divide with concanavalin A (Con A) (Liburdy, 1994). The increase was also shown to correlate with an increase in steady state c-*myc* transcript levels. Other studies also showed that increased c-*myc* transcript levels were dependent on the presence of external Ca^{2+} (Karabakhtsian *et al.*, 1994).

Based on these studies, it has been hypothesized that EMFs alter the surface of the cell. It is thought that it is the change in surface charge that influences receptor binding activity either directly or indirectly through changes in the calcium flux patterns. Calcium ions are known to play an essential role in transmembrane signaling by activating enzyme cascades, thereby amplifying the triggering signal (Adey *et al.*, 1994). The proposed signaling cascade is utilized by several mitogens including 12-O-tetradecanoylphorbol13-acetate (TPA). TPA activates diacylglycerol (DAG) and protein kinase C (PKC). PKC is involved in calcium recycling and the activation of the oncogenes c-*myc*, c-*fos* and *c-jun*. Activation of PKC by EMF was reported by Luben (1994a), which substantiated the proposal that a signal transduction mechanism was utilized by the fields within the cells.

II. Rationale

One problem in EMF research has been that no proposed or viable mechanism for EMF interactions in the cell has been unequivocally proven. The lack of a plausible mechanism has created a barrier for acceptance of data that showed EMFs affected cellular functioning. A major problem associated with proposed mechanisms in EMF research is that the frequencies of the elf fields, unlike higher frequencies, do not break chemical bonds or cause tissue heating. In point, the energies associated with EMF are so small that they are dwarfed by the natural thermal noise of the tissue itself (voltage fluctuations resulting from normal cellular and molecular interactions) (Adey *et al.*, 1994). The signal transduction mechanism provides a non- linear mechanism that is able to explain the bioeffects seen in the cell by very weak elf EM fields through amplification. Several investigators have tried to answer the question of which potential mediators of the elf EMF interaction on the membrane exist, implicating change in charge, frequency dependent resonance and receptors on the membrane as possible trigger points of EMF (Carpenter *et al.*, 1994).

There are several means by which a relationship between signal transduction and EMF exposure could be deciphered. One is to attempt to trace a pathway from the cell surface into the cell. This is difficult, considering pathways are interconnected and that a given cell type has multiple overlapping pathways.

The second method is to determine if EMFs influence the regulation of the genes themselves and then trace the steps backwards from the gene. This study used the latter method by investigating regulation of the c-*fos* gene.

The basis for the present study was the observation that EMFs affected chloramphenicol acetyl transferase (CAT) expression in transfected cells containing constructs of c-*myc* promoter regions (Lin *et al.*, 1994). This was the first indication that EMF exposures resulted in increased steady state transcript levels by affecting regulation. To investigate if the increase in steady state transcript levels of c-*fos* gene after EMF exposure was due to the effect on regulation, studies were initiated using the c-*fos* promoter. The fos gene was deliberately chosen since it provides a system where the regulatory regions are well defined.

The *c-fos* gene plays a pivotal role in the cell's development and growth. It has also been shown that transcript levels of the gene are increased after short EMF exposures (Phillips *et al.*, 1992). C-*fos* is characterized as a "primary response gene" or early/immediate gene; it is one of the first genes to respond to external stimulation (Greenberg *et al.*, 1984). It is expressed rapidly in the cell, but the expression degenerates rapidly. As an example, increases in c-*fos* transcripts were observed after five minutes following stimulation by serum (Deschamps *et al.*, 1985). Maximum response to serum was reached in 30 minutes; *c-fos* transcripts reached basal levels within two hours following stimulation. The c-*fos*

gene response to external stimuli occurred in the presence of cycloheximide (Greenberg *et al.*, 1986), indicating that protein synthesis was not required for a response, a characteristic of a primary response gene.

The c-*fos* gene is also responsive to several stimuli such as TPA (Fisch *et al.*, 1987), insulin, platelet derived growth factor (PDGF), estrogen growth factor (EGF) and nerve growth factor (NGF) (Doucet *et al.,* 1990). C-*fos* regulatory regions have been extensively studied (Prywes *et al.*, 1988) and regions that respond to specific mitogens have been mapped (Figure 3). Several proteins down regulate the promoter, including retinoblastoma, retinoic acid and the phosphorylated fos protein (Ofir *et al.*, 1990; Busam *et al.*, 1992; Sassone-Corsi *et al.*, 1988). Autoregulation could explain the appearance and disappearance of the c-*fos* RNA within hours after cell activation.

The Fos protein contains 380 amino acids. The protein is phosphorylated at its serine residues (362 and 364, at the carboxyl terminal) and has a MW of 55-62 KD (the molecular weight depends on the number of residues phosphorylated). Fos protein is regulated by proteins in the PKA pathway (Curran *et al.*, 1988). Synthesis of the *c-fos* protein follows the mRNA profile; maximal levels are obtained at 1-2 hours post induction and decay occurs with a half life of approximately 2 hours (Curran *et al.*, 1987). Fos protein, in conjunction with Jun, gives rise to AP1 transcription binding complexes (Hirai *et al.*, 1994). These complexes in turn cause an increase in expression of other proteins such as the

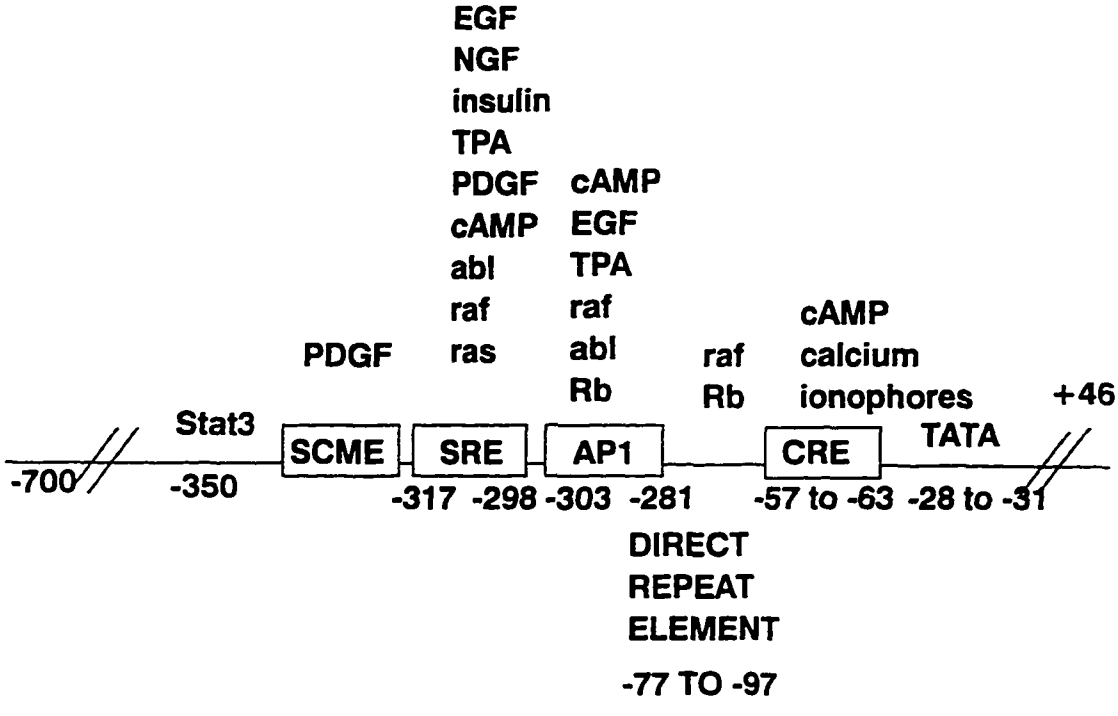


Figure 3. Schematic diagram of the *C-fos* promoter regions from -700 to+42.

The sequence elements involved in regulation; sis conditioned mediating element (SCME), serum response element (SRE), the AP1 binding site, C-AMP response elements (CRE) are listed. Factors that bind or affect these elements are listed above the appropriate region.

an increase in expression of other proteins such as the differentiation marker, intermediate filament protein TROMA-1 (Muller *et al.*, 1984). It has been hypothesized that the Fos-Jun products act as *third messengers* coupling a cell surface phenomenon to the regulation of genes, which is ultimately responsible for the cell's long term phenotypic response to stimulation. This cascade effect makes the Fos-Jun complexes extremely important for growth and cell proliferation. The present study focused on the effect of elf EMF on the regulation of the c-*fos* gene and the mechanism of interaction, with the ultimate goal to defining a pathway of interaction for EMFs within cells.

III. Research Findings

The investigation was divided into three interrelated studies. The first was to determine the effect of EMF exposure on the CAT driven expression by the c-*fos* upstream regulatory region. The second was to determine which region of the promoter was responsive to EMFs. The third was to determine if other mitogens acted in a synergistic fashion with EMF and thereby try and decipher some portion of the pathway by which EMFs interacted with the cells.

The first part of this study was based on the fact that c-*fos* steady state transcript levels increase following exposure to EMF (Phillips *et al.*, 1992). To determine if the response to EMFs was due to regulation at the gene level, c-*fos* upstream regulatory regions were studied. A construct containing the region from

-700 to +42, attached to the CAT gene, was transfected into HeLa cells. HeLa cells were exposed to an environmentally relevant EMF at 60 Hz (60 mG). The results showed that the upstream regulatory region was involved in the cell's response to EMFs, as measured by an increase in CAT activity. The response was substantiated by determining that a similar increase was observed in CAT mRNA. The response to the field was specific to the upstream regulatory region of the c-*fos* gene as a CAT construct with the herpes thymidine kinase promoter (with no enhancer elements) did not respond to EMFs.

The second part of the study determined which region of the upstream regulatory region was responsible for the cell's sensitivity to EMFs. Deletion analysis of the promoter revealed that CAT expression following EMF exposure requires the region from -363 to -289. This region, containing the SRE/AP1 site, is responsive to serum and the AP1 transcription factor. The region of the promoter that is responsive to EMF exposure is also responsive to other mitogens, such as TPA (Fisch *et al.*, 1987).

The third part of this investigation focused on determining if TPA acted synergistically with EMFs. TPA activates the enzyme PKC, which serves as its receptor. Other studies have demonstrated that EMFs and TPA could function in a similar manner (see Discussion). Transfected cells were exposed to EMF and TPA. Two different experimental tactics were used. First, the cells were treated with TPA followed by EMF exposure. In the next approach, the cells were treated

with EMF followed by TPA. Both experimental approaches showed that the response of cells to a combination of treatments was not additive. This suggested that TPA and EMF do not act synergistically.

PKC activation by TPA transiently modulates mRNA for two of the regulatory subunits of PKA. PKA mediates most c-AMP actions by phosphorylation (Tasken *et al.*, 1992). To study the role of PKA and PKC on the EMF response, transfected cells were treated with chelerythrine and H-89 inhibitors, of PKC and PKA, respectively. The experiments showed that the response to EMF exposure (relative to CAT expression) was negated by the presence of chelerythrine or H-89. This indicated that EMFs stimulation, like TPA induction, required both PKC and PKA enzymes to elicit a response. An indirect approach was also used to determine if correlated cellular pathways were involved. In these studies, we determined whether EMFs caused post-translational modifications of the c-*fos* protein as seen by other mitogens, including TPA. Immunoprecipitation of labeled Fos protein, was followed over a time course, demonstrated that exposure to EMFs increased the uptake of ^{32} P by the Fos protein.

The results of this study confirm the hypothesis that EMF exposures can affect cells using a signal transduction pathway, and support proposed mechanisms which invoke signal transduction pathways as a means for amplification of EMF produced signals at the cell surface.

MATERIALS AND METHODS:

Abbreviations:

ATCC (American Type Culture Collection)

BMB (Boehringer Mannheim Biochemicals)

DDT (Dithiothreitol)

DMEM (Dulbecco Modified Eagle Media)

DMSO (DiMethyl Sulfoxide)

EDTA (EthyleneDiamine TetraAcidic disodium salt)

MOPS (Morpholinopropanesulfonic acid)

OPNG (o-nitrophenyl-b-D -galactopyranosidase)

PBS (phosphate buffer saline)

PMSF (Phenyl Methyl Sulfonyl Floride)

SDS (Sodium dodecyl sulfate)

TAE (Tris/ Sodium Acetate/ EDTA)

TBE (Tris/ Boric acid/ EDTA)

TBS (Tris Buffered Saline)

TE (Tris/EDTA)

TEMED (NNNN-tetramethylethylenediamine)

TMPD(2,6,10,14-tetramethylpentadecane)

TPA (Tumor promoting agent) 12-O-tetradecanoylphorbol-13-acetate

UV (Ultraviolet)

Reagents and Solutions:

LB medium: 1.0% Bacto-tryptone (Difco), 0.5% bacto-yeast extract (Difco), 100mM NaCl, adjusted to pH 7.5 with NaOH.

LB plates: LB medium with 1.5% bactoagar (Difco).

DMEM medium: *Dulbecco Modified Eagle Medium* [containing glucose (4500mg/L); L-glutamine (584mg/L); sodium pyruvate (110mg/L)].

RPMI 1640 medium: RPMI-1640 medium containing L-glutamine.

MOPS(1X): 40mM morpholino propanesulfonic acid; 10mM sodium acetate; 1mM EDTA (pH 8.0).

PBS: (1X): 150mM NaCl; 150mM Na phosphate pH 7.2.

SDS (20%): 20 gms of SDS in 100ml of distilled H_2O.

SSC (20X): 3M sodium chloride; 0.3M sodium citrate in 1000ml of H_2O.

RNA lysis buffer: 0.8ml of 0.1M Tris (pH 9.0); 0.1M NaCl; 20mM EDTA; 0.1% sarkosyl.

Sevag's solution: 24:1 chloroform:isoamyl alcohol.

TAE buffer (10X): 400mM Tris; 200mM sodium acetate; 1mM EDTA, pH 7.2.

TBE (10X): 0.89M Tris; 0.89 boric acid; 0.1mM EDTA pH 8.0.

TBS: 8 gms of sodium chloride, 0.2 gms of potassium chloride in 800 ml of d. H_2O; 0.015 g of phenol red, adjusted to pH 7.4.

TE buffer: 10mM Tris, pH 8; 1mM EDTA, pH 8.

Bromophenol blue: 40% Sucrose in d. H_2O; 0.255 bromophenol blue.

1X trypsin-EDTA: 0.25%trypsin; 1mM EDTA (Gibco).

RIPA buffer: 10mM Tris, pH 7.5; 150mM NaCl; 1% Nonidet P-40; deoxycholate; 1% Aprotinin.

DTT (1M): 3.09 gms in 20mls of sodium acetate, pH 5.2.

Lysis buffer for proteins: 10mM Tris pH 7.5; 0.05M NaCl; 1% deoxycholate; 1% Nonidet-P40; 1mM Aprotinin; 1mM fresh PMSF.

SDS gel Loading Buffer: 50mM Tris-Cl ph 6.5 ; 100mM dithiothretiol; 2% SDS; 0.1% bromophenol blue; 10% glycerol

EDTA: 18.6 gms EDTA in 100ml H_2O, pH 8.0

Gas mixture: 5.0% CO_2, 20% O_2, 75%N_2, (T.W.Smith)

CAT buffer: 40mM Tris-Cl, pH 7.4; 150mM NaCl; 1mM EDTA

100X Mg solution: 0.1$MgCl_2$; 4.5 M β- D- mercaptoethanol

P1: Suspension Buffer: 50mM Tris-HCl, pH 8.0; 10mM EDTA and 100 µg of RNAse A

QF: Elution Buffer: 1.25M NaCl, 50mM Tris -HCL, pH 8.5, 15% ethanol

P2: Lysis Buffer: 200mM NaOH, 1%SDS

P3 : Neutralization Buffer: 3.0M potassium acetate, pH 5.5

QBT: Equilibration Buffer: 750mM NaCl, 50mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100

QC: Wash Buffer; 1.0M NaCl; 50mM MOPS, pH 7.0;15% ethanol

I. Cell Lines: Tissue Culture

A. Cell line. HeLa cells, a fibroblast cell line derived from squamous carcinoma cells, were obtained from ATCC. HeLa cells have several distinguishing morphological traits, including a tetraploid karyotype, which is consistent with its tumor morphology. HeLa cells show an unusual pleomorphism and are irregular, indicating that they are a group of immature cells. They also have an unusual nucleus with a high nuclear over cytoplasm ratio. HeLa cells were used in transfection studies as they readily assimilate foreign DNA and give a high transfection efficiency.

B. Tissue culture. HeLa cells were grown in DMEM media which was supplemented with 10% serum (obtained from SIGMA; this is purchased from a yearly stock which insures that the conditions used in the experiments are comparable) and 1% antibiotic (antimycotic antibiotic, Gibco-BRL). The cells were grown in 5% CO_2 environment. The HeLa cells were split and fed after 48 hours and were kept in a semi-confluent state. Since HeLa cells are fibroblasts, they were removed from the surface by using trypsin-EDTA (Gibco BRL).

II. EMF Exposure Conditions

a. Composition of electromagnetic fields. HeLa cells were exposed to EMFs at 60 Hz [60 mG (rms)]. The calculated corresponding induced electric field was 11 μV/meter (Bassen *et al.*, 1992). Control and experimental cells were subjected to the same environment within a double door incubator In all

experiments. Cells for control and experimental determinations were derived from the same "parent flask" (T-75) to ensure that the transfection efficiency was the same for both unexposed and exposed cells. The flasks (T-25) were exposed simultaneously to either no field or EMFs at a concentration of 6-8 x 10^5 cells. The magnetic fields in the growth incubator have been measured over a period of 5 years at 2 mG.

Early experiments, which include lag time determinations required to maximize protein synthesis, experiments involving the time dependent response of the promoter region -700 to +42, and portions of the deletion analysis, used a continuous sinusoidal 60 Hz field generated by a pair of Helmholtz coils designed by Electro Biology Inc. (EBI), Parsippany, NJ (Goodman *et al.*, 1992). All samples were coded using an alphanumeric code. All other experiments, including TPA induction, inhibition and phosphorylation used a double blind Helmholtz Coil Exposure System designed by Electric Research and Management, Inc (ERM) (these coils are double wound). 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.

B. Conditions of EMF exposures. The sinusoidal field was generated by a Wavetek function generator (Wavetek model 21) connected to a power regulator (EBI). The exposure system used a pair of 13 x 14 cm Helmholtz -aiding

coils with 8 cm spacing. The coils were constructed of 164 turns of 19 gauge copper wire around a 13X14 cm plexiglass form (EBI or ERM) (Figure 4a).

During exposures, the coil was positioned so that the magnetic field was horizontal. The induced electric field is dependent on the depth of the medium, which in these experiments was 0.6 cm. T-25 flasks, containing the cells, were placed horizontally on a plexiglass stand in an area of the coil shown to have a uniform magnetic field. During exposures, the Helmholtz coils were shielded in a mu metal container positioned within the 37.5^{o}C incubator (Figure 4b). The function generator and the power regulator remained outside the incubator. Measurements of the signal parameters were made with a calibrated coil probe (EBI). Temperatures in the medium were monitored using a fiber-optic probe that detected changes of <0.1^{o}C (Physitemp). The magnetic field was monitored prior to and after experiments using a calibrated coil probe (Monitor Industries). Control cells were in a different compartment of the same incubator as the experimental cell to minimize thermal differences that could arise by using separate incubators.

III. Transformation and Transfection Experiments

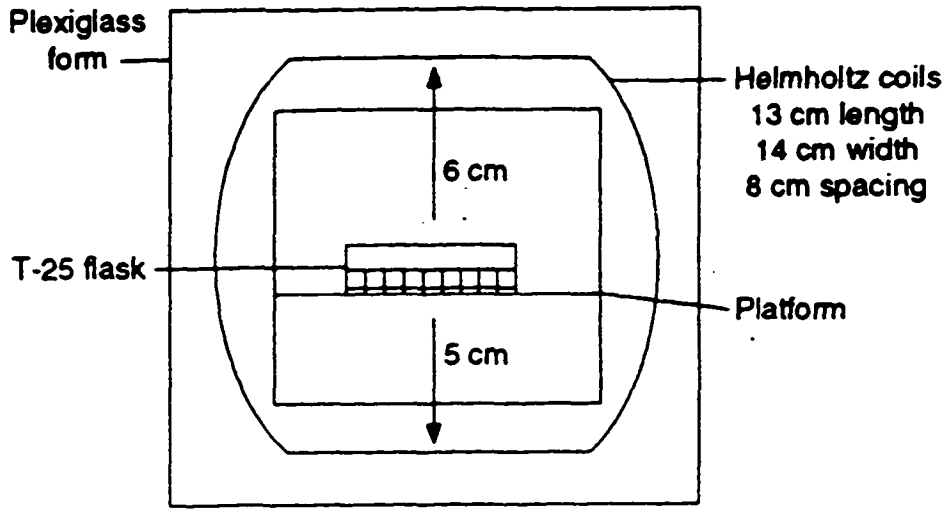
a. Constructs of the c-*fos* upstream regulatory region. The plasmid, pFC -700, is a PUC19 plasmid containing the upstream regulatory region of the c-*fos* promoter attached to the CAT gene. The plasmid was obtained from Dr. David Foster, Hunter College. The identity of the plasmid was verified by constructing a restriction enzyme map. All constructs were exposed to same

Figure 4. The EMF exposure system.

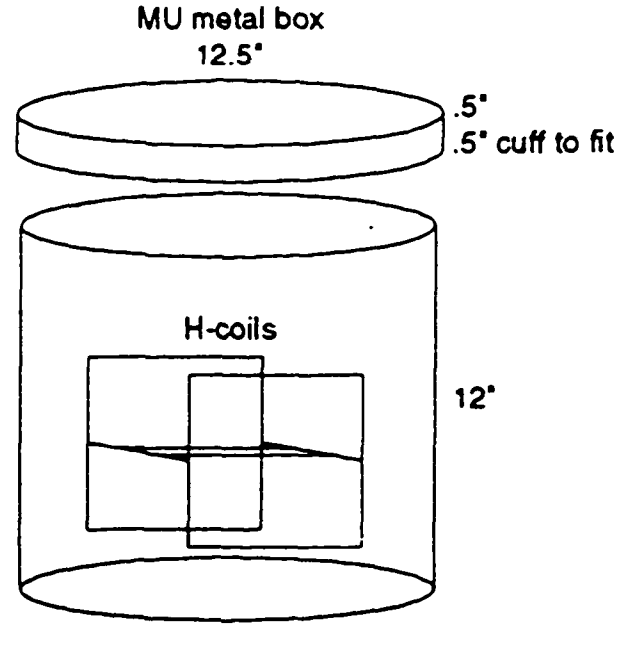
a. Helmholtz coil configuration: The Helmholtz-aiding coils (13x14cm and 8 cm spacing) are composed of 19G wire bundles wound 164 times around a plexiglass form. T-25 flasks containing cells are placed horizontally on a plexiglass stand. The portion of the flask containing the cells is a 5x6 cm square. The bottom of the flask is 2 cm below the axis level. The height from the flask bottom to the top surface of liquid is about 1.1cm. The height of the liquid is 0.6 cm.

B. Helmholtz coils are shielded in a mu metal container within a 37.5 ^{o}C incubator. Mu metal shielding is used as precautionary measure to ensure that magnetic field fluctuations in the laboratory or building do not affect experimental conditions. The container also prevents other cells in the incubator from possible exposure.

A



B



scrutiny. The plasmids pFC. 700/225 (bp -700 to -225), pFC.-363 (bp -363 to +42), pFC. -225 and pFC. -99 (bp -225+42 and -99 to +42, respectively) were also cloned into a PUC 19 plasmid. The constructs were obtained from Dr. R. Prywes at Columbia University. The constructs p-350ttk, p- 250ttk and ptk-CAT were cloned into PUC18. The constructs p- 350 and p- 250 had c-*fos* promoter regions from bp -700 to -350 and -350 to -225, respectively, attached to the herpes thymidine kinase promoter. The ttk -CAT construct contained the thymidine kinase promoter attached to the CAT gene. This construct was used to ensure that the response of the other two constructs resulted from the c-*fos* promoter. These three constructs were obtained from Dr. Harel Bellan (Institue Gustave Roussy, Villejuif, France).

B. Transformation experiments. The plasmids were transformed into the HB101 strain of *Escherichia coli*, followed by a large plasmid preparation and the introduction of the plasmid into mammalian cells.

1. Preparation of competent cells. HB101 cells were grown in 50ml LB broth for 24 hrs at 37°C. 50ml of LB media was inoculated from the overnight culture and grown for 2-4 hrs at 37°C. Three mls of culture were placed on ice for 10 mins and the cells were collected by centrifugation at 4,000g for 5 minutes at 4°C. The cells were suspended in 1.5ml of cold sterile solution of 50mM $CaCl_2$, 10mM Tris -HCL, pH 8.0 and incubated in an ice bath for 15 minutes. Cells were collected by centrifugation at 4°C and suspended in 0.2mls of cold sterile solution

of 50mM $CaCl_2$, 10mM Tris-HCL (pH 8. 0). The cells were placed at 4°C for 24 hrs and then stored at -70 °C.

2.Transformation of bacterial cells. Competent cells were kept at -70 °C and placed on ice before transformation. For each transformation, 0.2mls of competent cells were mixed with 1 µg/µl of plasmid and placed on ice for 30 minutes. The samples were heat shocked by placing them in a water bath at 42°C for 2 minutes. This was followed by the addition of 0.8 ml of LB broth containing 60 µg/ml of ampicillin The cells were incubated for 2 hrs at 37°C with shaking, and then plated on agar plate containing ampicillin and another plate that did not contain the antibiotic (used as a control). The plates were incubated at 37°C overnight.

3. Large Scale plasmid DNA isolation. The method for large scale plasmid extraction is described as "maxi prep" in Qiagen Plasmid Handbook, Spring 1994 (Qiagen). The pelleted bacterial cells were dissolved in buffer P1 or suspension buffer. 10mls of the buffer P2 or lysis buffer was added to the above suspension, which was then mixed gently and placed at room temperature for 5 minutes. After 5 minutes, 10mls of buffer P3 (neutralization buffer) was added to the suspension. The cell suspension was kept on ice for a additional 20 minutes before it was centrifuged at 27,000g for 30 minutes at 4°C. The supernatant was loaded onto a Qiagen column which had been equilibrated by buffer QBT(equilibration buffer). After loading the supernatant, the column was washed

twice with buffer QC (Wash Buffer) to remove any impurities. The plasmid was eluted from the column by the addition of buffer QF (elution buffer). The plasmid solution was precipitated by the addition of 10.5 isopropanol and centrifuged at 27,000g for 30 minutes at 4°C. The pellet was dissolved in TE buffer. Concentration and purity were determined in a manner similar to that used for DNA.

C. Transfection into mammalian cells. Transfections were performed using the lipofectin method as described in Ausubel *et al.* (1994). HeLa cells were at 60-70 % confluency for transfections. 10 µg of the plasmid DNA was dissolved in 100µl Opti-Mem (Gibco BRL). 50µl of lipofectin reagent (Gibco -BRL) was dissolved in a tube containing 100µl of the Opti-Mem. The two tubes were mixed together and maintained at room temperature for 15 minutes. The mixture was suspended in 10 ml of the Opti-Mem and added to the flasks containing washed HeLa cells. Cells incubated in this media for 12-16 hrs and then media was changed to normal DMEM media contain 10% serum. Protein was extracted from the cells and tested for the CAT activity after 48 hrs.

IV. Protein Extraction and Estimation

a. Protein extraction. The "freeze thaw" method was employed for the extraction of proteins from transiently transfected cells. The method is described in Ausubel *et al.* (1994). The cells were washed once in 1X PBS followed by a wash in CAT buffer. The cells were then dissolved in 400 to 500µl of 0.25M Tris-

HCL (ph 7.8). They were then placed at 37°C for 10 minutes followed by incubation at 4°C for 5 minutes. This procedure was repeated four times. The samples were centrifuged at 14,000g for 10 minutes. The supernatant was collected and stored at -70°C.

B. Protein quantitation. Determination of the amount of protein was performed using a Kit and procedure recommended by the manufacturer Bio-Rad or the micro-titer protocol. Each sample was measured in triplicate. The protein standard was gamma globulin. Spectrophotometric readings were done at wavelengths of 590nm.

V. CAT assays

a. Chromatographic method. The CAT assays were performed using two different methods. The first one was the "chromatographic method." The method is described in Ausubel *et al.* (1994). 50 µg of protein taken from each sample was used. 50µl of 1M Tris-Cl pH 7.8, 20µl of 4mM Acetyl CoA and 0.5µl of C^{14} labeled chloramphenicol (0.025mCi, Dupont NEN) was added. The total volume was adjusted to 100µl by adding distilled water. The samples were placed in a 37°C water bath for 1 hr, after which, 1ml 100% ethyl acetate was added to the samples. The samples were vortexed and centrifuged for 30 seconds. The supernatant was removed and speed vacuumed for 45 minutes. 30µl of ethyl acetate was added to the dry tube to redissolve the chloramphenicol. The ethyl acetate was spotted on a baked TLC plate (silica plate from Baker). The plate was

placed in a chamber containing a solution of chloroform/ methanol (95:5) for 2 hrs. The plate was allowed to dry and exposed to a X-ray film overnight.

Relative CAT activity was determined by the ratio of acetylated chloramphenicol to total chloramphenicol. The acetylated and non- acetylated spots were cut from the plates and counted by liquid scintillation.

B. Liquid phase method. Another method used to determine CAT activity was the liquid phase method. The advantage of this method was its extreme rapidity and the capacity for analysis of multiple samples at the same time. Similar concentrations of protein were used as those given above. 5µl of 1M Tris HCl at pH 8.5µl of butyryl CoA and 0.5µl of C^{14} labeled chloramphenicol was added to this solution and the total volume was adjusted with distilled water to 50µl. Each set of experiments in this method had a negative control or blank (contained no protein), to determine background values. The samples were kept at 37°C for 1 hour. Following this incubation, the acetylated product was extracted by the addition of 400µl of TMPD/Xylene (2:1). Samples were vortexed vigorously and centrifuged for 30 seconds, the organic phase removed, dissolved in 10mls of Ecoscint, and counted in the scintillation counter. CAT activity values from the two methods were not different.

VI. Transfection Efficiency

Transfected cells were combined and divided into different flasks. This ensured that the transfection efficiency was the same for all samples to be tested.

Transfection efficiency was also determined in some experiments by using the reporter gene, β-galactosidase.

a. β-Galactosidase assay. The β-galactosidase assay, was performed as given in Maniatis *et al.* (1989). Each assay had a negative control containing no protein. Each sample had the following mix of reagents 3µl of 100x Mg solution, 66µl of 1x ONPG, 200µl of 0.1M sodium phosphate pH 7.5 and 30µl of the protein. After 1 hour incubation, the samples were read in the spectrophotometer at 420nm (the linear range is 0.2-0.8 OD). The total light emission is proportional to the β-galactosidase activity of the sample, which in turn provides an indirect estimate of the transcription of the β-galactosidase gene. Transcription efficiency was directly related to β-galactosidase activity, which was between 30 to 45% on a per cell basis.

VII. Nucleic Acid Isolation

A. RNA extraction. RNA extraction used a modified version of the method given in Maniatis *et al.* (1989). The cells were lysed with 500 to 1000 µl of RNA lysis buffer and passed through a 1cc/#21G needle syringe for complete lysis. The cell lysate was extracted three times with an equal volume of saturated phenol: chloroform: isoamyl alcohol (freshly prepared), and once with chloroform: isoamyl alcohol. Ice cold 100mM LiCl in 95% ethanol was added to the aqueous phase to precipitate the nucleic acids. The samples were stored at -70°C for 30 minutes and then centrifuged at 10,000 g at 4°C for 30 minutes. The pellet was

air dried for 3 to 5 minutes. The dried pellet is dissolved gently in 600 µl of Tris -Cl buffer at pH 8.0. 6 µl of 1M MgCl (to 10mM) was added to the solution, followed by 10 µl of DNase I (1 µg/ml). The solution was placed on ice for 60 minutes (with gentle mixing every 10 to 15 minutes). After DNase digestion, 12 µl of 0.5M EDTA and 60 µl of 3M NaAc was added to stop the reaction. After mixing, the RNA was extracted twice with phenol-chloroform and chloroform: isoamyl alcohol. The aqueous phase was then precipitated in 95% ethanol and kept overnight at -70°C. The RNA was collected by centrifugation at 10,000 rpm for 30 minutes at 4° C. The pellet was dissolved in 50 µl of TE buffer.

RNA was quantitated by spectrophotometric readings. The concentration was determined using the standard, OD_{260} of 1= 40 µg/ml RNA. The OD ratio of OD_{260} and OD_{280} was determined. The ratio helped to determine the protein contamination of the sample. Samples with an OD ratio of 1.8 to 2 were used. Fidelity of ribosomal RNA was determined by agarose gel electrophoresis where the 28S, 18S and 5S units were visualized by ethidium bromide. The RNA samples were stored in TE buffer at -70°C.

B. Restriction endonuclease digestion. Plasmid DNA was digested overnight in the presence of buffer as recommended by BMB (a 1X buffer concentration was used). The time (16-20 hrs) was longer than the time suggested by the company to ensure complete digestion. Reactions involving more then one enzyme preceded with the enzyme requiring the lower salt

concentration, followed by adjusting the salt concentration to medium and high value and the addition of the second enzyme.

VIII. Agarose Gel Electrophoresis

A. DNA electrophoresis. Agarose gels were prepared by dissolving agarose into 1X electrophoresis buffer, TAE, and heating in a microwave until the agarose dissolved. The agarose solution was poured into a horizontal plate and then a well forming comb was placed gently into the tray.

DNA (1 µg/µl) was heated for 10 minutes in a 68°C bath and quick chilled on ice for 5 minutes before loading onto the gel. This process ensured the elimination of DNA secondary structures. The voltage and time were adjusted on the basis of the size of the DNA fragment being studied. Gels with small DNA fragments less then 1Kb were run at 12-15 V/cm (volts per centimeter), whereas gels used for larger DNA separation were run at 2-3 V/cm. The gels were run until the bromophenol blue had migrated 3/4 of the gel.

Staining of the gel was performed in two ways. The first was the addition of 30 µl of the ethidium bromide directly into the agarose solution. This method stained the nucleic acid directly making visualization simple. The second method was to place the gel was placed in a 0.5 µg/ml solution of ethidium bromide for 10 to 15 minutes, followed by a wash with distilled water for 20 minutes. We found that this method gave superior results and hence was used more often. The gels were visualized on a long wave UV transilluminator (Spectroline) and

photographed using a Konica instant camera with Polaroid type 667 Land film. The size of DNA fragment were determined by DNA ladders (Gibco -BRL).

B. RNA gel electrophoresis. RNA gels were visualized in a similar manner to that used for of DNA gels. RNase free agarose (2%) was dissolved in distilled water. The volume of the distilled water and weight of the agarose was dependent on the volumes of the samples being loaded into the gel (the agarose was dissolved in a microwave oven). The gel was allowed to cool to 60°C and then 1X MOPS and 37% formaldehyde was added to the gel.

Samples were prepared in a sample buffer with a final concentration of 50% formamide, 6.48% formaldehyde and 1X MOPS buffer. Samples were heated in a water bath at 55°C for 15 minutes, to ensure that any secondary RNA structures were eliminated. RNA (15-20 µg per well) was loaded into the gel following the addition of the loading buffer and the gel was run at a voltage of 3.5 V/cm for 3-5 hours or until the dye has migrated 3/4 of the gel distance. RNA was visualized by staining with ethidium bromide (0.5 µg/ml) for 15 minutes followed by washing with distilled water for 20 minutes.

IX. Nucleic Acid Hybridizations

A. Northern blots. RNA formaldehyde gels were transferred to a nylon membrane following the procedure described in Ausubel *et al.*, 1994. The gel was run with a marker (Gibco BRL) which was stained and photographed. Transfer was allowed to occur for at least 16 -24 hours. The time of transfer depended on

the molecular size of the RNA to be studied. After transfer, the membrane was marked to indicate the side containing RNA and then the membrane was washed twice in 2xSSC. The membrane was dried for 10 minutes at room temperature followed by 5 minutes at 80°C. UV light cross linking was for 5 minutes. The membrane was placed in a heat sealed plastic bag for 1 hour in prehybridization solution at 40 to 42°C. The prehybridization solution contains 50% formamide, 1% dextran sulphate, 1% SDS and 1M NaCl. Single-stranded salmon DNA, which had been heated at 100°C for 10 minutes (to ensure that the DNA is single stranded) and placed on ice for 5 minutes, was added to the mixture in the concentration of 1000 µg/ml. After an hour, the prehybridization solution was removed and a new prehybridization solution (lacking the salmon DNA) was added along with the radioactive probe. The bag was resealed and placed in a shaking water bath (40 -42°C) for 12 -16 hrs. The probe was heated at 100 °C for 10 minutes and then 5 minutes on ice before being added to the hybridization solution. Following hybridization, the membrane was washed twice in 2x SSC/0.1% SDS at room temperature. This was followed by two washes in 0.2x SSC /0.1%SDS. If there was a high background (checked by the Geiger counter and by using the phosphoimager) additional washes in the above solutions were performed at 42°C. The membrane was then dried and exposed to a X-ray film and/or the cassette for phosphoimaging (PDSI ImageQuant™ Molecular Dynamics).

X. Radioactive Labeling of Probes

A. Random primer method. The random primer extension method was used (Feinberg and Vogelstein, 1984). Labeling with ^{32}PαCTP (Dupont NEN) was performed with a kit (Amersham cat. #RPN1600Y). 25-40 ng of CAT RNA (Ambion) was used for labeling and the reaction was allowed to proceed overnight at room temperature for the best results. Unlabeled radionucleotide was removed by passing the probe through a Quick Spin G-50 Sephadex column (Gibco-BRL). The incorporation of the probe was determined by placing 1µl of the reaction mixture in 10 ml Ecoscint. This ^{32}P incorporation was measured by scintillation countering. The specific activity of the probe was calculated by multiplying the number of counts by the total volume of the probe, divided by the number of micrograms (SA = cpm/µg).

XI. "In-vivo" labeling of Hela cells

The labeling of cells was as described in Tratner *et al.*, (1990).

a. Dialyzed serum. Cellophane tubing was heated for 10 minutes in 100°C water bath that contained a trace of EDTA to inhibit nuclease activity . The serum was put in the bag, secured and placed in distilled water with stirring. After an hour, the water was removed and fresh water was added. Dialysis was for 17 hours.

B. Labeling cells and extraction of proteins for Immunoprecipitation.

Cells at a concentration of 0.3-0.4 x 10^6 cells/ml were aliquoted into different flasks. After 24 hours, the cells, which were quiescent, were grown in DMEM which contains 10% dialyzed serum. After an hour, the cells were washed in 1X TBS (Tris buffered saline) buffer. New media (DMEM with 10% dialyzed serum), along with ^{32} P (orthophosphate, 2 mCi, Dupont NEN), was added to the cells. Cells were kept in this media for 3 hours before being exposed to an EMF for 20 minutes. Following EMF exposure, protein was extracted after different incubation times (0, 20, 40, 60 and 120 minutes). The cells were washed once in 1 X PBS, followed by 2 washings in 1x TBS. The cells were then suspended in 400 µl of lysis buffer for proteins, lysed with a 1cc syringe (27G needle), and 50 µl of Pansorbin (Calbiochem) was added. Samples were placed on ice for 30 minutes, followed by centrifugation for 60 minutes at 14,000 rpm and 4°C. The supernatant was collected and protein estimation was performed as described above except the Bio-Rad DC (detergent compatible) kit was used, since the lysis buffer contained the detergent, P Nonidet. Assays were performed using the microtiter procedure. Gamma globulin was dissolved in lysis buffer and used as the standard.

C. Immunoprecipitation. 2 mg of protein was incubated with 5 µg of c-*fos* polyclonal antibody (Santa Cruz Laboratories) for 12-16 hrs at 4°C. Samples were then mixed with 50 µl of Pansorbin and kept on ice for 60 minutes. The antibody -fos protein -pansorbin conjugate was precipitated following centrifugation at

27,000g for 15 minutes at 4°C.

The pellet was washed three times with RIPA buffer The samples were suspended in loading buffer, and heated for 5 minutes at 100°C. The samples were loaded on to a SDS gel.

D. Polyacrylamide gels. Polyacrylamide-SDS gels were prepared as described in Maniatis *et al.* (1989). The gel was run at 100 V for 2-3 hours, fixed by washing in acetic acid: methanol: water solution (10:20:70) for 30 minutes, and dried in a gel dryer (80°C for 2 hrs under a vacuum). The dried gel was exposed to X-ray film (Kodak) and processed after 24 hrs. The densitometer (PSI ImageQuant ™ Molecular Dynamics) was used to determine the relative quantity of modified c-*fos* protein.

XII. TPA Induction and Inhibition Studies

TPA, dissolved in DMSO (1.6μM dissolved in 0.01% of DMSO), was introduced into flasks containing transfected cells at the concentration of 162nM (Trouche *et al.*, 1990; Stumpo et al.,1988). The corresponding control flasks had a similar volume of DMSO (10μl of 0.01% DMSO). This ensured that any effect was that induced by TPA. Similarly, in inhibition studies, the cells were preincubated with the inhibitor or in the case of the control cells, with just the solvent (chelerythrine, a PKC inhibitor was dissolved in water; H-89 was dissolved in DMSO).

XIII. Statistical Analysis

Data was analyzed statistically by using the Microsoft Excel Description Analysis program. Means were calculated by using the standard formula; Mean= $\mu = \sum x/N$, were $\sum X$ is the sum of sample values and N is the number of samples. The standard error of the means (SE) was obtained by dividing the standard deviation of the population by the square root of the number of items in the population($SE= \sigma/\sqrt{n}$ were σ is the standard deviation of the population and n is the number of items in the sample). The SE value was used to determine the reliability of the sample mean in estimating the true mean (Bahn, 1972).

The student t- test assumes that the means of both data sets are equal and is referred to as a homoscedastic t- test. The t- test was used to determine if the differences between the two samples (the population exposed to the fields and those not exposed) was significant. The test is based on the Null Hypothesis which assumes that no difference exists between the two samples. If the probability of a outcome or the p values obtained is greater then 0.05, then, by convention, the hypothesis is true. The t-test is calculated from $t=\bar{x}-\mu/\sigma/\sqrt{n}$, where $\bar{x}$ is the mean for one of the data sets and μ is the mean from the other data set, σ is the sample variance and n the sample size (Microsoft Excel Users Guide 2, 1992).

RESULTS

Overview. The aim of this study was to determine if EMF exposure affected c-*fos* regulation and test whether a signal transduction process could be linked to EMF exposure. Inherent in the research design was to develop sensitive systems of measurement.

The results are discussed below in three sections. The first part of this study examined whether the increase in steady state transcripts previously observed for c-*fos* were due to an effect of EMF on gene regulation. The second part links a specific regulatory region within the fos promoter to the effects observed following EMF exposure. The identification of a specific region that could drive CAT expression provided a link to signal amplification pathways used by other mitogens. The third section tested proposed pathways that could be involved in the EMF response.

Analysis of transfected cells which contained bases -700 to +42 of the upstream regulatory region of the c-*fos* promoter showed that CAT expression, driven by the c-*fos* promoter, was responsive to EMF. Analysis of the CAT expression driven by different portions of the promoter complex to EMF pinpointed the SRE/AP1 region as necessary to realize an effect of EMF exposure. The SRE/ API site is sensitive to mitogens, including TPA. To confirm that pathways used in TPA induction could be also used by EMF signals, direct and indirect approaches were taken. Direct studies involved a comparison of the

effects of TPA and EMF. The results showed that the response of the cells following exposure to TPA or EMF was similar, but they were not synergistic. This suggested the possibility that they used the same or overlapping pathways. The use of PKC and PKA inhibitors demonstrated that both enzymes were needed for the increase in CAT expression driven by the c-*fos* promoter in the presence of EMF. The application of EMF increased the ^{32} P uptake by the c-*fos* protein, in a manner similar to other mitogens, including TPA. These results demonstrated that EMF exposure affected c-*fos* gene regulation and that the mechanism by which it did so was similar to that of TPA.

PART ONE: EMF EXPOSURE AND THE REGULATION OF THE c-*Fos* GENE

The experimental goals were two-fold. Initial experiments set control conditions. Subsequent experiments dealt with the kinetics of CAT expression driven by the c-*fos* promoter following EMF exposure.

Setting Conditions. CAT assays. HeLa cells were analyzed for possible contaminating background expression that would affect the measurement of CAT activity. This control ruled out the possibility that any other enzyme expression would interfere with the CAT activity measurements. Protein was extracted from HeLa cells which were "mock" transfected. The results showed that there was no endogenous expression of enzymatic activity that would interfere with the reaction (Table 1a). The level of CAT activity in HeLa cells was compared to the activity

of the blank, a sample which contained the assay reagents without the protein. As shown in Table 1a, expression of mock transfected cells was the same as that of the control, *i.e.*, HeLa cells do not show a basal expression for CAT or any other activity that could complicate the analysis of CAT activity. Cells transfected with a construct containing the CAT gene, but without a promoter region, expressed CAT activity similar to that of the blank (containing no protein). A summary of the measurements of CAT activity is given in Table 1b.

Setting Conditions; Positive control for CAT expression. TPA induction was used as the positive control since it induces a measurable response in CAT expression driven by the c-*fos* promoter. The level of increase seen in CAT expression driven by the c-*fos* promoter to TPA was lower then the expression of c-fos mRNA to TPA even though both had the same promoter (Fisch *et al.*, 1987). The authors based this on the relative stability (as compared to the c-*fos* mRNA) of the CAT mRNA, such that natural levels of CAT mRNA are measurable in uninduced cells, making any increases after induction difficult to detect. *C-fos* mRNA, on the other hand has a very low basal expression in the cell. Induction by TPA increases the steady state levels of *c-fos*, which is easily measurable when compared to the uninduced cells. Other research which used CAT protein activity as a marker showed an increase following exposure to TPA after 4 to 6 hours (Trouche *et al.*, 1990). TPA concentration of 162nM was used, based on

Table I

Analysis of CAT activity in non transfected HeLa cells

A. Background CAT expression in HeLa cells.

	Total counts (CAT activity)		
	Blank	Samples without plasmid	Samples with plasmid
Exp 1	158	159	35980
Exp 2	158	170	40933

B. CAT expression without the promoter.

	Total counts (CAT activity)		
	Blank	Samples Plasmid with CAT only	Samples Plasmid with Promoter + CAT
Exp 1	148	150	41534
Exp 2	145	146	30703

assays and previous studies (Stumpo et al., 1988).

CAT expression following EMF exposure. HeLa cells were transiently transfected with the construct pFC 700. Following transfection, the cells were divided into control and exposure flasks to ensure that the transfection efficiency was the same for both determinations. "Control cells" were defined as those cells not exposed to EMF, "exposed cells" were defined as those exposed to 60 Hz EMF (60 mG) for 20 minutes. This time corresponded to the time when c-*fos* mRNA showed maximum expression after EMF exposure (experiments by R. Karbakistian). Protein was extracted after different time periods following exposure to EMF (0, 20, 40 and 60 minutes and 120 minutes) by the freeze- thaw method. This method ensured that the CAT enzyme would be stable for a longer time (detergent lysis since detergent decreases the stability of the CAT enzyme). The Sigma computer program (4.1) was used to construct standard plots (Figure 5). The results of the chromatographic CAT assay are given in Figure 6a. The amount of acetylated and nonacetylated chloramphenicol was determined by both scintillation counting and. The ratio of the acetylated form to total chloramphenicol was used to calculate the relative % activity of the CAT enzyme. A measurable CAT response was seen after 60 minutes of EMF exposure (Figure 6b). In subsequent experiments that measured CAT activity, there was a 60 minute interval between exposure and protein extraction.

Kinetics of c-*fos* promoter driven CAT expression; The effect of time

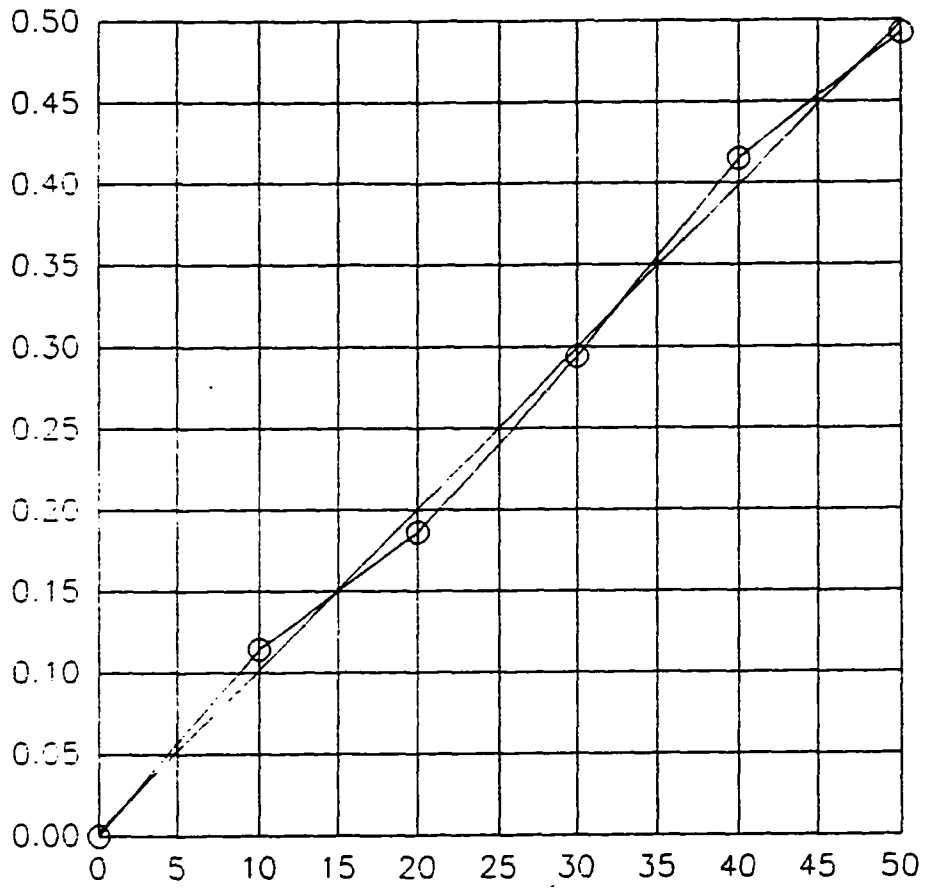


Figure 5. Standard protein concentration plot.

Concentration was plotted by using the regression analysis of the Sigma 4.1 computer program. The standard protein used was gamma globulin (O). Optical density (at 590nm) was plotted on the Y axis and concentration on the X axis (µg/µl). The protein samples were represented as means of triplicate readings. Concentrations of the protein samples were determined by correlating OD readings of the samples with standard curve and extrapolating it on to the X axis.

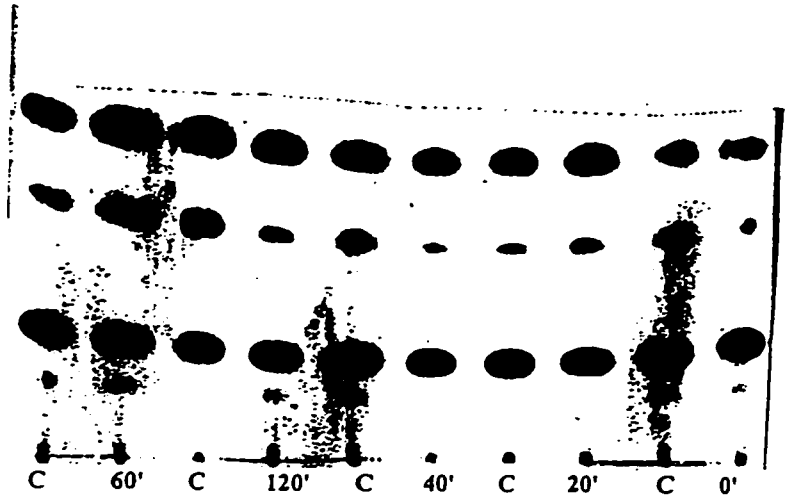
Figure 6. CAT expression driven by the *c-fos* promoter

A. CAT expression was determined by the chromatographic method. The top two spots represent the acetylated chloramphenicol while the bottom spot is the non-acetylated chloramphenicol. CAT expression was determined for the control (C), and the experimental samples (0, 20, 40, 60 and 120 minutes).

B. The "lag time" for the induction of the CAT protein following 20 minute exposure to EMFs was 60 minutes. HeLa cells, transfected with pFC-700, were incubated for different times (0, 20, 40, 60 and 120 minutes) following 20 minute exposure to EMF of 60 Hz and 60mG. The numbers in each column represent the number of times the experiment was repeated.

C. CAT activity values for the controls and time point (0,20,40, and 60 minutes) are represented as %. CAT activity for 120 minutes incubation was measured by the liquid phase method and represented as cpm. The bars represent the standard errors of means of the control. Each experimental (control and experiment) set is represented as a separate unit due to different transfection efficiencies.

A



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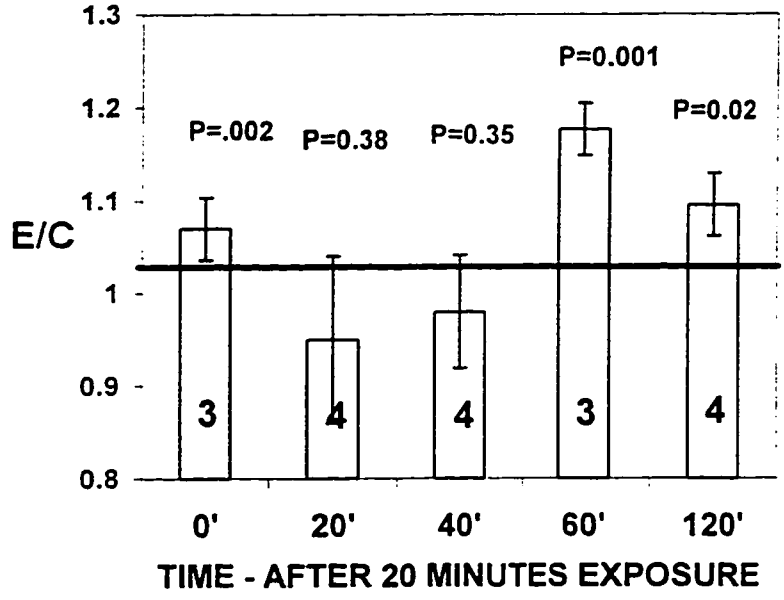

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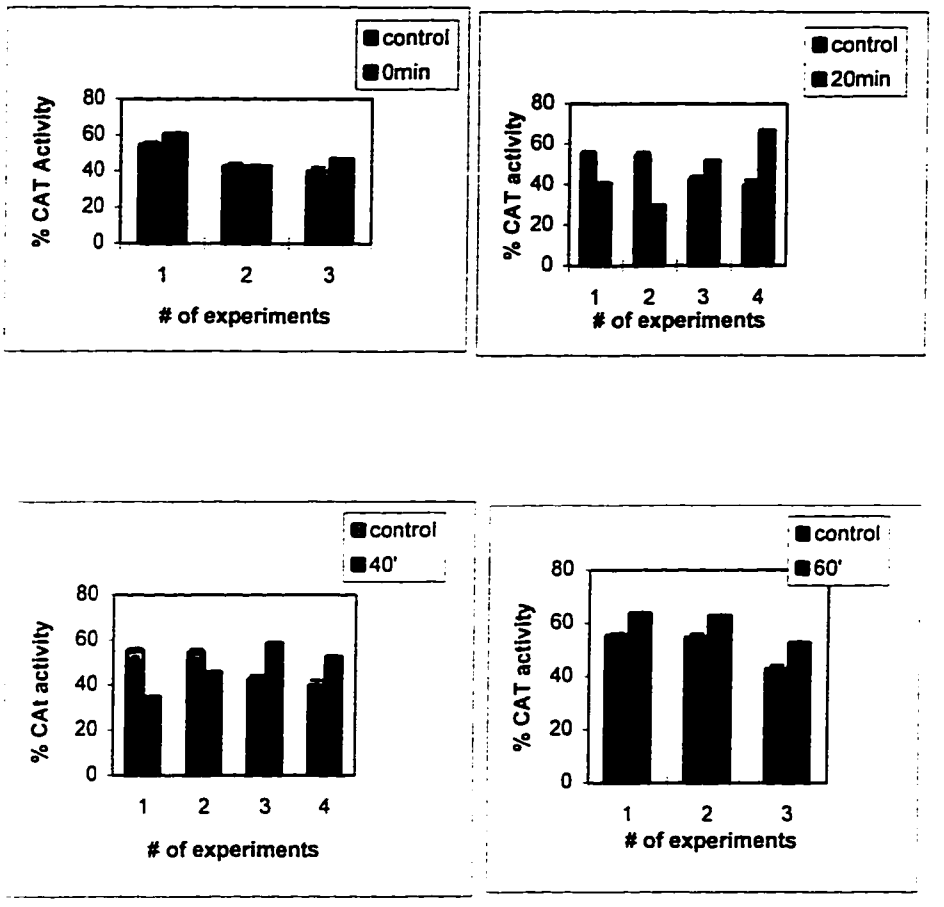
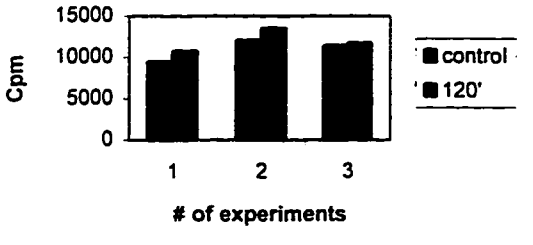




of exposure. EMF exposure resulted in a rapid, and transient increase, in the expression of the c-*fos* transcript. CAT expression was increased following EMF exposure in a time dependent manner. Transiently transfected cells were exposed to EMFs for time intervals of 5, 10,15, 20, 25, 30 and 40 minutes. CAT expression driven by the c-*fos* promoter responded within 5 minutes of exposure similar to steady state mRNA expression following EMF exposures (Figure 7). The response to EMF was seen for up to 20 minutes of exposure, where the maximum peak was about ~25% greater than control values. CAT activity fell rapidly; it reached basal levels within 25 minutes and no further increase was seen even with longer exposures (up to 40 minutes). The increase was relatively small as compared to responses seen using other mitogens. To ascertain that the response seen was due to EMFs and not due to unknown factors, "sham- sham" experiments were performed.

Sham- sham experiments. "Sham-sham" experiments showed that the increase in CAT activity was due to EMF exposure and not the experimental design or other external factors, such as handling of the cells. Transiently transfected cells were placed in the mu metal box for 20 minutes, but no field was applied. No measurable increase in CAT activity was observed (Figure 8).

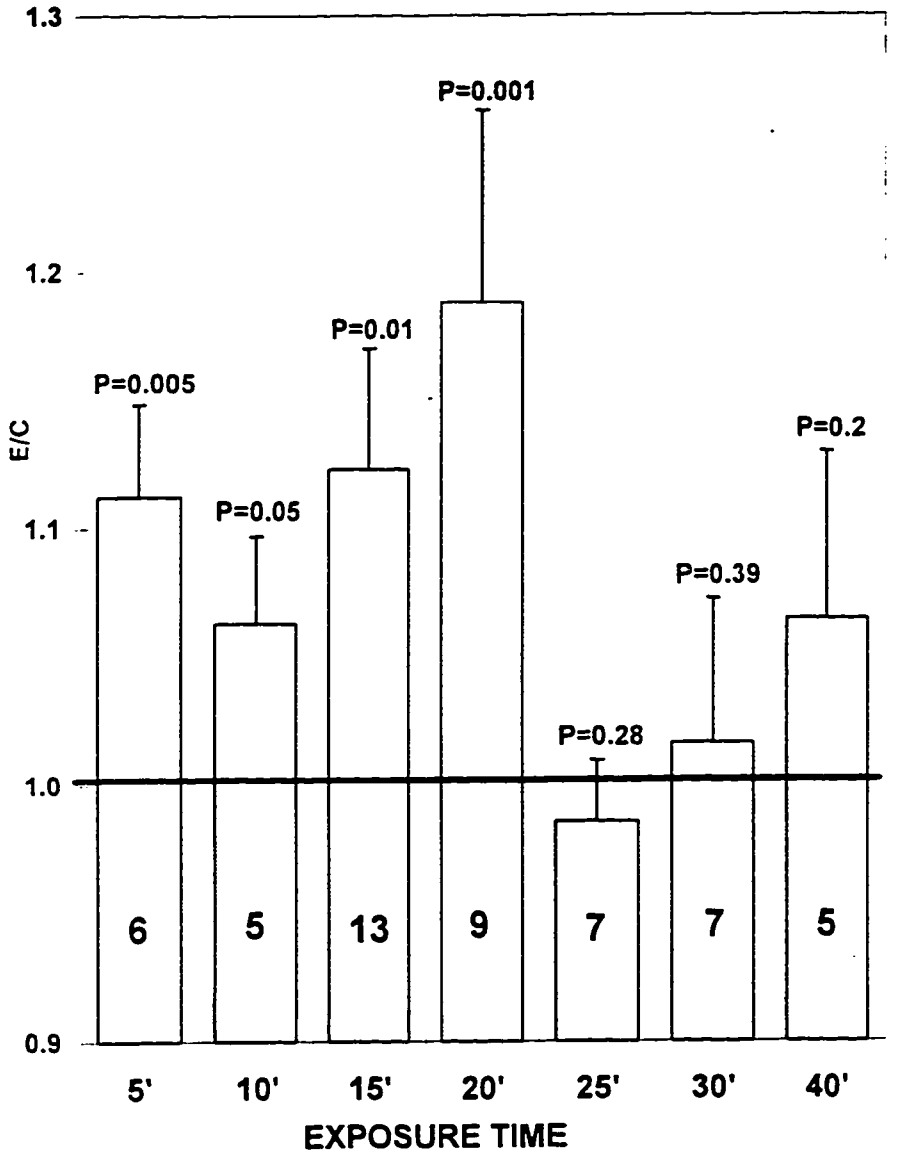
Measurement of CAT transcripts. To ensure that the increases seen in expression of CAT were due to the effect of EMFs on the regulation of the gene and not a downstream effect, the level of steady state CAT mRNA was analyzed.

Figure 7. Time dependent induction of CAT expression following EMF exposure.

A. Transfected HeLa cells were exposed to EMF for different lengths of time (5, 10, 15, 20, 25, 30 and 40 minutes) and the cells were harvested for CAT following 60 minutes of incubation. A student (two tailed) t-test was performed to determine the significance of these increases against basal expression (p values are shown on the graph). The t-test between time points, used values from all experimental samples. The t test between 5 minutes and 20 minutes gave a p value of 0.02, 20 and 15 minutes gave a p value of 0.02. This suggests that there is a significant difference in CAT expression between these two time points.

B. CAT values represented as% activity for individual experiments. Bars represent the SEM values.

A



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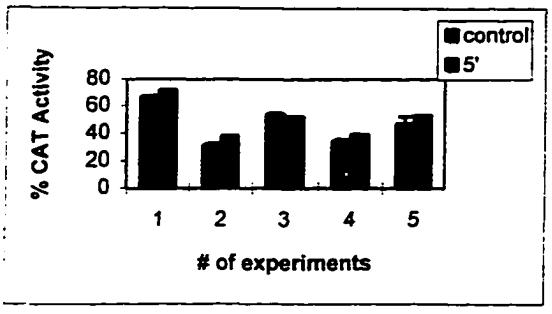
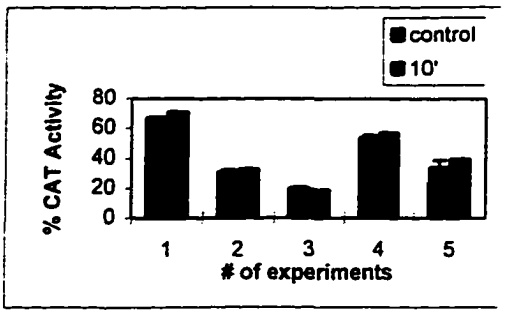
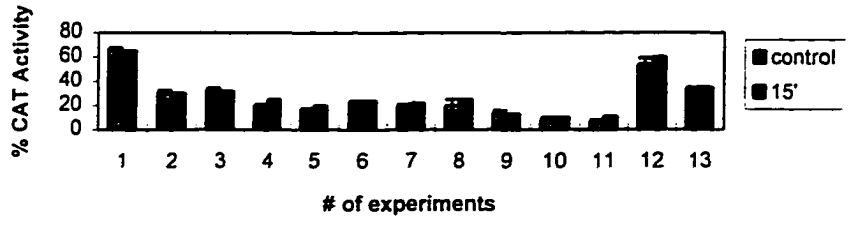
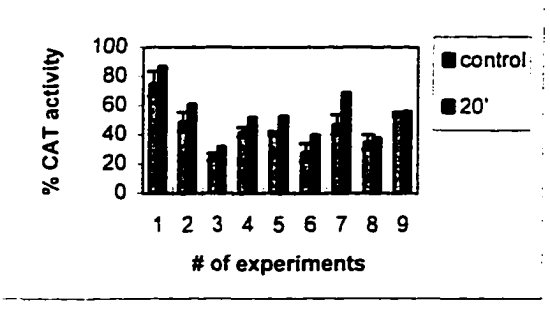
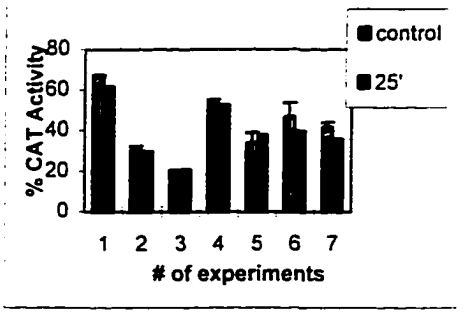
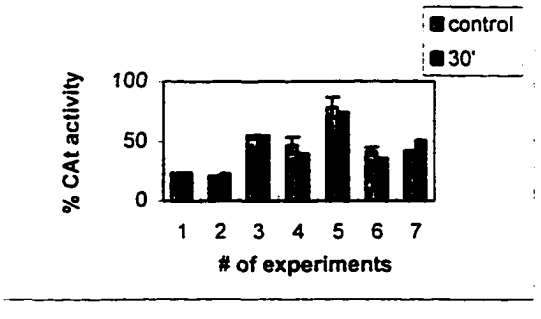












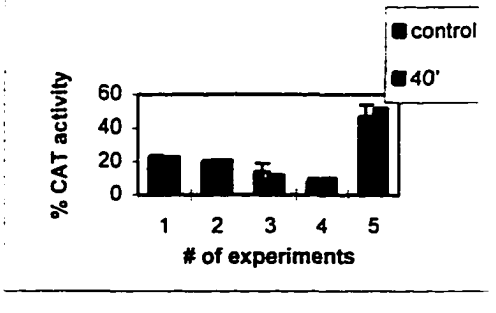
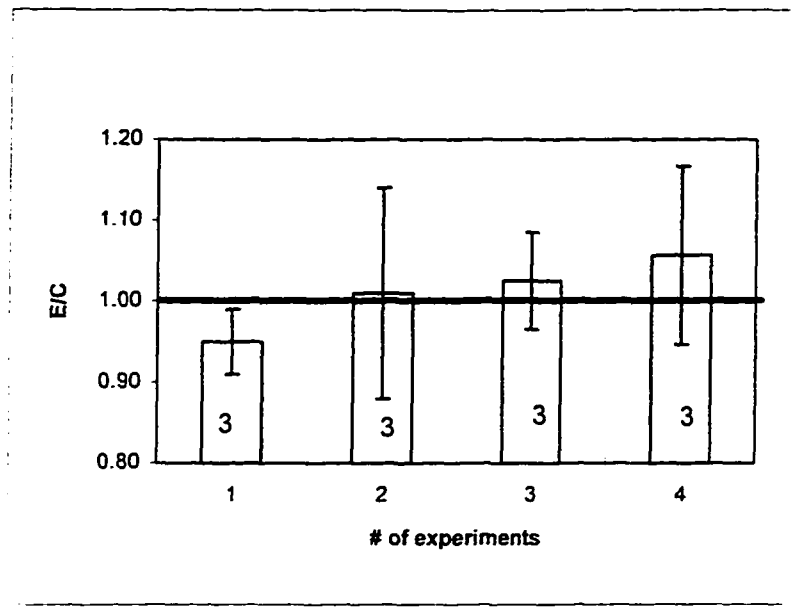


Figure 8. Sham- Sham experiments

A. Transfected cells were treated under exactly the same conditions as those for exposure, except no field was applied. Each experiment contained three sets of control and exposure flasks. No increase in CAT expression was observed, demonstrating that the increases seen in CAT expression were due to EMF exposure.

B. CAT activity is represented in cpm for the controls and experiments, as the liquid phase method of CAT activity was utilized for this experimental setup. SEM values are represented by bars.

A



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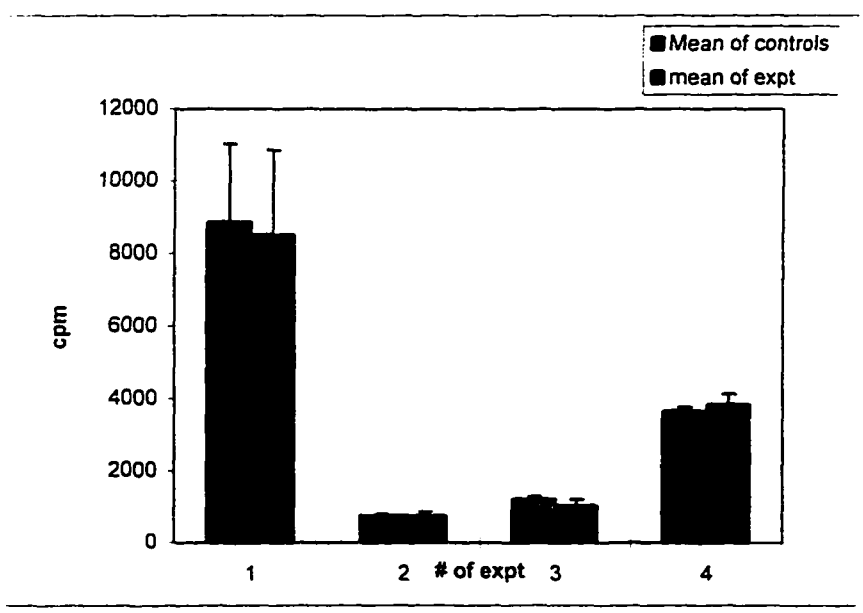


Total RNA was extracted from transiently transfected cells which had been exposed to an EMF for 20 minutes (Figure 9a). The level of mRNA was comparable to the level of CAT expression. The CAT mRNA was quantitated using phosphoimaging or densitometry of negatives produced from Northern hybridizations (Figure 9b).

Specificity of CAT expression following EMF exposures. To confirm that the increase in CAT expression observed after EMF exposure was dependent on the c-*fos* promoter, CAT expression driven by the thymidine kinase promoter was analyzed after EMF exposure. The thymidine kinase promoter was studied as the thymidine kinase gene is a essential housekeeping gene with a well characterized promoter complex. The construct ptk-CAT contains promoter regions of the herpes thymidine kinase gene without the enhancer regions. The construct showed no increase in CAT expression following EMF exposure. This comparison indicates that the increase in the CAT expression is specific (Figure 10).

PART TWO: ANALYSIS OF *c-fos* UPSTREAM REGULATORY REGIONS.

The c-*fos* promoter is sensitive to several mitogens and has been extensively studied. The next series of experiments were designed to determine which region(s) of the promoter complex were driving the increased CAT expression in the presence of EMFs. The results showed that the SRE/AP1 site of the *c-fos*

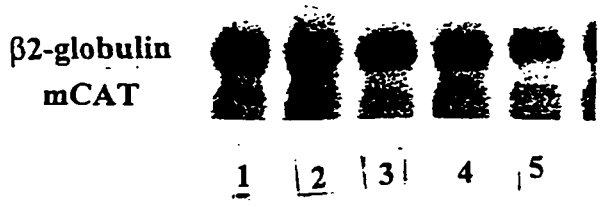
Figure 9. Analysis of CAT mRNA by Northern Blots

A. Northern blot hybridization against ptri-CAT (RNA probe from Ambion) was measured in transiently transfected cells. RNA was extracted immediately from HeLa cells after exposure to a field of 60 Hz (60 mG) for 20 minutes. The odd numbered lanes represent the RNA from unexposed cells; the even lanes represent the RNA from exposed cells. Hybridization was performed at 40°C for 12 hours and the membrane was washed at room temperature (10 minutes) twice, with 2 xSSC, 0.1% SDS, 0.2 x SSC and 0.1% SDS. The membrane was exposed overnight at -70°C. β2-microglobulin was used as the internal control.

B. Comparison of CAT mRNA and CAT expression. E/C values were determined by phosphoimaging. Local background values were taken and subtracted from the counts. E/C of CAT was divided by the E/C values from β2-microglobulin to give the final E/C values.

C. Control and experimental values (cpm) have been represented as a ratio of the CAT and β2-microglobulin. The values were corrected for background.

A



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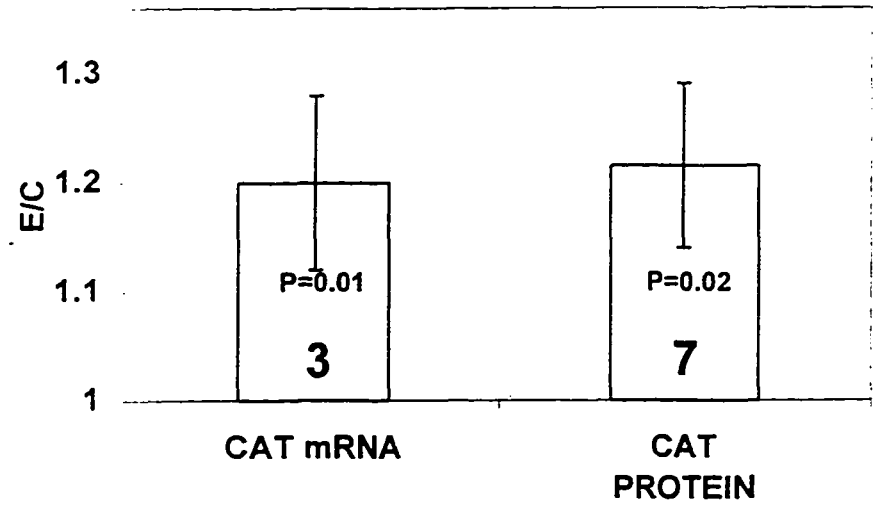

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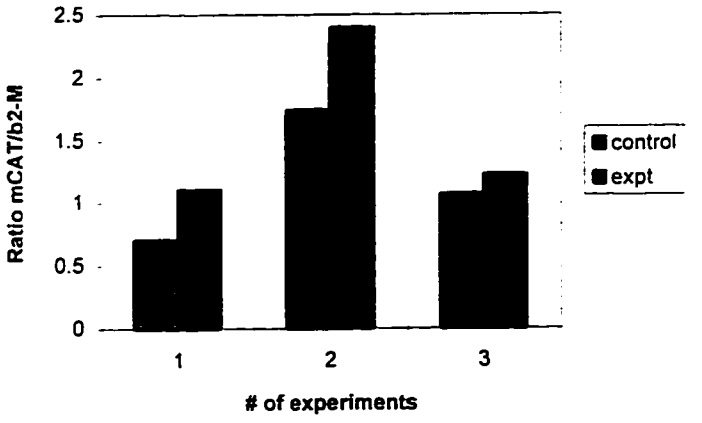
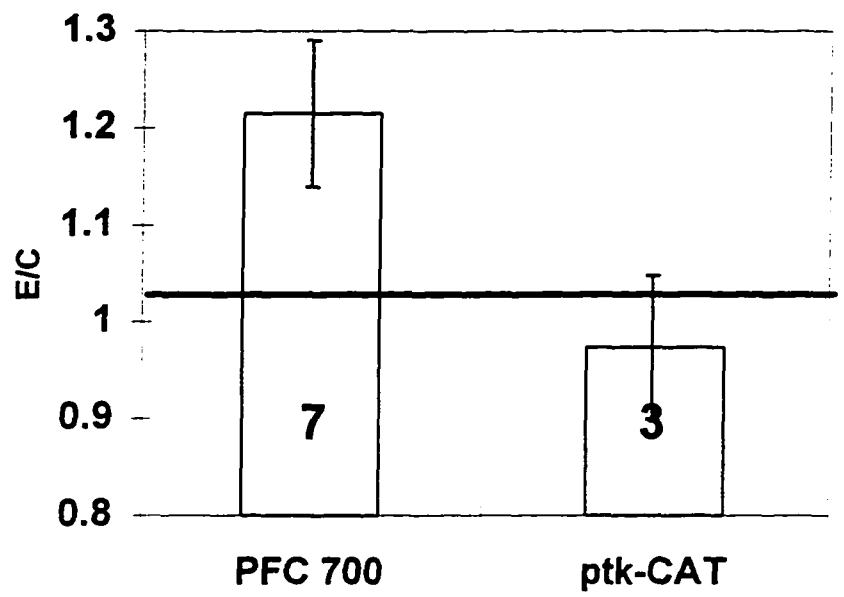


Figure 10. CAT activity in cells transfected with the pFC 700 and ptk-CAT constructs.

A. The construct ptk-CAT contains the herpes thymidine kinase promoter without enhancer regions. The thymidine kinase promoter is not responsive to EMF.

B. Individual experiments graphed using cpm of C^{14} labeled chloramphenicol.

A



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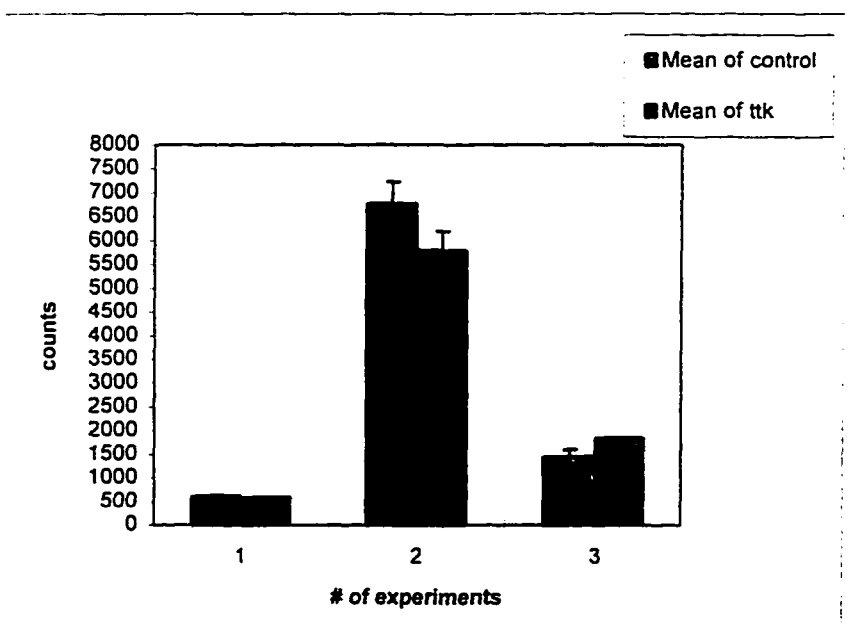

promoter was responsible for the increase seen in CAT expression following EMF exposure.

CAT expression driven by upstream bases from -700 to -225. The upstream bases from -700 to -225 include the SRE/ AP1 site (-298 to -317). Transfection of this construct into HeLa cells resulted in a strong response to EMF on the basis of CAT expression. CAT expression was 37% above basal levels (E/C =1.37, SE<0.03) (Figure 11). The results show that the region containing bases -225 to +42 was not necessary for increased CAT expression in the presence of EMFs.

CAT expression driven by the bases -225 to +42 or -99 to +42. The construct pFC.225 contains bases -225 to + 42 of the c-*fos* promoter directly attached to the TAATA box. This region includes the c-AMP responsive element (CRE), the direct repeats (DRS) and a region responsive to Ca^{+2} ionophores (Figure 12a). The construct pFC 99, includes bases from -99 to +42. This construct lacks the CRE site, but the direct repeat elements are still present (Figure 12b). Deletion analysis showed that neither of the constructs transfected into HeLa cells increased CAT expression following EMF exposure. This confirmed earlier results using the construct (pFC700/225).

CAT expression using constructs pFC. 363, p-350 or p-250. The construct pFC. 363 contains bases -700 to -363, and includes SRE/AP1, as well as CRE and DRS. Expression of CAT activity in cells transfected with this

Figure 11. CAT activity in cells transfected with the pFC 700/225 construct.

The construct pFC700/225 has the bases-700 to-225 directly attached to the TAATA box. This construct contains the SRE/AP1 site. The * represents the E/C values for the complete construct (pFC700). E/C values of CAT activity for the construct pFC 700/225 was comparable to the complete construct, indicating that this region is responsive to EMF.

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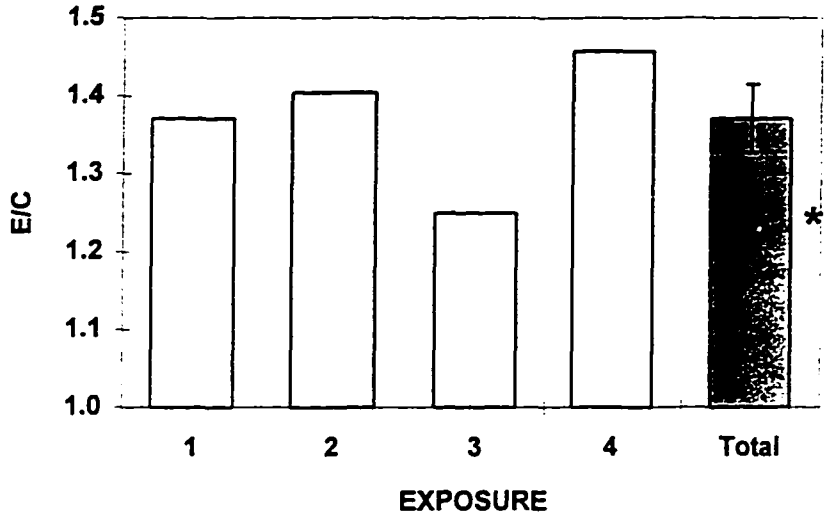

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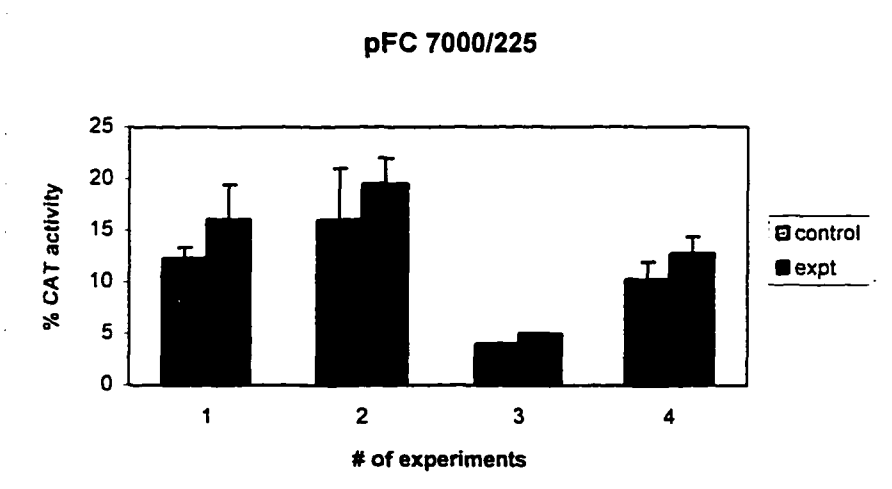


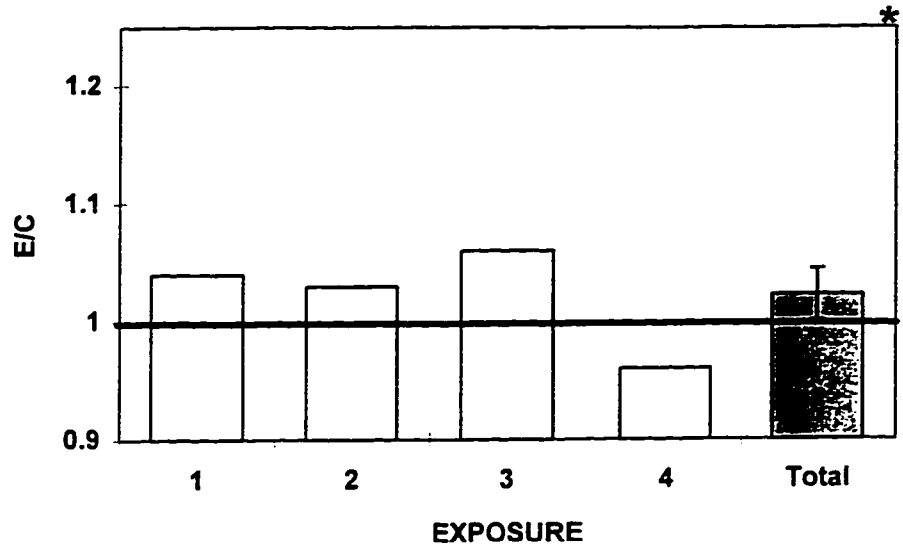
Figure 12. CAT activity in cells transfected with the pFC225 and pFC99.

A. The pFC 225 construct contains the promoter regions from bases-225 to+42. CAT expression driven by this region did not increase significantly above basal values after exposure to EMF for 20 minutes; indicating that it does not respond to the fields.

B. The construct pFC 99 has the bases -99 to+42 of the *c-fos* promoter attached to the CAT gene. CAT expression driven by this region did not increase significantly above basal levels.

C. CAT activity is represented in percent for HeLa cells transcfected with pFC 225(1) and pFC99(2) but not exposed to EMF (controls) and those exposed to EMF for 20 minutes (experimental)

A



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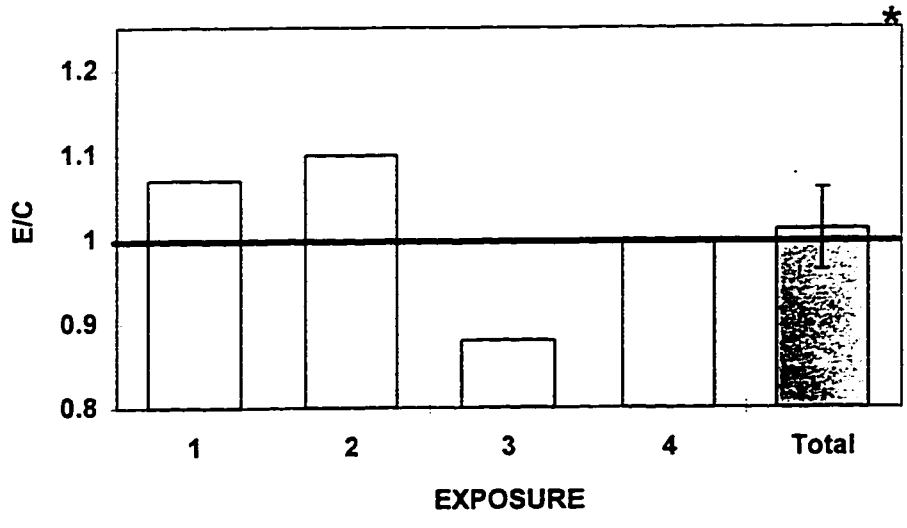

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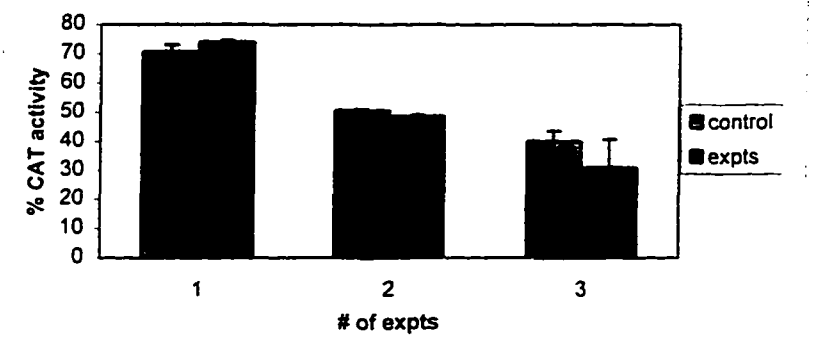

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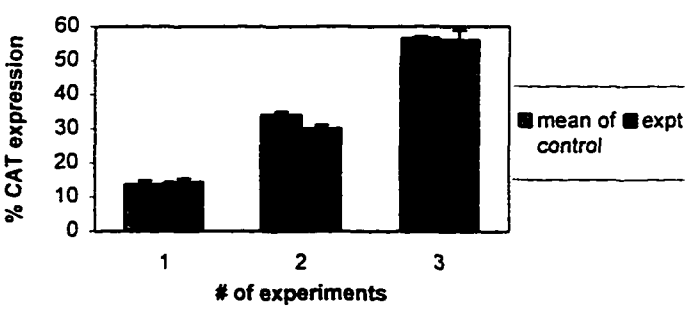


construct shows that the activity does not rise above basal levels (Figure13). Since the construct containing the portion of the promoter from -700 to -225 increased CAT expression after EMF exposure, it was tentatively concluded that the region containing bases -700 to -363 was responsible for increased CAT expression. To verify these results, we studied a construct which contained bases -700 to -363 directly attached to the TAATA box (p-350). The plasmid containing this construct was different from those used previously.

The promoter region containing bases -700 to -363 was attached to the CAT gene in a PUC-18 plasmid. In this plasmid, the c-*fos* promoter insert was separated from the CAT gene by the herpes thymidine promoter (which does not contain enhancer regions). Analysis of the plasmid ptk -CAT, which contains only the thymidine kinase promoter attached to the CAT gene, showed that it does not respond to EMF (Figure 10).

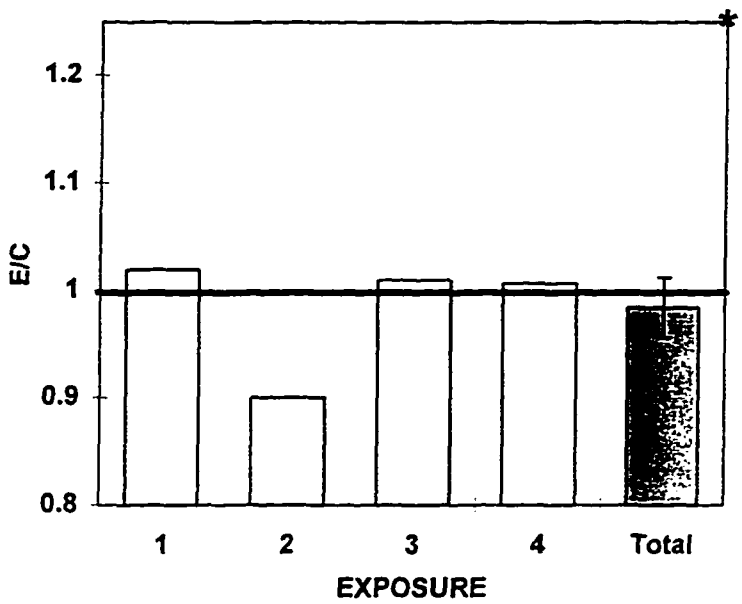
This ensured that the any increase seen in CAT expression with EMF exposure driven by the p350 or p250 construct was a direct result of the c-*fos* promoter insert. Analysis of CAT activity using the p-350 construct was not different from the control (Figure 14); our tentative conclusion that the bases -700 to -363 were necessary for the response, was in error. Based on the above studies, two conclusions could be reached. Either bases from -700 to -225 are necessary, or that the bases -363 to -225 responded to the fields but the presence of the downstream bases -225 to -53 negated the response. To

Figure 13. CAT activity in cells transfected with pFC 363

A. The pFC 363 construct has the promoter region containing bases -363 to +42 which encompasses the SRE/AP1, CRE and DRE sequences. The CAT activity driven by this promoter region did not increase after EMF exposure of 20 minutes at 60 Hz and 60 mG.

B. Actual CAT values of control and experiments also show no increase similar to E/C data.

A



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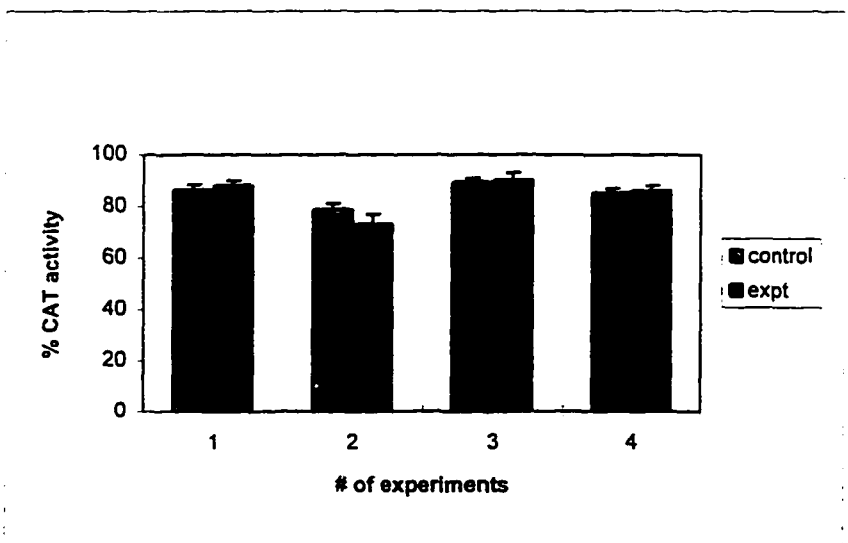
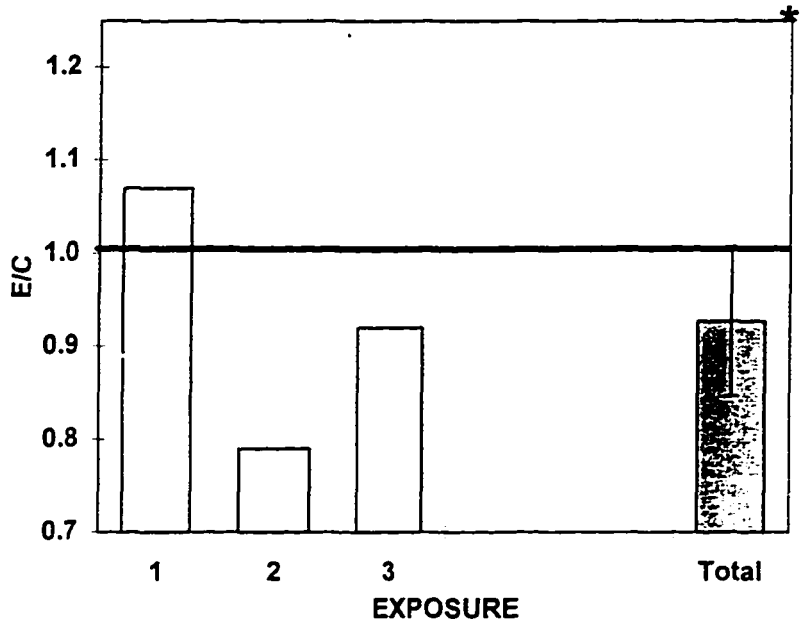


Figure 14. CAT expression in cells transfected with the p350-ttk construct.

A. The construct p350 contains the regions-700 to-363 attached directly to the TAATA box in a PUC 18 plasmid. CAT expression (E/C values) driven by this region of the promoter did not increase following EMF exposure.

B. No difference is seen between actual CAT activity values of control and experimental samples, indicating that the construct p350 does not respond to EMF.

A



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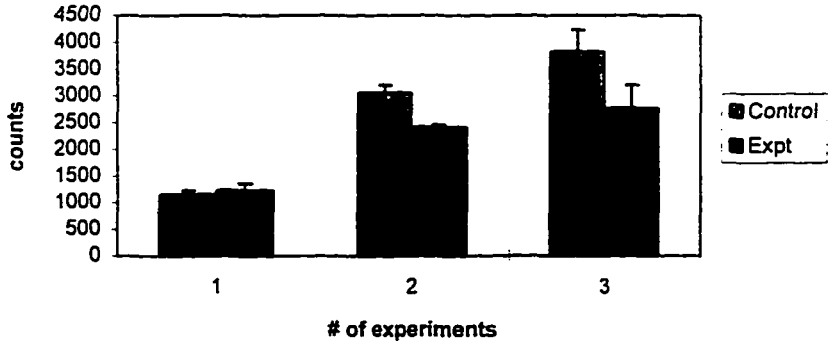


determine which of the two scenarios was correct, a construct containing bases -363 to -225 directly attached to the TAATA box (p250-ttk which lacks downstream region from -225 to-53) was analyzed. There was a difference between control and exposed cells (Figure 15), indicating that the bases in the region -363 to -225 were responsive to EMF exposure and that the response could be negated by the downstream regions (bases -225 to -53). It also showed that the promoter portion encompassing bases -700 to-363 helped to overcome the negative inhibition seen from the bases -225 to -53, even though it did not increase CAT expression following EMF exposure directly, explaining the response seen from the complete promoter. These results indicate that spatial organization of the c-fos promoter is critical to its correct function. Natesen *et al.*, (1995) showed that the transcription factor YYI binds to the CRE and TAATA box causing the DNA to bend. Bending of the DNA represses transcription from the *c-fos* promoter. One possibility is that the presence of the upstream region from -363 to-700 prevents the bending of the DNA at the CRE site by blocking the binding of the transcription factor and hence inhibits repression of the promoter following EMF exposure, explaining the response seen from the complete promoter. It could also be proposed that the binding of the upstream region to the CRE site brings the SRE region closer to the start site of transcription, thereby facilitating promoter expression (Natesan et al., 1993; Tijan et al., 1994).

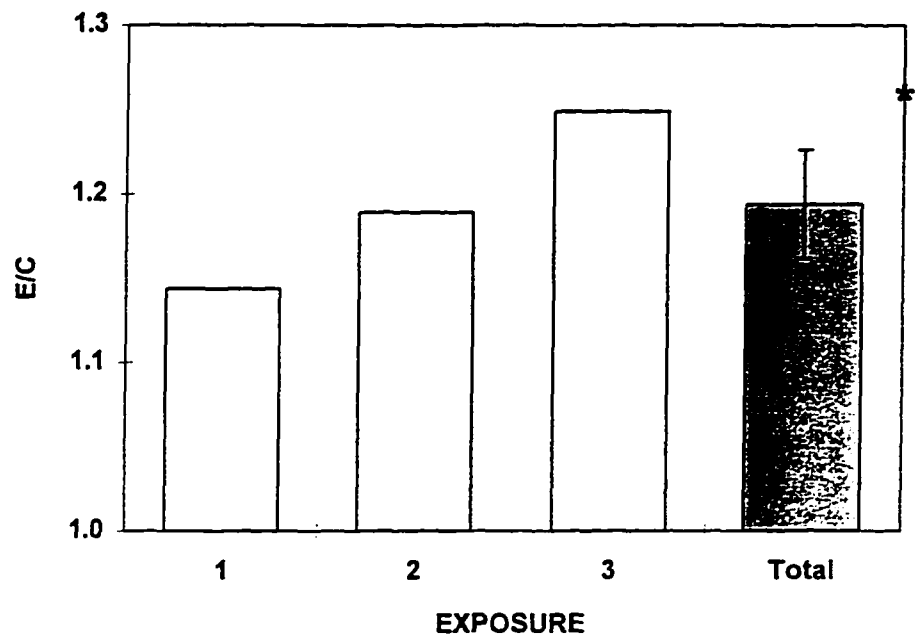
CAT expression driven by the SRE region. The construct pFC. 53E was used

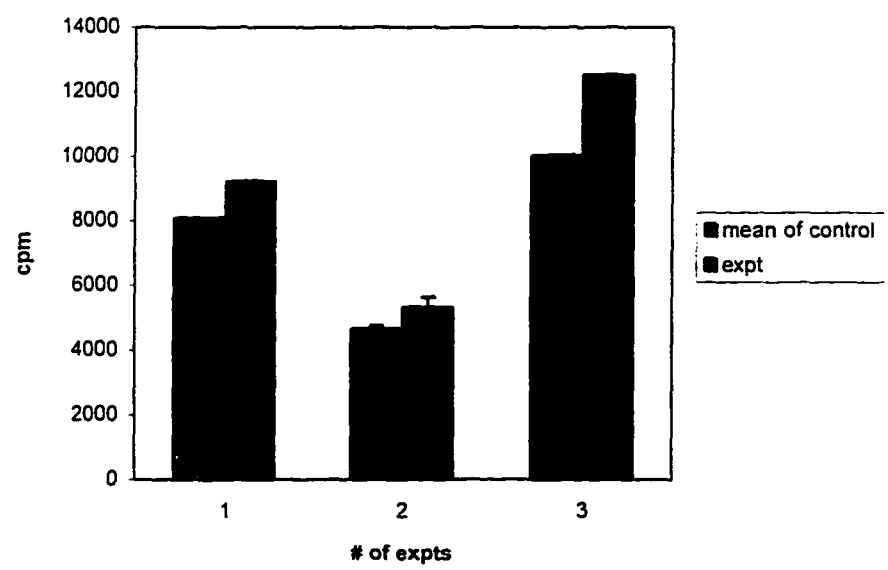
Figure 15. CAT expression in cells transfected with the construct p250-ttk .

A. The construct p250 has the regions-363 to-225 attached directly to the TAATA box. This region contains the SRE/AP1 sites. CAT expression driven by this construct following EMF exposure was comparable to the increase in CAT expression seen from exposed cells transfected with the complete promoter.

B. Cat activity shows that for transfected cells exposed to the field was higher then the control. The liquid phase method was used to analyze CAT activity, which is depicted in cpm.

A





to determine the exact portion of the promoter complex within the region containing bases -363 to -225 that regulated increased CAT expression. This construct has the SRE site attached to the TAATA box directly. The construct alone was not adequate to induce CAT expression above control levels (Figure16). SRE inducibility is sensitive to its spatial organization, shown by the loss of inducibility to PDGF when it was moved to a different position (-100 or -57) (Wagner et al., 1990). Others have shown that in some cases the region -60 is required for the efficient induction of SRE (Fisch et al., 1987). It can therefore be inferred that the presence or absence of other elements within the promoter determines the response of the SRE to a given stimulus.

From the above experiments we conclude that the SRE/AP1 region is responsible for the increase in CAT expression seen after exposure to EMF. These experiments are summarized in Figure 17.

PART THREE: MECHANISM(s) BY WHICH EMFs AFFECT CELLS

The SRE/AP1 region which is responsible for the increase in CAT expression in cells exposed to EMF is also sensitive to TPA. Further research investigated the possibility of overlap in the signal transduction processes activated by EMF and TPA. The results indicated that the EMF and TPA act along similar pathways within the cell to activate the *c-fos* promoter.

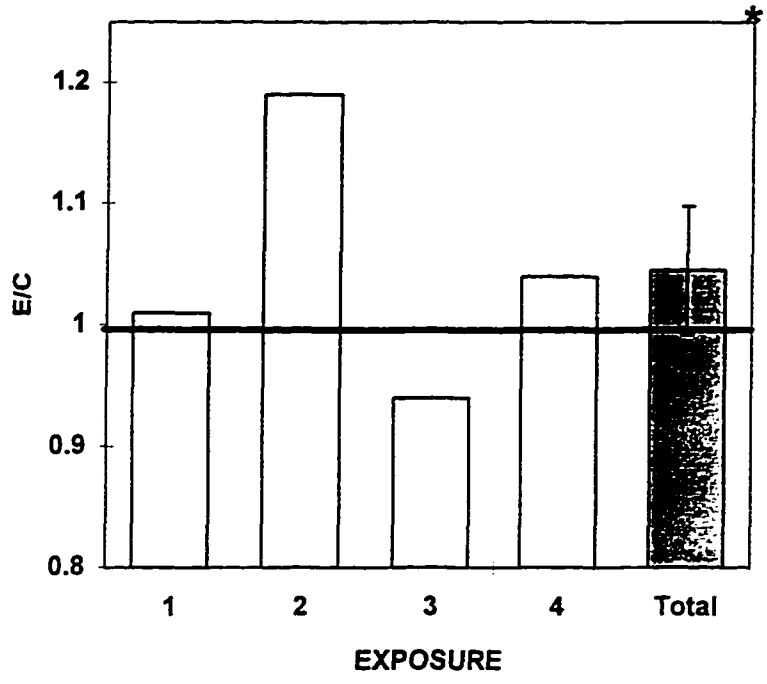
Comparing effect of TPA and EMF on the cells. To investigate the possible

Figure 16. CAT activity in cells transfected with the construct pFC 53 E.

A. The construct pFC 53E contains 27 base pairs of the oligonucleotide TF4, spanning the human *c-fos* SRE (-319 to-298) cloned at -53. The E/C ratios from cells transfected by this construct after EMF exposure was not significantly different from basal levels.

B. CAT activity values for each experimental series show show no significant differences.

A



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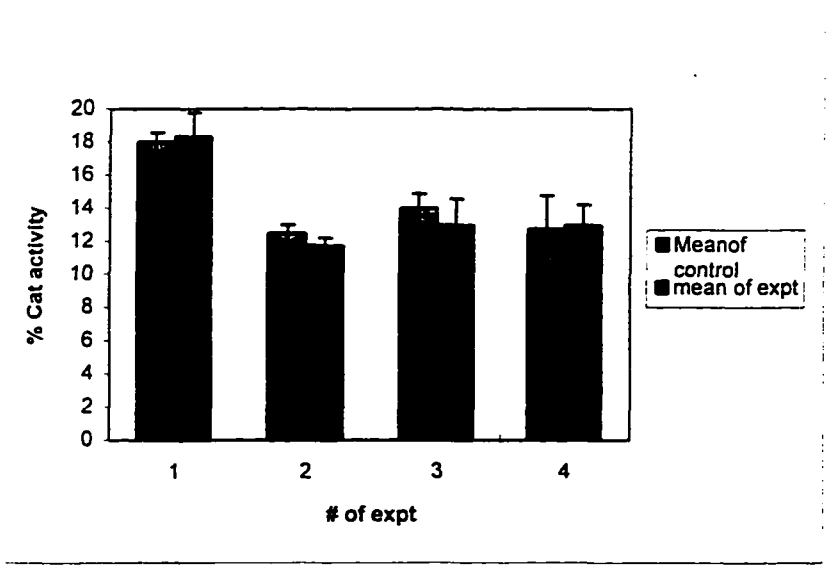
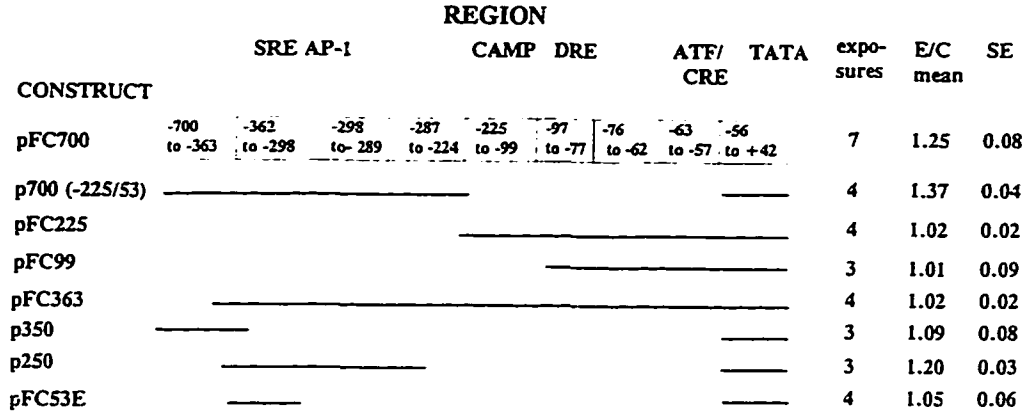




CONSTRUCT	expo-sures	E/C mean	SE
pFC700	7	1.25	0.08
p700 (-225/53)	4	1.37	0.04
pFC225	4	1.02	0.02
pFC99	3	1.01	0.09
pFC363	4	1.02	0.02
p350	3	1.09	0.08
p250	3	1.20	0.03
pFC53E	4	1.05	0.06

Figure 17. Summary of the deletion analysis.

The boxes denote regulatory regions, SRE, AP1, CRE, DRS and C-AMP. Constructs were -700 (pFC700), -363 (pFC363), -225 (pFC225) and -99 (pFC99), all extending to+ 42. The construct p53E the regions -319 to 297 attached to the TAATA box. pFc 700/225 has the promoter regions spanning from-700 to -225. p 350-ttk and p250-ttk contain the regions from-700 to -362 and -362 to-223, respectively.

mechanisms used by the cell following EMF exposure, the effects of TPA and EMF induction were compared. These experiments showed that the increases in CAT expression driven by *c-fos* promoter were not additive following application of both EMF and TPA. This suggested that EMFs and TPA activate similar signal transduction pathways.

Cells, transfected with the pFC700 construct, were divided into three sets (designated 1,2,3); each set contained a control and experimental flask. The experimental flask from the second and third sets was exposed to TPA. After an hour, the experimental flask from the first and second set were exposed to EMF for 20 minutes. Protein was extracted from all three sets one hour following EMF exposure.The experimental design is summarized in Table II. The kinetics of this experiment was especially important, since the response to EMFs is rapid and transient. The experimental approach was to provide conditions that were similar those used in earlier EMF experiments (the conditions under which maximum CAT expression was observed after EMF exposure). These conditions were not ideal to TPA. Transfected cells exposed to TPA, followed by EMF exposure, showed CAT expression similar to that of the cells exposed only to TPA (Figure 18). These results demonstrated that TPA and EMF did not give an additive effect under the conditions used. The response after the exposure to TPA alone was seen to be smaller than that observed following EMF exposure because the conditions used for TPA were not optimal (maximum response is seen 6 hours

Table II

Experimental procedure for comparing the CAT activities generated by the addition of TPA and EMF to transfected cells.

TIME	O HR	1 HR	2 HR
SET #	TPA	EMF	PROTEIN EXTRACTION
1		+	+
2	+	+	+
3	+		+

after induction). This result demonstrated that once the mechanism is initiated by TPA, EMF exposure cannot elicit a further response from the cell. It can be argued that CAT expression in cells exposed to both TPA and EMF should be similar to that of cells exposed to only EMFs, but the results showed that this is was not the case. To ensure that EMFs and TPA activate similar pathways, EMF and TPA were applied to the cells in the opposite order, that of EMF followed by TPA. Similar to the previous comparison, the transfected cells were divided into three sets each containing a control and exposure flask. The first and second set of flasks were exposed to EMF for 20 minutes at 60 Hz and 60mG. After exposure, TPA was added to the second and third sets. Protein was extracted two hours after TPA induction (Table III). The results showed that transfected cells exposed to EMF and TPA did not show an additive effect (Figure19). The response of all three sets of cells is the same. It can be concluded that EMF and TPA act along a similar pathway (s).

PKC and PKA inhibition of CAT expression. These experiments analyzed the role of PKC and PKA in the cell's response to EMF exposure relative to CAT expression driven by the c-*fos* promoter. Inhibition studies demonstrated that EMF response required the activity of both enzymes.

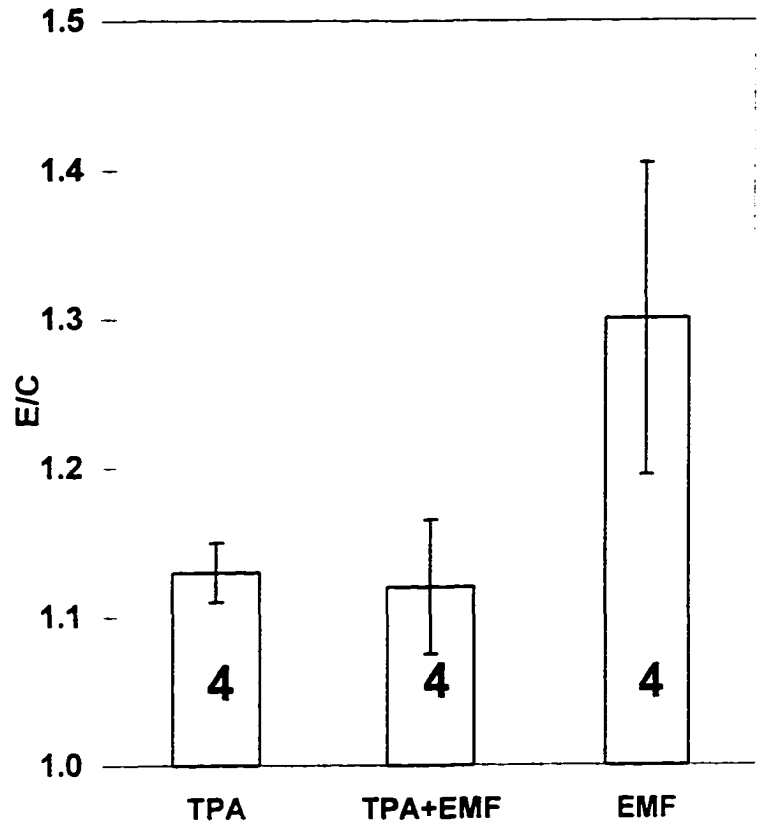
TPA activates both PKC (directly) and protein kinase A (PKA) (indirectly). HeLa cells, transfected with the pFC 700 construct, were divided into four sets, each containing an experimental (flask containing cells exposed to EMF) and

Figure 18. CAT expression following exposure to TPA and EMF.

A. HeLa cells transfected with the pFC 700 construct were treated with 162nM TPA. After an hour, the cells were exposed to EMF for 20 minutes. CAT expression was determined one hour following EMF exposure. Cells exposed to both EMF and TPA did not show a additive effect.

B. CAT activity was measured using the liquid phase method and is represented as cpm. Control and experimental values for each set have been depicted separately as the treatment for the controls was different.

A



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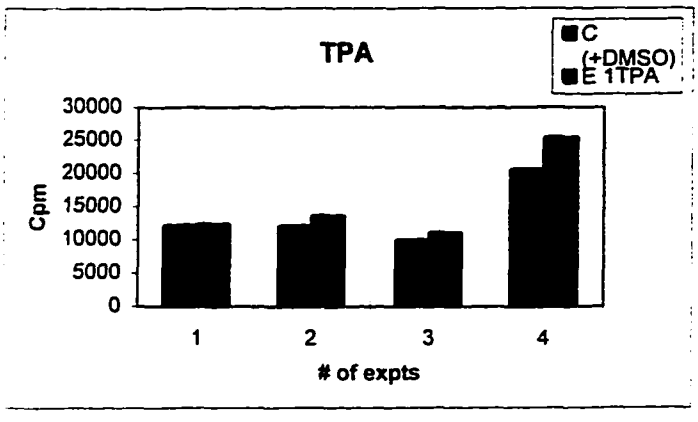
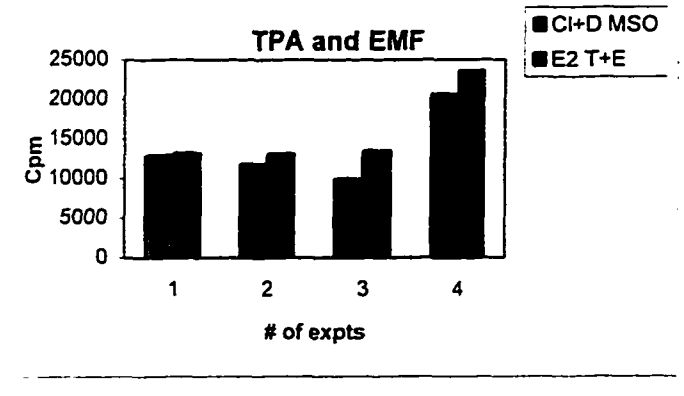




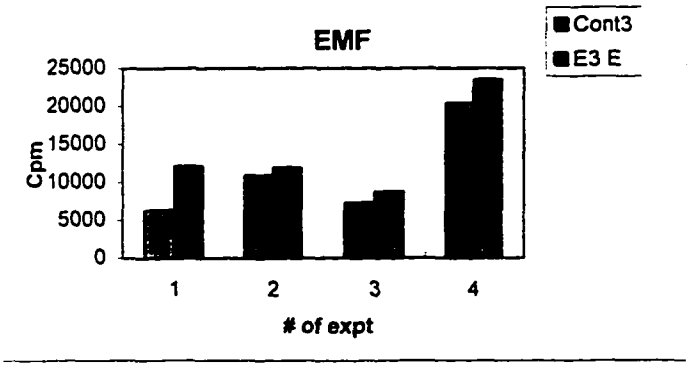


Table III

Experimental design for comparing the CAT expression from transfected HeLa cells exposed to EMF followed by TPA.

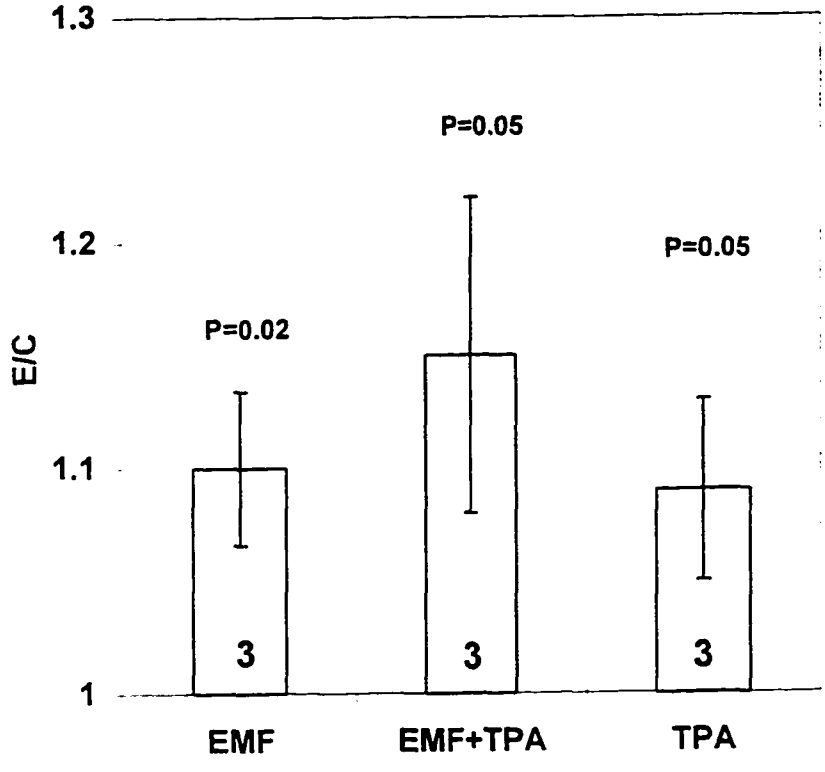
TIME	O HR	20 MIN	2 HR 20 MIN
SET #	EMF	TPA	PROTEIN EXTRACTION
1	+		+
2	+	+	+
3		+	+

Figure 19. CAT activity of transfected cells exposed to EMF, followed by TPA.

A. Transfected HeLa cells were exposed to an EMF of 60 Hz and 60 mG for 20 minutes, followed by the addition of 162nM of TPA. CAT activity was measured 2 hours after TPA addition. Cells exposed to both EMF and TPA did not show an additive effect.

B. CAT activity represented in cpm. Controls for TPA treated cells were treated with DMSO, the TPA solvent. Each experimental condition is depicted separately similar to Figure 18.

A



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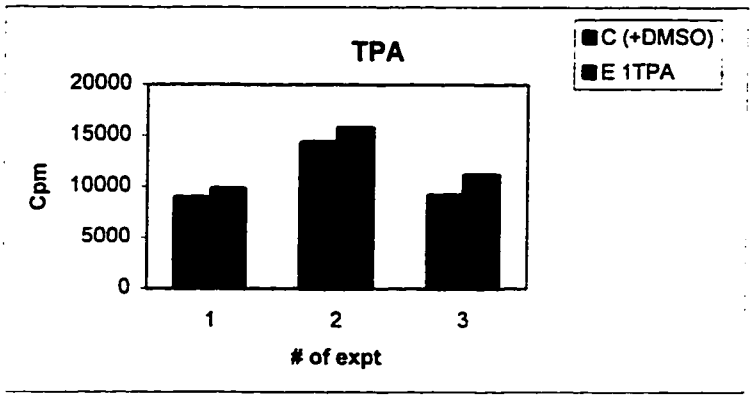
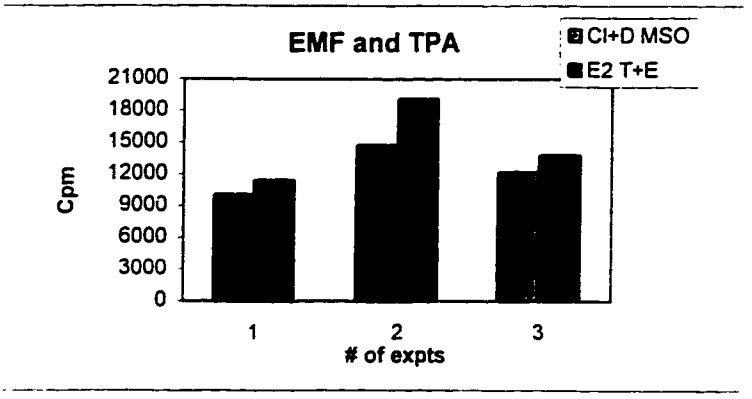
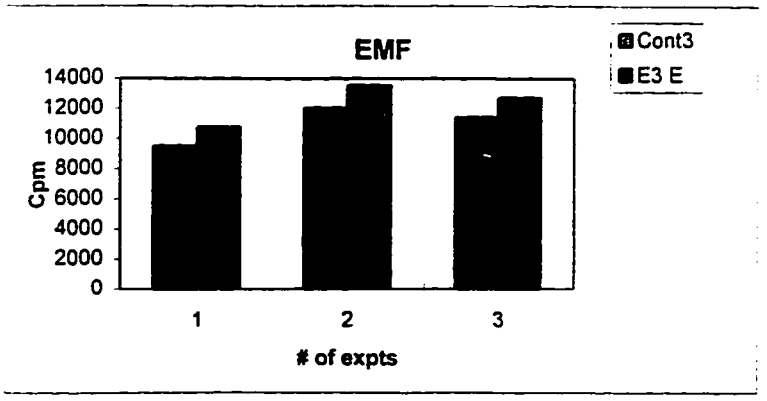






control flasks. Table IV describes the experimental procedure.

Chelerythrine, a benzophenanthridine alkaloid, is an antagonist of the Ca^{+2} phospholipid- dependent protein kinase (Herbert *et al.*, 1990). The inhibitor interacts with the catalytic domain of PKC and acts as a competitor to the phosphate acceptor. The alkaloid is non-competitive with ATP. On the other hand, the PKA inhibitor H-89 (isoquinolinesulfonamide) inhibits PKA activity by competing with ATP (Chijiwa *et al.*, 1990). Both inhibitors are highly specific and extremely potent. Preincubation of one hour is sufficient to block enzymatic activity (Herbert *et al.*, 1990; Chijiwa *et al.*, 1990). Transfected cells were exposed to the inhibitors for one hour, followed by EMF exposure were not sensitive to EMF exposure, implicating both PKC and PKA in pathways induced by EMFs (Figure 20).

Phosphorylation of the c-*fos* protein. Fos protein, along with the c-jun gene product, forms the AP1 binding protein. The Fos protein is modified giving rise to a different binding capacity and function (Abate *et al.*, 1990). Several mitogens, such as TPA (Tratner *et al.*, 1990), were shown to cause an increase in phosphorylation of the Fos protein. Phosphorylation of the c-*fos* protein results in it binding to its promoter and down regulating it. The effect of EMF exposure on the uptake of ^{32}P by the Fos protein was studied. The results indicated that the fields increased the relative amount of uptake of ^{32}P by the Fos protein.The Fos protein was identified on SDS gels (Figure 21a), and the relative uptake of ^{32}P

Table IV

The experimental procedure used for analyzing the effect of PKC and PKA inhibitors on CAT expression. [1]

SET #	chelerythrine	H89	EMF	TPA
1			+	
2	+		+	
3		+	+	
4				+

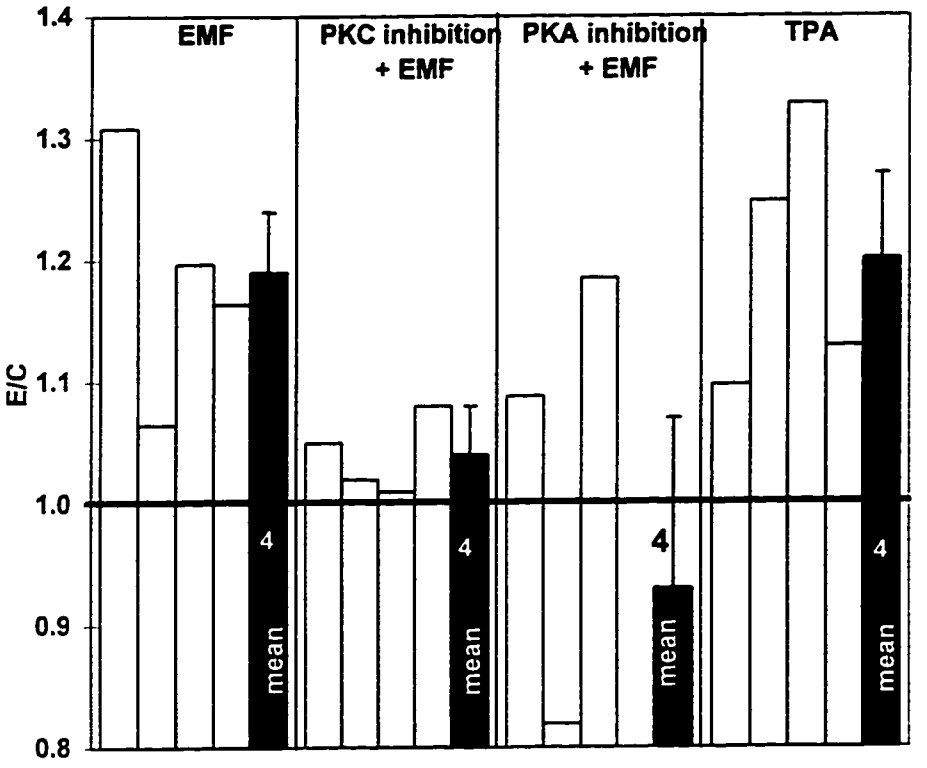
[1]Sets 2 and 3 were exposed to the inhibitors an hour prior to exposure to EMF. Set 1 was a EMF control and set 4 was a TPA control.

Figure 20. Measurement of CAT expression to EMF in the presence of PKC and PKA inhibitors.

HeLa cells transfected with pFC 700 construct were either exposed to 1μM Chelerythrine (PKC inhibitor) or 30 μM of H-89 (PKA inhibitor) for an hour before exposure to a EM field of 60 Hz and 60 mG for 20 minutes. TPA was used as a positive control for these experiments. The results showed that the inhibitors blocked the EMF induced increases in CAT expression.

B. CAT activity was measured by the liquid phase method. The conditions for each control unique, each condition is graphed separately: the controls were treated with the same solvent in which the inhibitors or TPA was dissolved.

A



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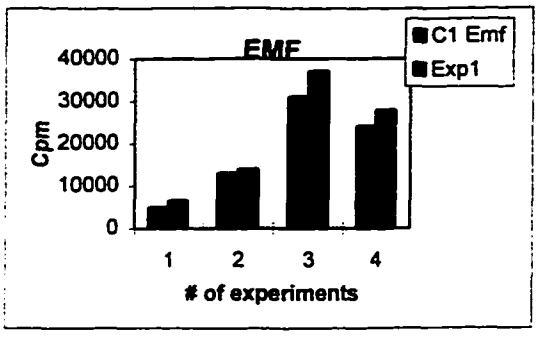
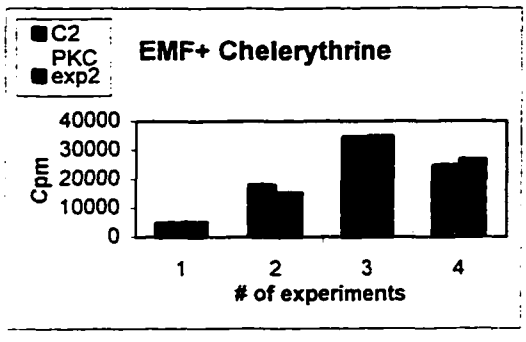
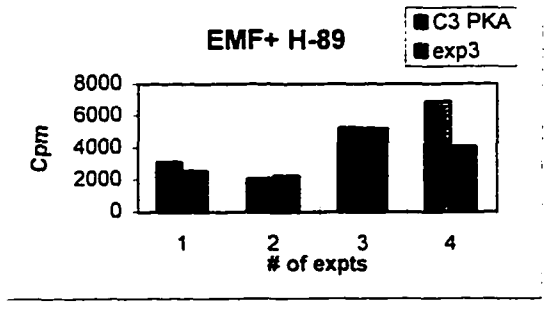






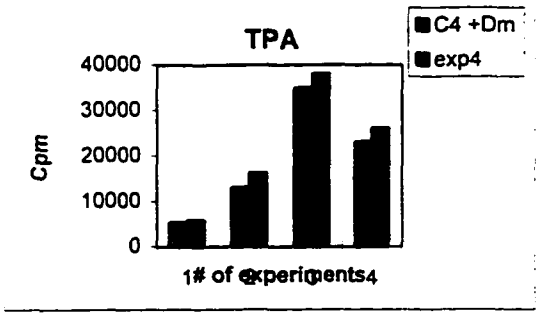


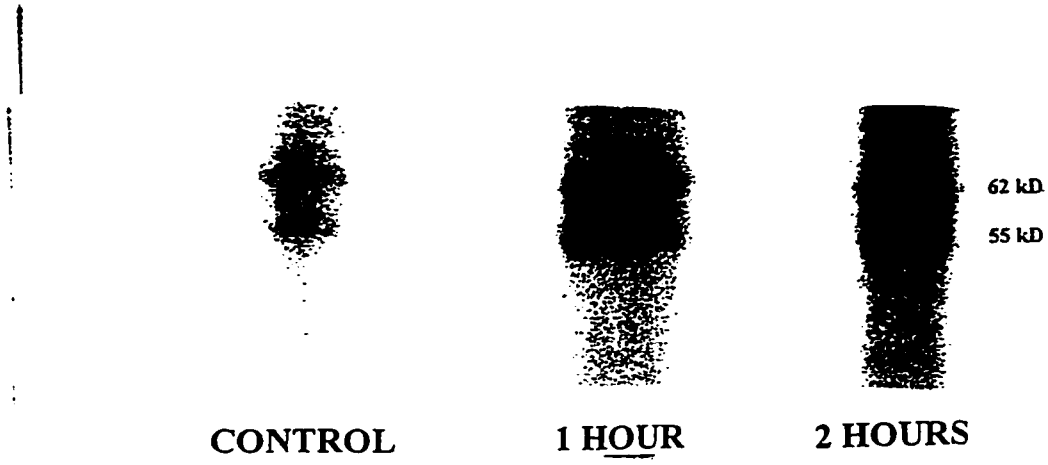
Figure 21. Time dependent expression of phosphorylated Fos protein following EMF exposure.

A. Immunoprecipitation was performed on protein extracts from radio labeled HeLa cells. Fos protein was immunoprecipitated with the *c-fos* polyclonal antibody (Santa Cruz Laboratory) and run on a 10% SDS gel. The gel was dried and exposed overnight at -70°C. Fos protein bands corresponded to the MW of 55 to 62 KD depending on the uptake of ^{32}P. The above gel depicts the uptake of ^{32}P by Fos protein after 1 hour and 2 hours of incubation following a 20 minute EMF exposure.

B. Time dependent increase ^{32}P uptake by the Fos protein 0, 20, 40, 60 and 120 minutes following a EMF exposure of 20 minutes is seen. Maximum uptake is seen at 120 minutes, showing that EMF increases the amount ^{32}P uptake into Fos protein.

C. P^{32} uptake was measured by using a densitometer PSI Image Quant. Control and experimental values for each time point were obtained after background noise correction.

A



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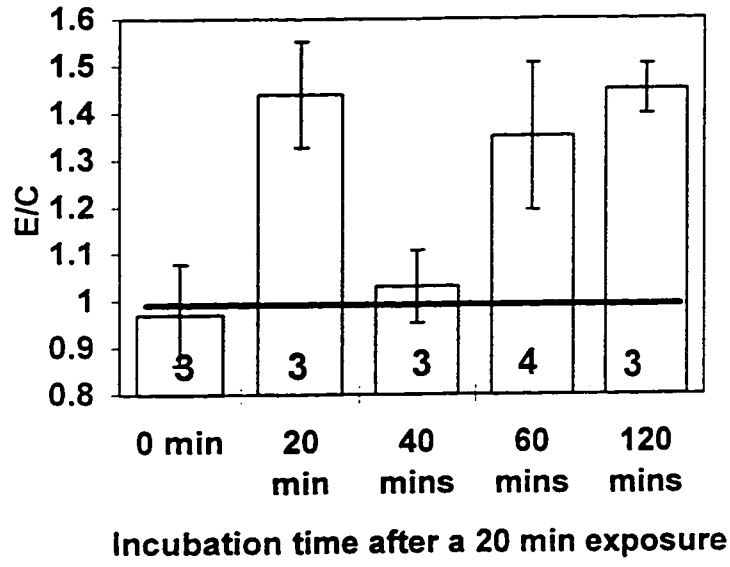

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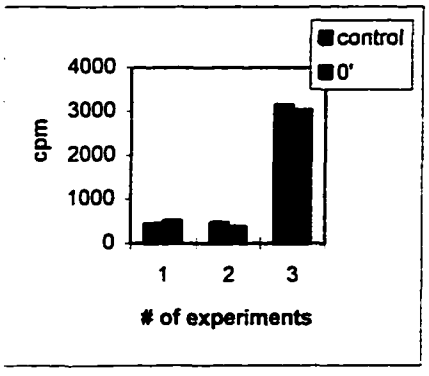

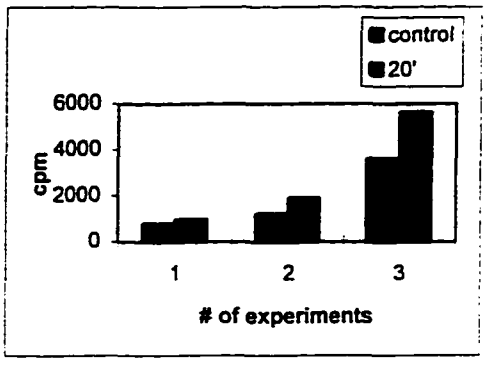

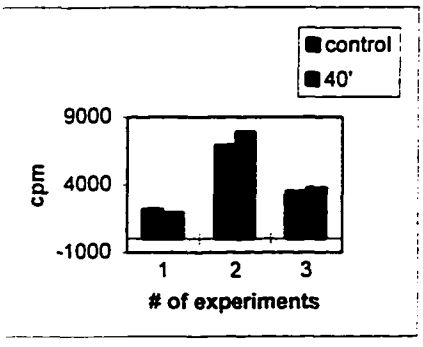

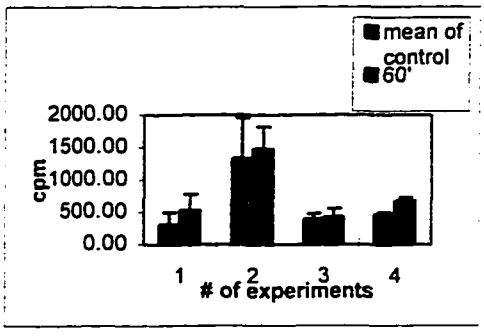

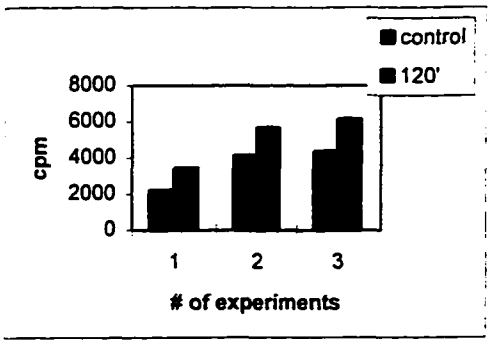

was estimated by the use of a densitometer. The time-dependent increases in uptake of ^{32}P of the Fos protein was unexpectedly bimodal (Figure 21b). An increase in ^{32}P uptake was seen after 20 minutes following exposure (40%), but after 40 minutes the uptake of ^{32}P was at basal levels. Uptake of ^{32}P increased after one hour and reached an increase of about 50% after 2 hours following exposure. The bimodal expression of the uptake of ^{32}P suggest that EMF activated kinases might be sensitive to state of the Fos protein (bound, unbound) and depending upon the state of the protein, uptake of ^{32}P is enhanced or eliminated. The experiment, however, did demonstrate that EMFs cause an increase in the uptake of ^{32}P by the FOS protein, consistent with that observed under conditions of TPA induction (Tratner *et al.*, 1990). The increase in the uptake of ^{32}P might be due to the increase in total FOS protein following EMF exposure or just an increase in the phosphorylated protein population. Since the purpose of the experiment was to measure the uptake of ^{32}P, the source of the increase was not investigated.

CONCLUSIONS

This research demonstrated that EMF affects the regulation of the c-fos gene, resulting in the amplification of its message. The effects of the fields were further confirmed by deletion analysis which demonstrated that the SRE/AP1 site opportunity to study a system that responded to both TPA and EMF. Studies, which compared the mechanism of interaction of both TPA and EMF,

demonstrated that is responsible for the increase in CAT expression seen after EMF exposure. The SRE/ AP1 site is also responsive to TPA. The promoter system provided a uniquemodel on how the fields interacted with the cells in a manner similar to that of TPA.

Further studies showed that, like TPA, EMF required the activity of the PKC and PKA enzymes. Similarities in the interaction of these two agents were not restricted to the promoter itself as increases in the phosphorylated protein after EMF exposure corresponded with those elicited by TPA (the degree of phosphorylation differed). It therefore can be concluded that EMF activates signal transduction processes in a manner similar to that of TPA.

DISCUSSION

Overview. The ultimate goal of this research is to find out if cancer-related changes occur in human cells exposed to EMF. The experiments described here were initiated on the basis of epidemiological data which indicates a relationship between EMF exposure and elevated risks to cancer, particularly lymphoma, nervous system tumors and childhood leukemia. The present research built on the hypothesis that altered signal transduction processes result from EMF exposure. Implications of this hypothesis are that unregulated expression in critical transcripts could conceivably augment a propensity to neoplastic disease.

The present study was also designed to develop an endpoint for measurement in cells exposed to electromagnetic fields (EMFs) that was sensitive, unambiguous and could be correlated with what is known about inductive pathways in the cell. The design also provided a means of testing possible mechanisms for EMF-cell interaction.

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 effect of EMF on regulation was analyzed indirectly by monitoring the expression of CAT driven by the c-*fos* promoter. C-*fos* upstream regulatory regions are necessary to be EMF response which was time dependent, and similar to effects elicited by mitogenic and other inducing factors. Deletion analysis of the upstream regulatory

regions showed that the response of cells to EMF exposure is mediated at the SRE/AP1site.

One model for this study is the effect of phorbol esters (the most intensively studied of cancer promoters), which can cause differentiative changes in HL-60 cells. Without prior initiation, tumor promoters do not necessarily affect cells permanently; following initiation, they can set in motion irreversible alterations in cells. This scenario is consistent with proposed mechanisms for the putative role of EMF exposure in malignancy.

The SRE/AP1 region is sensitive to TPA, and a comparison was made between the effects of TPA and EMF. TPA and EMF driven CAT expression were not synergistic. Once the pathway was initiated by either TPA or EMF, the other factor was unable to elicit a response. Since PKC and PKA are activated by TPA, the functions of these two enzymes in EMF response were analyzed. Specific inhibitors negated the EMF response, indicating that both enzymes were necessary. Other similarities in EMF and TPA induction were determined in post-translational modification studies.

EMF and tumorigenesis. A direct association between EMF exposure and malignancy requires experimental substantiation that cell function(s) known to be altered during the course of malignant progression or transformation is also altered by exposure. How cells could translate "information" from EMF exposure to a neoplastic condition as a part of a multi-step progression remains an enigma.

Yet, the possibility of a health risk provides strong motivation to provide a possible mechanism by which EMFs could alter cell function in a manner consistent with tumor promotion and /or progression.

A long held tenet in experimental oncology is based on the theory of multistep transformation resulting from the broad categories of initiation and promotion. Initiation is usually associated with heritable changes in DNA. This research plan assumed that it was improbable that EMF exposure could initiate tumor development. If EMF exposure can play a role in carcinogenesis, exposure will promote the process once initiated; *i.e.*, increase the chance that a neoplastic phenotype will be expressed. The failure to show genetic changes or chromosomal anomalies, DNA crosslinks, changes in DNA repair, or sister-chromatid exchanges in cells exposed to EMFs, suggests a possible role in promotion, rather than initiation (Nair *et al.*, 1989).

The role usually designated to EMF exposure is that of co-promotion (Cain *et al.*, 1993; Stuchly *et al.*, 1992). EMF exposure affects normal growth and differentiation, which is consistent with copromotion. Observations in the growth characteristics of cells following EMF exposure include increased DNA synthesis, developmental rates, and steady state transcript levels of some genes (Byus *et al.*, 1988; Delgado *et al.*, 1987 and Phillips *et al.*, 1992).

Whether the bioeffects caused by EMF could be directly related to transforming characteristics or the augmentation of pre-existing neoplasia, is still

unclear. Our results, however, are consistent with the hypothesis that de- or unregulated expression can occur following EMF exposures. This could possibly augment a neoplastic progression.

Experimental Designs. One of the major problems in EMF studies has been designing system(s) that are sensitive enough to measure small changes following EMF exposure. Further, there are many factors that effect expression including cell type, cell density, frequency range and exposure systems, as well as unknown factors. It was imperative to set up a system which used extremely stringent conditions and had well-defined parameters since virtually every study on the effects of EMF exposure is subject to controversy.

The debate which has arisen from EMF exposure studies results, in part, from lack of a proven mechanism (see Bennett, 1994), but also from variability in both epidemiological and molecular data (Saffer *et al.*, 1995: Lacy-Hulbert *et al.*, 1995). The mechanisms by which cells recognize EMF is still far from being solved. Some of the problems stem from the weak energies generated by EMFs which could be swamped by natural fields generated by the cell. A membrane potential of 0.1V creates an enormous electric barrier of 10^5 V/cm, obviously greater then the weak component of environmental electromagnetic fields.

At the experimental level, it is critical to utilize both positive and negative controls. The use of positive controls (TPA) in the present experiments confirmed that the design was sensitive to experimental changes. Cells were obtained from

a single flask to ensure that the comparisons between experimental and control flasks were valid. This, along with sham -sham experiments, ensured that the increase in CAT expression was not due to external factors, such as other exogenous fields or handling of cells.

Unlike usual research which studies induction, studies with EMFs have to consider the parameters of the exposure systems. These parameters include measurements of field strength, cellular geometry and exposure geometry. It is still unproven if the magnetic, electric field, the induced electric field or any combination is biologically the effective agent. It is generally considered that the magnetic field is the critical element which is responsible for the bioresponse, as the electric field is easily shielded by the plasma membrane. This remains to be proven, however.

A further complication arises from persuasive experiments which support a "window" phenomenon. Bassett *et al* (1994) hypothesized that certain thresholds should be met by specific field parameters. These thresholds depend on the cellular targets(s) and tissue environment. The most commonly studied parameters for induced response have been field strength and the time of exposure. Setting up ideal expression conditions was imperative to the success of the present experiments. Determining the time dependent course of CAT expression defined one window. Another parameter, that of field strength, had been previously defined for *c-fos* transcripts, and was based on a minimum field

strength (at environmental levels) to achieve a statistically significant effect.

Another of the fundamental problems of EMF research that has been debated is what constitutes an appropriate internal control. This is not normally a problem where the degree of change in a transcription assay is large. It is necessary to have the least variable internal control possible where the difference between unexposed and exposed samples are expected to be small. Different genes have been tested, including actin, histones, rRNA and GAPDH. None of these is absolutely satisfactory (Kochlaty *et al.*, in manuscript). In this study, β2-microglobulin (β2-M) was used as an internal control for steady state transcript analysis. It has been demonstrated that the use of this as an internal control could increase the variability of the results, but β2-M was used here for lack of a more appropriate control.

The *c-fos* promoter complex. The *c-fos* promoter region is well-studied. It is sensitive to several mitogens and the responsive regions have been mapped. This provided an opportunity in the present experiments to indirectly test whether EMF exposure could affect cell regulation via signal transduction pathways. The SRE/AP1 site was found to be necessary for an increase in CAT expression following EMF exposure. The SRE/AP1 region of the promoter is sensitive to several environmental cues and is pivotal in the regulation of the gene. This region is responsive to nerve growth factor (NGF), insulin, serum and TPA (Stumpo *et al.*, 1988; Visavader *et al.*, 1988; Prywes *et al.*, 1988). It is also a

region for binding of the retinoblastoma protein and phosphorylated Fos protein, both of which repress promoter expression (Robbins *et al.*, 1990; Sassone-Corsi *et al.*, 1988). The SRE/API site is sensitive to retinoic acid, which down regulates the promoter (Busam *et al.*, 1992).

EMF and signal transduction. The present study was based on several experimental observations which support the hypothesis that EMF exposures affect intracellular functioning by using a signal transduction pathway(s) (rev. in Liburdy *et al.*, 1994). The primary biological effect of nonionizing electromagnetic radiation has to be indirect and initiate at the cell membrane. The membranal component of the cell performs a triple role- in signal detection, signal amplification and signal transduction into the cell interior (Adey *et al.*, 1992). A likely scenario is that the signal on the cell surface is recognized by a receptor. This is translated into calcium influx, amplifying the signal and activating several enzymes, including PKC and PKA. Enzymatic activation progresses through the cell by a signal transduction mechanism ultimately inducing the production of the protooncogenes c-*myc*, c-*fos* and c-jun, among other genes. Acceptance of this scenario has not been universal, as it has not been conclusively demonstrated. The present results, however, are consistent with this scheme.

A comparison between TPA and EMFs in the present experiments allowed a comparison of changes in cell state consistent with *in vivo* promoting activity. Earlier studies indicated that phorbol esters and EMF act as co-promoters (Cain

et al., 1993; McLean *et al.*, 1991). TPA is a potent promoting agent. It acts on the cell membrane and activates the positive, but not the negative feedback of PKC. The positive forward action of PKC activates protooncogenes and interleukin receptors. Negative feedback of PKC is extended to long term responses, such as cell proliferation. PKC phosphorylates the receptor and functionally down regulates it. Prolonged TPA exposure causes persistent activation of PKC and thereby its depletion. Removal of PKC relieves the negative feedback control exerted by the enzyme and leads to uncontrolled cell proliferation.

Promotion studies in cultured cells and animal studies have examined the joint action of phorbol esters and EMF, since both act on cell membranes. TPA association with EMF was first demonstrated by Cain *et al* (1993) who showed that TPA and EMF increased co-foci density, which was not seen by the addition of EMF alone. Normal cells (C3H/10T1/2 fibroblast cells) and their UV mutated daughter cells, which behave as cancer cells, were co-cultured or placed together in cell culture. Contact with the parent cells inhibits the unregulated growth of the daughter cells. Addition of TPA and a 60 Hz (0.1mT) field for 1 hour unbalanced the contact equilibrium allowing the return of unregulated growth of daughter cells and the formation of tumors (foci) in the culture dish by 60%. Cancer promotion studies on mice skin treated with dimethylbenzomtherene (DMBA) showed a greater number of tumors when treated with TPA and EMF together, as compared with treatment with TPA alone (McLean *et al.*, 1991). These studies suggested

that TPA and EMF act as co-promoters. It must be emphasized, that in these studies EMFs had no effect on the cells alone. It therefore could be concluded from these experiments that EMF enhanced TPA function by initiating systems outside the cell, but not the signaling processes itself (as the fields by themselves did not activate the response).

Other studies focused on PKC activity in relation to TPA and EMF. Luben *et al.*, (1994), compared the action of 60Hz, 0.1mT sinusoidal magnetic field with that of TPA. Both produced rapid transient increases of PKC enzymatic activity in the cell membrane, while reducing activity in the cell cytoplasm. Turning off the field led to a recovery of PKC in the cell, as determined by 4-5 fold increase in total cell PKC activity over a 4 hour period.

Our results showed that the increase in CAT expression after EMF exposure requires the SRE/AP1 region, which is responsive to TPA. A comparative study was done to determine if the pathway(s) activated by EMF and TPA overlap. The results demonstrated that EMFs and the tumor promoting agent utilize similar pathways in the activation of the c-*fos* regulator. Inhibition studies further confirmed these results by showing that PKC was needed for EMF-induced increases in CAT expression driven by the c-*fos* promoter.

There are several different isoforms of PKC, which are divided into two groups. PKC Group A contains the subspecies α, βI, β II and γ, whereas Group B contains the subspecies δ, ϵ and ζ (Nishizuka *et al.*, 1988). The tissue and

intracellular distribution of the various subspecies is different and is dependent on the state of proliferation of the cells (Makowske *et al.*, 1988).

The activation of different isoforms by EMF exposure or TPA could explain, in part, the fact that the present results are somewhat inconsistent with earlier data that indicate that EMF and TPA act as copromotion agents. In the copromotion systems, EMF alone was unable to enhance detectable cellular changes. The researchers proposed that EMFs might activate undefined processes outside the cell that enhance TPA activity. It also should be noted that in the co-promotion experiments, long term events were being monitored. In this study, the effects that were monitored were short term, and capable of activation by EMF alone.

Although there is strong evidence that EMF and TPA act along similar pathways, there are differences in the kinetics of the response they elicited relative to CAT protein expression. The response to EMF is extremely rapid and gives about a 25% increase in the expression of CAT protein. The response to TPA, on the other hand, is seen after 2 hours of exposure and reaches a maximum value of 50% after 6 hours. These differences could be reconciled if EMF activates a subset of the TPA receptors. For example, while TPA is able to activate all isoforms of PKC, thymeleatoxin, another phorbol ester (Thy) can activate only group A PKCs (MacDonald *et al.*, 1994). Consequently, TPA demonstrated a 40-fold more potent activation of DNA synthesis than Thy. This

indicated that different stimuli are selective in the PKC isoforms they activate and elicit different potentials of the same response (MacDonald *et al.*, 1994). The comparison between EMF exposure and TPA in the present experiments suggests the possibility that EMF activates some of the receptors activated by TPA, but not all of them, resulting in different intensity of the same response.

It has also been demonstrated that different isoforms of PKC demonstrate different kinetics in activation by several stimuli. Insulin and TPA activate similar PKC isoforms, yet they have distinctly different effects on the subcellular distribution and activation of the isoforms. Insulin, but not phorbol ester, caused the translocation of PKC beta to the plasma membrane, whereas phorbol ester-induced redistribution of PKC alpha to the plasma membrane was greater then insulin (Farese *et al.*, 1992). Similarly, different subspecies of PKC are activated by a series of phospholipid metabolites, such as diacylglyerol, arachidonic acid and lipoxin A, that are produced in successive phases of the response of the cell to the stimulation of cell surface receptors. These observations, when taken together, indicate that each isoform of PKC has a distinct set of characteristics, kinetics and functions. The kinetics of the TPA and EMF reaction also suggests that the putative isoform activated by EMF reacts rapidly and does not elicit a very strong response.

EMF and post-translational modification. In a series of related experiments, post translational modification of c-*fos* was analyzed following EMF

exposure. Experiments with the PKA inhibitor showed that EMFs activates the enzyme PKA. PKA has been shown to phosphorylate several different proteins, including the Fos protein. Fos protein is also phosphorylated by several kinases such cdc2, casein kinase II (CKII) and glycogen synthase kinase. These kinases are selective in their recognition and phosphorylation of the bound, unbound and dimerized states of the Fos and Jun proteins (Abate *et al.*, 1993). *In vivo* labeling studies showed that there was an increase in radioactive ^{32}P in Fos protein following EMF exposure. The kinetics and the magnitude of the increase vary from that of cells exposed to TPA. A maximum increase was seen 2 hours following exposure similar to TPA. The time dependent increase following EMF exposures produced a bimodal curve. The bimodal response might be due to kinases that are activated following EMF induction that are unable to phosphorylate the Fos protein at 40 minutes since it could be in the bound state. The lack of phosphorylation response at 40 minutes could also be due to the instability of the phosphorylated Fos protein (Doucet *et al.*, 1990). Nevertheless, the increase in ^{32}P uptake into *c-fos* protein following EMF exposure showed that EMFs may induce the autoregulatory system of the Fos protein. This explained the rapid increase and decrease previously observed in the steady state transcript levels of c-*fos*.

Conclusions. 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. 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. Portions of our research findings are consistent with this working model. The most persuasive finding is an involvement of the SRE/AP1 site following EMF exposure. THE SRE/AP1 site is directly involved in the response to TPA. Exposure to TPA results in the translocation of conventional PKC isoforms from cytosolic to the membrane fractions (Blumberg *et al.*, 1988). Translocation of PKC results in transcriptional activation, leading to early expression and regulation of some genes, including Fos (Hirai *et al.*, 1994). Even though there is strong indication that EMFs use signal transduction processes in the cell, its identification as a health risk is still not universal and will require further investigation.

Future studies. There are still many questions to be answered, including the relationship between time and dose to TPA versus EMF exposure. Other problems include the identification of the isoform(s) that are affected by EMFs. This identification would also help to better define the kinetics of the expression elicited by EMFs from the cells. One of the primary targets activated by TPA is the AP1 factor. The role of the AP1 factor in the initiation and activation of the EMF induced signal transduction should be identified. Further, this study showed that

the EMF induced increases in CAT expression driven by the *c-fos* promoter required the SRE/ AP1 site. It would be important to see if binding of the AP1 (by using the gel mobility shift assay) to this region was increased after exposure to EMFs.

It is clear that other factors must be investigated before any conclusive evidence can be drawn about the pathway used by EMFs. There is also no means of knowing the effect of what is a very transient response on the part of cells exposed to EMF relative to the potential for participation in malignant progression. Proof of a general mechanism for EMF effects on the cell, or as health risk for the population, will require addressing these problems as well as further investigations as to feasible means for the interaction of EMFs at the cell membrane level.

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