Order Number 9521274

Effects of extremely low frequency electromagnetic fields on transcription and translation of a transformed human fibroblast cell line

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City University of New York, 1995

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EFFECTS OF EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS ON TRANSCRIPTION AND TRANSLATION OF A TRANSFORMED HUMAN FIBROBLAST CELL LINE

By

Steven Gold

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

1995

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

EFFECTS OF EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS ON SELECTED TRANSCRIPTION AND TRANSLATION OF A TRANSFORMED HUMAN FIBROBLAST CELL LINE

By

Steven Gold

Adviser: Professor Ann S. Henderson

The purpose of this study was to examine the transcriptional and translational responses of an SV40-transformed human fibroblast cell line, HAL, exposed to a 20-minute, 60Hz electromagnetic field. SV40 mRNA levels were found to increase in both Helmholtz-aiding and solenoid exposure systems based on measurements of the viral large-T antigen. The translational product of large-T antigen was seen to increase in a Helmholtz-aiding system.

Large-T antigen is known to increase heat shock 70 (hsp70) transcription. Using a reverse transcription-polymerase chain reaction (RT-PCR) protocol, a comparison of the heat shock and electromagnetic responses in the HAL cell line was undertaken based on earlier work which suggested a correlation between

electromagnetic exposure and heat shock in cells. Transcript levels of hsp70, c-*fos*, c-*myc*, p53 and c-*jun* were measured in a 20-minute, 60Hz field. HAL cells were heat-shocked for periods of 4 hrs and 20 minutes, respectively, with subsequent measurements made of hsp70, c-*fos*, c-*myc* and c-*jun* transcript levels. EM-exposed cells elicited a notable increase in c-*myc* and p53 mRNA levels and essentially no change in hsp70 levels; heat-shocked cells resulted in large increases of hsp70 following either 4hr or 20-minute incubation. In summary, the transcriptional patterns observed in cells exposed to electromagnetic fields and those that were heat shocked differed, although evidence is presented that electromagnetic exposure represents a form of cellular stress.

Acknowledgements

The age of the solitary, self-sufficient researcher, working in blissful isolation from society, is long since past. With this thought in mind, what follows is a list of those who have assisted me during the past several years.

First, I wish to thank the members of my committee for their encouragement and guidance: Dr. Shirley Raps, Dr. Arthur Pilla and Dr. Rivka Rudner. I wish also to thank Dr. Harvey Ozer for providing me with the HAL cell line and monoclonal antibody which enabled me to perform several protein experiments.

Through the years I've received a great deal of technical assistance: Dr. Lois Resnick-Silverman taught me the Western blot protocol; Dr. Rouzan Karabakhtsian carried out the RT-PCR experiments on my RNA samples, using a method that was originally standardized by Dr. Natasha Broude; Drs. L-X Wei, Johann Bumann, Michael Hadjiargyrou, Phillipos Patsalis and Kathi McDowall demonstrated various laboratory techniques associated with molecular biology; Victoria Kaganovskaya provided tables and figures for this dissertation; Sharmila Rao, Natasha Shalts and Alun Uluc allowed me to share their goodwill and friendship.

During my tenure at Hunter, I was fortunate enough to get to know Dr. Reba Goodman, whose great good humor helped make the years pass far more quickly than they had any right to. For this reason, as well as her kindness toward me, I offer my heartfelt thanks.

Last, but far from least, I salute Dr. Ann Henderson, who, by agreeing to take me on as a student, now doubt rescued me from the clutches of juvenile deliquency. Adjie's patience and understanding--which were sorely tested in my case--provided a sturdy foundation for learning. I was very lucky indeed; I learned from the best.

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Introduction

Background

It is known that ionizing electromagnetic fields, *e.g.*, X-ray, UV or gamma rays, alter the biosynthetic apparatus of a cell in ways which are detectable to the researcher, and which are often deleterious to animal life in general [see Watson *et al.* (1987) for review]. It has also been established that strong nonionizing fields, such as those in the microwave range, can heat human tissue to levels sufficiently high to cause noticeable damage (Paulsson *et al.*, 1979). More recently, increasing attention has been focused on the relationship between weak nonionizing forms of radiation and biological systems. Energies in this range produce no heat and do not cause obvious biological damage (reviewed by Tenforde, 1993). The electromagnetic spectrum, with emphasis on its nonionizing components, is shown in Figure 1.

The most common source of environmental, nonionizing energy is the flow of electricity generated by an alternating current, the development of which began in earnest during the latter part of the previous century. The result is a world saturated with nonionizing radiation. The electric field produced by an alternating current generates a perpendicular fluctuating magnetic field. The electromagnetic (EM) field is described on the basis of the number of fluctuations-frequency-per second (**Hz**); the magnetic (B-field) component is measured in Tesla (**T**); and the

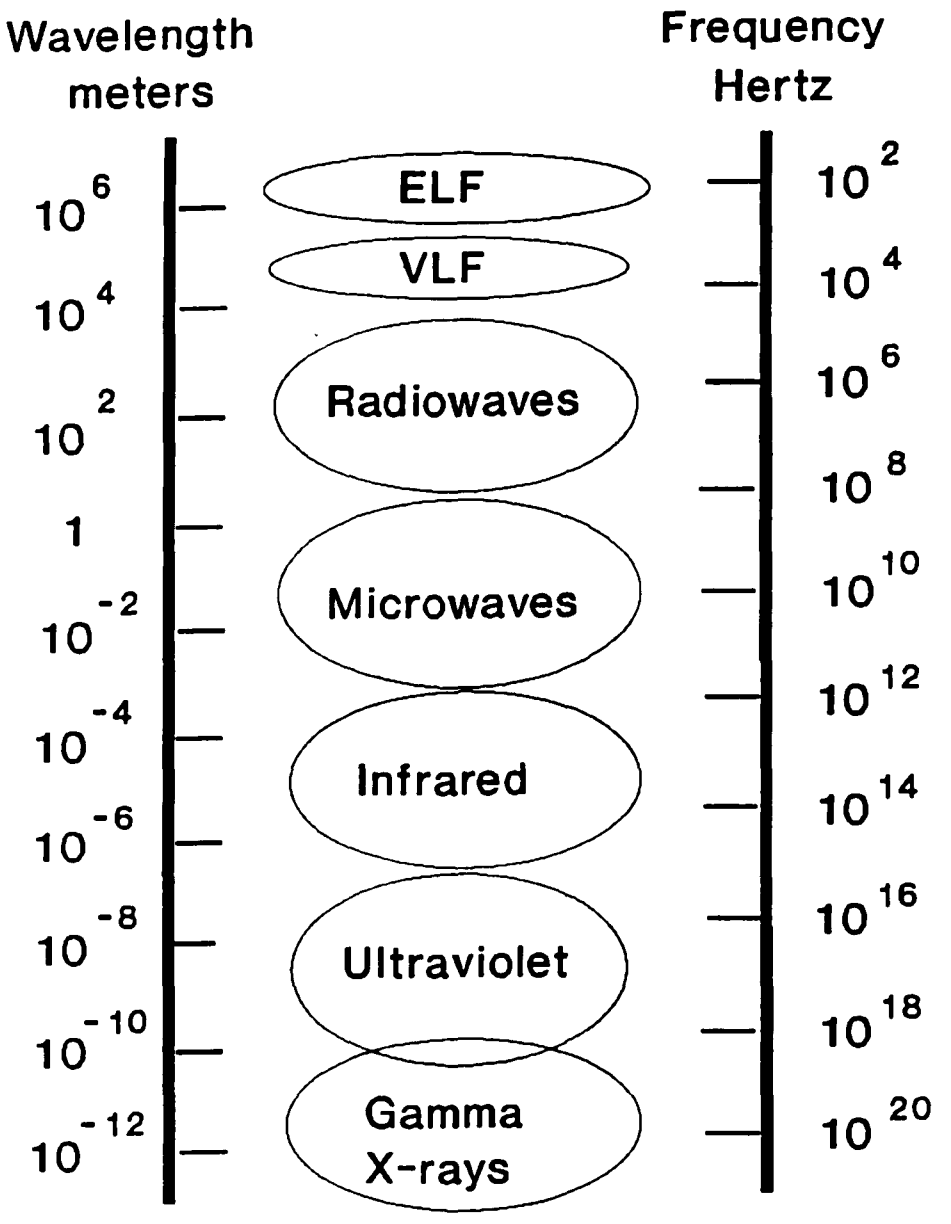


Figure 1. Scale of electromagnetic radiation

electric (E-field) component in volts per meter (V/M). Most artificially induced nonionizing radiation falls within the frequency range 0-300 Hz (Tenforde, 1993) and is designated "elf" (extremely low frequency). Many household appliances in the USA operate at a frequency of 60 Hz. The wave generated is sinusoid.

It is becoming increasingly important to determine whether or not exposure to EM fields could be harmful to human health. An unanswered question concerns dosimetry, particularly as it relates to risk assessment. Dose-response parameters have yet to be fully established, notwithstanding epidemiological data collected to date. This is one reason for the controversy surrounding the issue of human exposure to EM fields. Another reason, put forth by physicists, is that EMF amplitudes fall below the potential of the cell membrane [1.66×10^{-14} J/m (Joules/meter)] and are thus incapable of eliciting a biologic response. It has been argued, however, that cell-cell communication, in the form of gap junctions, may increase EMF sensitivity by several orders of magnitude (Pilla, 1993).

Organismic Studies

Results of whole animal studies provide evidence that EM exposure causes a measurable biologic response. Many of these investigations have used a particular type of exposure signal: asymmetric, time-varying pulsing

electromagnetic fields (PEMFs). Ottani *et al.* (1988) created surgical skin wounds in rats and subjected them to periodic 12-hr, 50 **Hz** (8mT) PEMF exposures. Treated animals showed enhanced vascular network regeneration in the damaged area compared to untreated animals, implying a more rapid recovery from the invasive surgical procedure. PEMFS in fact serve a clinical function as a means of facilitating proper healing in patients with bone non-unions (Bassett, 1989). Ryaby *et al.* (1993) showed a 76.6 **Hz** sinusoid field to be capable of preventing osteopenic bone loss in rats to a degree similar to that achieved with hormonal replacement, thereby demonstrating a possible therapeutic role in the treatment of osteoporosis in humans. Potts (1993) believes EM fields may serve a purpose in expediting delivery of pharmaceutical drugs to target tissues by adjusting endogenous field gradients to allow for increased efficiency of penetration.

Other investigations imply harmful effects of exposure. For example, de Jager and de Bruyn (1993) performed generational studies of mice continuously exposed to a 50 **Hz** (10K V/m) electric field. Their findings revealed a higher rate of death in the experimental group during a span of two generations. Changes in pineal gland activity in rats exposed to a Helmholtz-generated magnetic field were also seen; the field, an inversion of the earth's magnetic field, caused a decrease in serotonin levels (Lerchl *et al.*, 1990). Wever (1973) demonstrated

that exposure to a 10 Hz (40 μT) field decreased the length of circadian rhythms in humans. This led to the proposal that long-term exposure may promote depression in humans since a known symptom involves changes in circadian rhythms, which are regulated by the pineal gland (Wilson, 1988).

Epidemiology

Attempts to elucidate a mechanism(s) of elf interaction have been given increased urgency based on epidemiological reports linking a two- to three-fold increase in the occurrence of childhood leukemias in children living in proximity to power lines and substations (Wertheimer and Leeper, 1979). Milham (1988) examined the death certificates of almost 2500 amateur radio operators covering a five-year period and noted a 25% increase in the rate of death due to lymphatic cancer. On the other hand, Fulton *et al.* (1980) saw no connection between the incidence of childhood leukemias and exposure. Nasca *et al.* (1988) reached a similar conclusion in a study of childhood cancer patients from the perspective of paternal exposure to EM fields. Epidemiological data provide evidence for pathogenicity of nonionizing radiation although other factors, *e.g.*, simultaneous exposure to known carcinogens such as benzene or asbestos, cannot be ruled out as causes of increased cancer rates. In any event, there is no evidence that EM fields cause tumor synthesis *de novo* in animals (Reese *et al.*, 1988;

Rosenthal *et al.*, 1989); rather, they are thought to play a pathogenic role by enhancing the growth of malignant tumors already present (Byus et al., 1989).

A connection between particular types of cancer and specific job occupations would strengthen interpretation of epidemiological results heretofore obtained. Tynes and Andersen (1990) found a two-fold increase in male breast cancer occurrence among electrical workers. Demers *et al.* (1991) saw a six-fold increase in male breast cancer cases among electricians, telephone linemen and electric power workers, and a 2.9-fold increase among radio and communications workers. Indeed, an increase in malignant disease was found for any worker exposed to electromagnetic fields while on the job.

Cellular Investigations

A large body of literature is being created concerning cellular behavior vis-a-vis EM fields. A brief overview reveals the diversity of the response; in several cases the results are conflicting. For example, human lymphocyte proliferation was shown by Mehta *et al.* (1993) to increase in a 15 **Hz** (1G) magnetic field, an observation thought to be attributed to a concomitant rise in levels of the cytokine IL-2. An interesting finding by Fitzsimmons *et al.* (1993), and one with clinical implications as well, showed the IGF-II system, a regulator of bone growth, is responsive to a combined AC-DC field with a peak seen at 76 **Hz**. Significantly,

amplitudes below the thermal noise level, calculated to be 6.4×10^{-21} J for these experiments, increased bone cell growth *in vitro*. Cridland *et al.* (1993), however, did not observe a change in human fibroblast proliferation following exposure to a 50 **Hz** (2mT) magnetic field. Exposure parameters vary from study to study, thereby adding to the difficulty concerning data interpretation. Evidence nonetheless exists that EM fields act as a co-factor in stimulating cell growth. Cridland *et al.* (1993) noted an increase in proliferation of mitogen-stimulated fibroblasts placed in a 50 **Hz** magnetic field. Proliferation of PHA-induced human blood mononuclear cells was shown using radioactive incorporation assays (Cossarizza *et al.*, 1989). Seen in this light, EM fields may serve an enhancement role in promoting cell growth in the presence of an additional mitogenic factor.

Other studies have focused specifically on aspects of overall DNA synthesis as they relate to EM exposure. Takahashi *et al.* (1986) noted quantitative changes in DNA synthesis in mammalian cells exposed to pulsing EM fields of 10 and 100 **Hz**. Other studies have sought to link DNA and chromosomal aberrations to electric and/or magnetic fields. Results are inconclusive regarding promotion of chromosomal aberrations (Nordenson *et al.*, 1984; Nordenson *et al.*, 1988; Bauchinger *et al.*, 1981), and there is no evidence that changes occur in the single-strand DNA repair mechanism (Frazier *et al.*, 1990).

Any change in cell behavior would be due presumably to a component of the field itself, be it electrical, magnetic or both. Empirical data supports the view that each component, acting separately, can elicit a response at the cellular level (Marron *et al.*, 1988). Clinical experiments have in fact revealed the beneficial effects of separate electric (Lavine *et al.*, 1972) and magnetic fields (Weinstein *et al.*, 1990) in bone repair.

The principal challenge of cellular biology, as it relates to EM studies, is the delineation of a mechanism(s) of action in order to account for both clinical and epidemiological data. Further, it is desirable, if possible, to establish cellular threshold levels of exposure and thereby use these parameters as a guideline in quantifying proposed pathogenic effects at the organismic level.

Molecular studies

Concurrent with cellular investigations are those which attempt to understand the molecular biology of exposure. A seminal report found an overall transcriptional increase in insect salivary gland cells exposed to a pulsing EM field (Goodman *et al.*, 1983). A subsequent study, which used a symmetric sine wave as the source of exposure, likewise showed an increase in transcriptional activity (Goodman and Henderson, 1986). This work was expanded to include the human leukemia cell line HL-60, with enhanced amounts of transcript observed

for c-*myc*, β-actin and histone 2B in cells exposed to 60 and 72 **Hz** sine waves (Goodman *et al.*, 1992b). In addition, transcript levels of the mammalian tumor virus SV40 were found to be altered in a 60 **Hz** field based on a study using a virus-transformed human fibroblast cell line (Gold *et al.*, 1994).

Experiments with HL-60 cells have revealed the existence of exposure "windows" as well, the result of varying both frequency and time parameters (Goodman and Henderson, 1991a; Goodman *et al.*, 1992). There is no simple correlation between transcript level and exposure time or field strength. The studies of Goodman *et al.* (1991b) provide additional insight into the duration of the transcriptional response. An increase in c-*myc* mRNA levels was seen after four minutes exposure, with a maximum level reached at 20 minutes. Control levels, however, were observed at 4 hours in cells either exposed continuously or exposed for 20 minutes and held in the incubation chamber, with samples in each case taken at appropriate time intervals. Thus the response to exposure is rapid and the return to control levels after 4 hours implies the cells exhibit an adaptive mode. Adaptation could be accomplished either by "adjusting" transcription rate or rate of transcript turnover, or a combination of the two.

An alternative mode of investigation centers on the study of translational products of exposed cells. Murray and Farndale (1985) placed cultured fibroblasts in a 15 **Hz** (2.3 V/m) pulsing magnetic field and observed changes in collagen

production. A major protein product of SV40, large-T antigen, was increased in human fibroblasts exposed to a 60 **Hz** (7.5 uV/m) sinusoid field (Gold *et al.*, 1994). Exposure of murine L929 cells to a 60 **Hz** field was likewise shown to increase levels of the growth-associated enzyme ornithine decarboxylase (Byus *et al.*, 1987; Byus *et al.*, 1988). Mattsson *et al.* (1993) found an increase in ornithine decarboxylase (ODC) activity in HL-60 cells subjected to a 50 **Hz** sinusoid magnetic field. The latter results are notable in that increased ODC levels are linked to cancer (Heby and Persson, 1990).

Goodman and Henderson (1988) performed two-dimensional gel analysis of dipteran salivary gland cells that were placed in symmetric or asymmetric fields. Both qualitative and quantitative differences were evident. Proteins synthesized in exposed cells were not detected in controls. Interestingly, among those were five proteins normally made under heat shock conditions. Since the exposure signal is athermal, these data lend support to one view of biological response to EM fields as a stress event (Goodman and Henderson, 1990).

Proposed mechanisms

While there is general agreement that cells are affected by exposure to EM fields, there are many issues to consider, among them the physiological state of the cell at the time of exposure, the geometry of the flask containing the cell, the

frequency and/or amplitude, and the duration of exposure. These and other variables must be taken into consideration in proposing a mechanism of exposure. An additional matter concerns the contribution of the electric and magnetic components of the field. It is still not entirely clear which of these components, if either, takes precedence in eliciting a cellular response. Adey (1981) believes it is the electric field, by interacting with the cell surface, that is responsible for the biologic effect, as proposed in Figure 2. The electric field would produce a current in the surrounding fluid leading to changes in the surface charge of the cell. This in turn would result in receptor activation and a subsequent second messenger-type response. On the other hand, Marron *et al.* (1988) exposed the slime mold Physarum to a 60 Hz field (0.1mT; 1.0 V/m) and found both electric and magnetic components, acting separately, altered the cell surface. The electric field increased its negative charge and the magnetic field decreased its hydrophobicity. The latter effect could cause a change in membrane permeability such that hydrophilic molecules, formerly prohibited from crossing the membrane, would now be able to do so. It is possible the exposure field facilitates a realignment of membrane phospholipids, leading to the change in hydrophobicity.

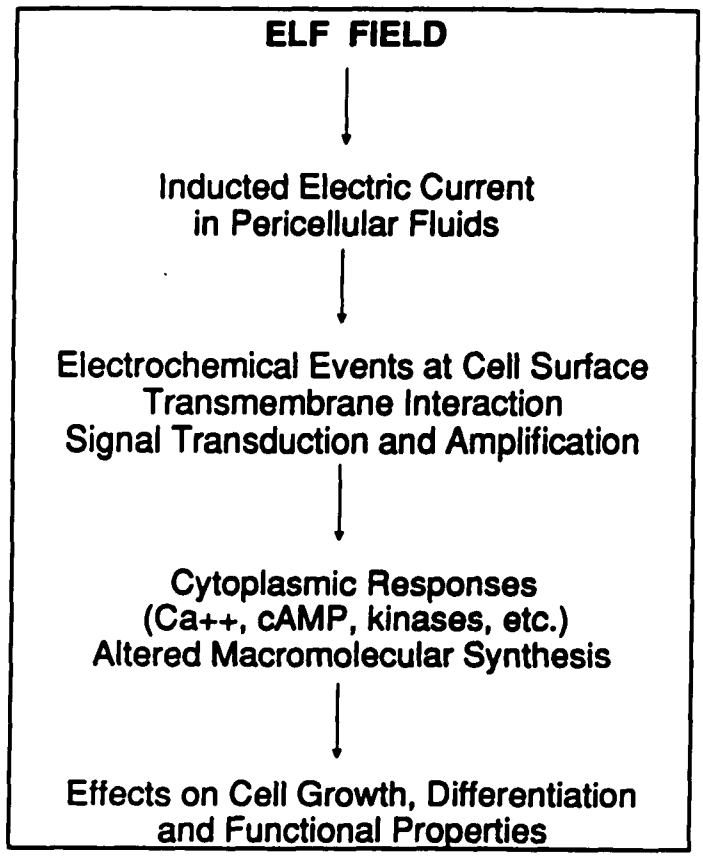


Figure 2. Proposed series of events resulting in cellular response to exposure. The cell membrane surface is theorized to play a key role in recognition of the signal.

Adapted from: T.S. Tenforde, (1993), Cellular and Molecular Pathways of extremely Low Frequency Electromagnetic Interactions with Living Systems. In M. Blank ed. Electricity and Magnetism in Biology and Medicine. San Francisco: San Francisco Press. p.5

There is evidence to support Adey's hypothesis of a secondary messenger-type response based on observed changes in intracellular calcium levels. Blackman *et al.* (1988) demonstrated increased levels of calcium uptake in brain tissue. Normal rat thymocytes exposed to a 60 Hz field were found to exhibit enhanced calcium uptake in the presence of the mitogen concanavalin A (Walleczek and Liburdy 1990; Liburdy *et al.*, 1993). These results implicate calcium

and, by extension, the cell membrane as mediators in the biological response to EM exposure. Broude *et al.* (1993) provided direct evidence for the role of the plasma membrane by demonstrating alterations in the stimulatory effects of c-*fos* and β-actin transcription in HL-60 cells. The effect is erased when the cells are treated beforehand with colchicine, which decreases the amplitude of plasma membrane fluctuations. Thus the membrane appears to play a role in the cell's response to exposure. Further, Karabakhtsian *et al.* (1993) demonstrated a role for calcium by conducting exposures in calcium-deficient media, the result being abolition of enhanced transcriptional activity of c-*fos* and c-*myc*. Blackman *et al.* (1991) discovered the presence of exposure "windows", as they relate to calcium transport across the plasma membrane; as with the results of transcriptional studies of Goodman and Henderson, these windows were frequency-dependent.

Calcium fluctuations may therefore constitute a form of cell recognition of EM fields.

The argument favoring calcium as a participant in the process is attractive given its role in a wide variety of cellular events, and this in turn may explain the varied cellular response to EM fields observed to date. The putative role of calcium in this process has led to the formulation of models for coupling EM fields to cells. Liboff and McCleod (1988) propose the Ion Cyclotron Resonance model (**ICR**), the basis of which involves an interaction between specific frequencies and membrane-sequestered ions, *e.g.*, calcium, in such a way as to affect uptake of the ion either in a positive or negative manner, depending on the frequency of exposure. This theory places greater emphasis on magnetic parameters than Adey's hypothesis, which assumes a less important role for the magnetic component of the field. Results of experiments designed to test the hypothesis when applied to combined AC-DC fields, however, are inconclusive (Parkinson and Hanks, 1989).

Lednev (1990) proposes that calcium-binding proteins, e.g., calmodulin, are the actual targets of EM field interactions, as opposed to any component(s) of the membrane itself, a theory designated Paramagnetic Resonance. Shuvalova *et al.* (1991) tested this theory by measuring the phosphorylation of light-chain myosin in solution in a magnetic field. This reaction is normally dependent upon

calcium-calmodulin interactions. They observed decreased phosphorylation of up to several hundred per cent at frequencies between 8 and 16 **Hz** for unpurified light-chain myosin and an increased amount of phosphorylation when purified light-chain myosin is used. They believe the level of available calcium is the determining factor in these results. Experiments utilizing an optical technique to measure calcium-calmodulin binding, however, were unable to provide evidence supporting Lednev's hypothesis (Bruckner-Lea *et al.*, 1992).

Rationale

This research is investigating selected transcriptional and translational responses of an SV40-transformed cell line (HAL) to defined elf EM fields. Our purpose is two-fold: 1) to measure gene expression and protein production of an integrated DNA, SV40, a virus which, among other things, causes increased transcriptional activity of the human heat shock 70 (hsp70) promoter by virtue of the viral large-T antigen, and 2) to compare the electromagnetic and heat shock response patterns of gene expression in HAL cells. The rationale for this set of experiments is based in part on SV40's influence on hsp70 expression, as well as prior evidence that EM exposure may facilitate a heat shock-type response in cells. In this way, information can be obtained concerning a mechanism of action in a cell line that was not previously studied in terms of EM exposure response.

Emphasis was placed on those genes involved in signal transduction pathways since our lab has done extensive work along these lines, albeit in a B-lymphocyte cell line, HL-60.

A. SV40 Biosynthesis

Uninfected wild-type fibroblasts normally manifest a limited life cycle in cell culture (Hayflick, 1961). There are, however, circumstances under which the barrier to cell proliferation is overcome, whether through spontaneous genetic mutation or infiltration into the cell of a foreign element such as the tumor virus SV40.

SV40 is a member of the papovavirus family of viruses. Its genome is composed of duplex DNA of approximately 5.2 Kb (reviewed in Tooze, 1981). Only simian cells are fully permissive hosts of the virus, with permissivity defined as the ability to propagate infectious viral particles (reviewed in Livingston and Bikel, 1986). Human cells can support DNA synthesis of SV40. Production of viral particles, however, does not occur. A Papovavirus can nevertheless cause the life span of certain cells, other than those of its natural host, to be extended indefinitely; the result is neoplastic transformation of the cell (reviewed in Tooze, 1981). For both permissive and nonpermissive cells, viral entry occurs via pinocytosis (Mattern *et al.*, 1966). This is followed by transport to the nucleus,

where the viral protein coat is shed. Most, if not all, aspects of SV40 assembly and biosynthesis occur in the nucleus (reviewed in Livingston and Bikel, 1986).

In permissive cells, SV40 synthesis proceeds along a pathway leading to lysis of the cell. When nonpermissive cells are infected with SV40, there occurs an alternative series of events, not entirely delineated, which culminates in neoplastic transformation. The basis of the transformed phenotype is integration of intact early region of SV40 DNA into a site in the host genome; specifically, that portion of the early region encompassing the amino terminal of large-T antigen (Rassoulzadegan *et al.*, 1982)

First isolated as a contaminant in polio vaccine preparations (Sweet and Hilleman, 1960), SV40 is a model system for study given its status as a small, well-characterized DNA tumor virus. The immortal phenotype of an SV40-transformed human cell line allows the researcher to perform an extended series of experiments. Further, in its integrated form the virus affords an opportunity to observe the behavior of what is in effect a genome within a genome. This simplifies the determination of specific mRNA and protein levels since the viral products are distinguishable from those of the host cell.

From a scientific perspective, the most intriguing product of SV40 is large-T antigen. It is a highly phosphorylated protein of 94Kd molecular weight which exists in monomeric or oligomeric form (reviewed in Livingston and Bikel, 1986).

In addition to its role in viral metabolism, large-T antigen functions as a DNA-dependent ATPASE (Giacherio and Hager, 1979: Tijian and Robbins, 1979) and exhibits serine/threonine protein kinase activity (Griffin *et al.*, 1979; Tijian and Robbins, 1979). Large-T can induce G_0/G_1 cells into S phase (Dulbecco *et al.*, 1965; Hiscott and Defendi, 1979; Hiscott and Defendi, 1981). It also enhances transcription of the human heat shock 70 (hsp70) promoter, perhaps by interacting with transcription factors required for hsp70 expression (Taylor *et al.*, 1989). Thus large-T antigen's presence may trigger a heat shock stress response at temperatures which would not ordinarily elicit such behavior.

The effect of EM fields on gene expression and protein production in a virus was not previously investigated. We chose to examine the DNA tumor virus SV40 for this and other reasons. First, it is well-understood in genetic terms, as shown in Figure 3. Within its promoter region are several copies of the consensus sequence TGACTA, a sequence shared by a number of genes involved in signal transduction pathways such as *c-jun* and *c-fos* (Lee *et al.*, 1987). These genes were shown to be responsive to EM fields (Phillips *et al.*, 1992) . It is possible SV40 is subject to the same transcriptional control based on the presence of the consensus sequence.

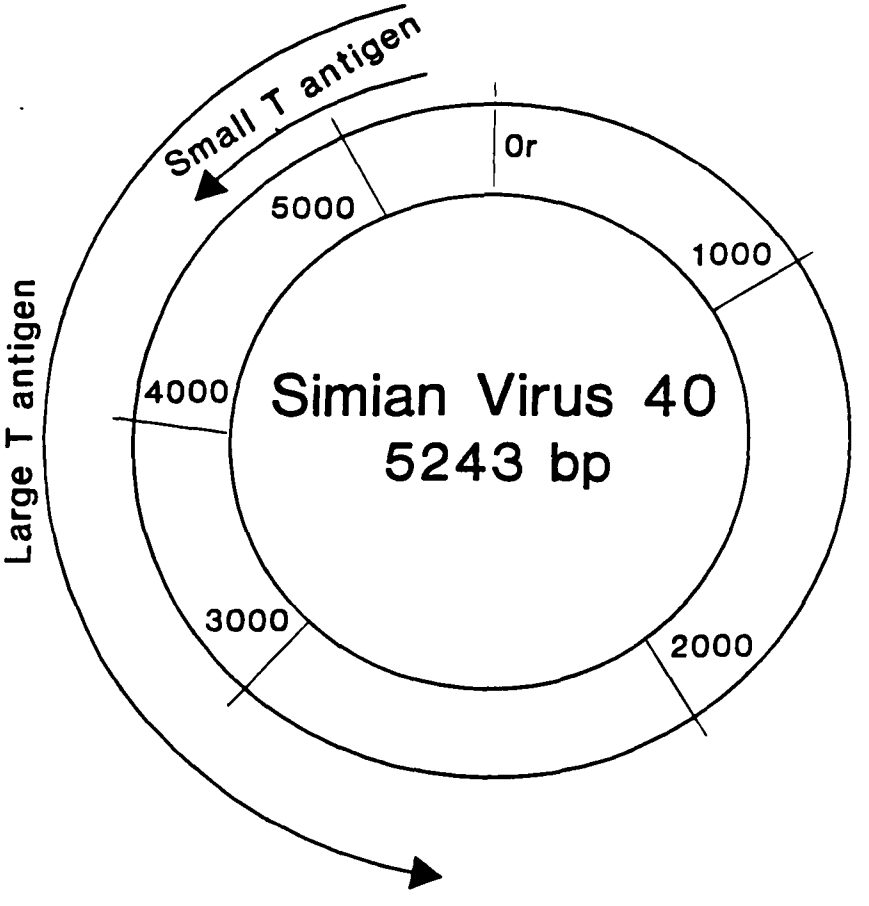


Figure 3. The genome of SV40. Double-stranded DNA approximately 5.2kb in size.

B. Comparison of electromagnetic and heat shock patterns of gene expression in HAL cells

Elucidation of a mechanism(s) of action of EM exposure is necessary in order to account for the observed and varied biological responses. Prior evidencesuggests cells react in a manner reminiscent of heat shock exposure (Goodman and Henderson, 1988). Experiments were conducted in order to expand on these earlier findings. To wit, the patterns of expression of EM-exposed HAL cells and heat-shocked HAL cells were compared. In examining cellular transcription, however, a problem arises in that the expression level of many genes in cell lines such as HAL, is insufficient to make dot blot analysis practical. Consequently we have utilized a novel, indirect means of measurement of gene expression by, first, reverse transcribing mRNA and subsequently amplifying the resulting cDNA for semi-quantitative purposes. The reverse transcription-polymerase chain reaction (RT-PCR) assay was utilized in the measurement of, among others, levels of several oncogenes as well as p53 and heat shock 70.

The heat shock stress response is widespread in nature. It results in induction of a related group of genes which code for proteins that serve a protective role (for review see Hightower, 1991). As with large-T antigen, c-*myc*

affects the hsp70 promoter and is thus a transcriptional regulator of this gene, although in this case it actually binds to the promoter (Taira *et al.*, 1992).

The work of Bukh *et al.* (1990) had shown c-*fos* and c-*jun* transcript levels to be elevated in human lymphoid cells incubated at 42°C for a period of 4 hours; a concomitant decrease in c-*myc* transcript levels was also noted. The purpose of the current experiments was to learn to what degree, if any, the heat shock and EM responses overlap in regard to transcription in HAL cells. In this way insight can be gained into what the cell "sees" when placed in an EM field.

Research Results

An SV40-transformed human fibroblast cell line, HAL, is responsive to an a 60 Hz EM field (Gold *et al.*, 1994). Evidence is offered that SV40 is altered at the transcriptional and translational levels based on measurements of a viral product, large-T antigen.

In addition, studies of a mechanistic nature reveal that the electromagnetic signal represents, in the case of HAL cells, a form of stress in some respects similar to, but ultimately exclusive of the heat shock response in the same cell line.

A. EM exposure and viral biosynthesis in HAL cells

Levels of the transcriptional and translational product of SV40, large-T antigen, are increased in an EM field. RNA dot blot analysis revealed an elevated transcript level, averaging 50%, of the early region of SV40, in exposed cells based on quantitative radioactive measurements. This region encompasses the coding sequence of large-T antigen. These results were obtained using a DNA probe composed of a 1 Kb portion of the large-T antigen coding region. Specificity of the probe was confirmed using Northern hybridization assay. Western blot analysis of HAL total protein lysates showed increased amounts of large-T antigen averaging 40-50% as shown by quantitation involving laser scanning densitometry.

B. RT-PCR studies of EM-exposed HAL cells

Using dot blot analysis, the biologic effect of EM fields on transcript levels of the oncogene c-*myc* in HL-60 cells had previously been demonstrated by Goodman *et al.* (1989) to be positively influenced. The RT-PCR protocol was employed in order to measure c-*myc* levels in HAL cells. An average increase of 42% was seen in exposed cells over controls, thereby confirming results of earlier work with the HL-60 cell line. We also measured transcript levels of the anti-oncogene P53 and likewise found an average increase of 40%. Transcript levels

of hsp70 in exposed cells, however, remained essentially unchanged compared to controls, indicating that if a relationship between exposure to EM fields existed, it was an indirect one.

The relationship between specific transcriptional components of signal transduction pathways and elf EM fields was explored using the RT-PCR protocol. Measurements were made of c-*fos* and c-*jun* transcript products using appropriate primers. The average levels of c-*fos* increased over 40% in exposed cells compared to controls. In addition, there was a small average increase in c-*jun* transcript levels.

C. Heat shock transcriptional response in HAL as measured by RT-PCR

Results of 4 hour (42°C) heat shock experiments showed approximately a 30% average increase in the steady state levels of c-*myc* transcripts. The steady state levels of Hsp70 transcripts increased an average of 53%, but there was only a 12% increase in c-*fos* transcript levels. The change observed for c-*jun* transcripts was about 20% (average increase).

HAL cells were also subjected to a 20-minute, 42°C heat shock exposure, with measurements of c-*fos*, c-*jun*, c-*myc* and hsp70 steady state transcript levels. Only Hsp70 expression was increased to any degree. In addition, an increase in c-*jun* (+12%), and no significant change in c-*myc* (+1%) values, were

also recorded. No increase in c-*fos* was seen; control levels in fact averaged 12% higher than that seen in exposed cells.

We conclude from these experiments that exposure to EM fields could represent a cellular stress, but if so, the mechanism is not identical to that of heat shock.

Methods and Materials

Abbreviations

EDTA (Ethylenediamine TetraAcidic Disodium Salt)

EtBr (Ethidium Bromide)

PMSF (Phenylmethylsulfonic Flouride)

MOPS (Morpholinopropanesulfonic Acid)

DDT (Dithiothreitol)

PBS (Phosphate Buffer Saline)

PCR (Polymerase Chain Reaction)

TEMED (NNNN-tetramethylethylenediamine)

D-MEM (Dulbecco's Modified Eagle Medium)

SDS (Sodium Dodecyl Sulfate)

NTP (Nucleotide Triphosphate)

Reagents and Solutions

Growth media: D-MEM/F12 media, 10% fetal calf serum, 1% penicillin/streptomycin.

SDS: 20 g SDS in 100 ml distilled water.

RNA lysis buffer (100 ml stock): 0.8 ml 0.1M Tris pH 9.0, 0.1M NaCl, 20 mM EDTA, 0.1% sarkosyl.

Sevag solution: 96 ml chloroform, 4 ml isopropyl alcohol.

TBE (10x stock; 1 liter): 108 g Tris, 55 g boric acid, 4 ml 0.5 ml EDTA.

TE: 1mM EDTA pH 8.0, 10mM Tris.

DDT (0.5M stock): 0.77 g in 10 ml distilled water.

PMSF (0.1M stock): 0.174 g ml in 10 ml isopropanol alcohol.

SSC (20X): 0.3M sodium citrate pH 7.0, 3M NaCl.

SSPE: 0.18M NaCl, 10mM Na2HPO4 pH 7.2, 1mM EDTA.

STE: 10mM Tris, 0.1M NaCl, 1mM EDTA pH 8.0

DNase I: 10 ug/ml concentration (BMB).

PBS (10X): 1.5M sodium phosphate pH 7.2, 1.5M NaCl.

SV40 DNA: approximately 0.5-1.0 ug/ul (BMB).

Bromophenol: 40% sucrose, 0.25% Bromophenol blue.

MOPS: 5mM sodium acetate, 20mM 3-[N-morphkllino]propanesulfonic acid, 1mM EDTA : adjusted to pH 8.0.

formaldehyde: 37% w/v (Sigma).

EtBr (stock): 0.25 g /500 ml distilled water.

Denhardt's solution (100x; 500 ml): 10 g Ficoll 400, 10 g polyvinlypyrrolidine, 10 g bovine serum albumen.

Tris-glycine (10X): 0.25 M Tris, 1.9 M glycine.

Polyacrylamide gel running buffer: 200 ml methanol, 100 ml 10X Tris-glycine; bring to 1 liter with distilled water.

TBS (5x): 100 mM Tris pH 7.5, 250 mM NaCl.

Cell Lines

A human fibroblast cell line, HAL (for "human acceptor line"), was used in the present study (Neufeld *et al.*, 1987). This cell line was constructed following transfection of a temperature-sensitive mutant of the DNA tumor virus SV40. A deficiency in the viral origin of replication is responsible for the mutant phenotype. The virus is stably integrated in the 5q21 region of the host genome (Hubbard-Smith *et al.*, 1992). As a result, HAL cells are immortalized. The particular strain of SV40 used for the transfection produces a heat-sensitive form of large-T antigen, the protein responsible for imparting the immortal phenotype to the host cell. Thus the cells are propagated at 35°C, the temperature at which immortalization is manifested. At 39°C, the cells revert to wild-type status as large-T antigen becomes biologically inactive, although the protein continues to be produced (L. Resnick-Silverman, personal communication).

For protein experiments, a mouse fibroblast cell line, PyT, is used as a negative control. PyT, which contains an integrated, defective strain of mouse polyoma virus, is used as a negative control.

Tissue Culture

Cells were fed two to three times weekly. The growth media consisted of D-MEM/F-12 media (Gibco), supplemented with 1.0% penicillin/streptomycin (Gibco) and 10% fetal calf serum (Sigma). When HAL cells attain confluence, they are removed from the surface of the flask by incubation in 1x Trypsin-EDTA (Gibco) and subsequently transferred, in equal amounts, to two new flasks containing fresh media. A digital cell counter (Royco) is used for quantitating cell number as a means of preparing for exposure. Culturing of PyT is accomplished under conditions described above for HAL cells, except PyT is grown at 37°C.

Transcription-measurement of steady state transcript levels

A. RNA Extraction

Total RNA is isolated as previously described (Goodman *et al.*, 1989). HAL cells from experimental or control cells are scraped and washed twice with (1X) PBS, lysed with 1.5 ml RNA lysis buffer and passed through a 5cc #21G needle syringe (Becton Dickinson) 5-6x to facilitate lysis. An equal volume of saturated phenol:chloroform:isoamyl alcohol (25:24:1) is added followed by extraction with chloroform:isoamyl alcohol (24:1). The nucleic acids are then precipitated with two volumes 0.1M LiCl (dissolved in 95% ethanol). After storage at -70°C for a minimum of 30 minutes, the sample is centrifuged for 30 minutes at 14,000 rpm.

The resulting pellet is washed once with 95% ethanol, centrifuged for 10 minutes at 14,000 rpm and air-dried for approximately 30 minutes. The pellet is dissolved in 300 µl 50 mM Tris-HCl, pH 8.0. To this solution is added 2.5 µl 1M $MgCl_2$ (final concentration of 3.3 mM) and 10 µl 100 ug/ml DNase I to a final concentration of 0.3 µg/ml (BMB). The sample is then incubated on ice for two hours. This is followed by addition of 2.5 µl 0.5M EDTA (to 4 mM) and 30 µl 3M NaOAc, pH 5.2 to a final concentraiton of 0.3M. The mixture is gently vortexed and extracted with an equal volume of saturated phenol: chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). Precipitation of the nucleic acid is accomplished by adding two volumes of 95% ethanol. The solution is incubated at -70°C for a minimum of 30 minutes. The sample is then centrifuged at 14,000 rpm for 30 minutes. The resulting pellet is washed once with 95% ethanol and air-dried, followed by suspension in 10-20 µl TE buffer. A Bausch and Lomb Spectronic 1001 spectrophotometer is utilized for measuring the concentration of each RNA sample, which is stored at -70°C.

B. Restriction endonuclease Digestion

The restriction enzyme HpaI was used for digestion of total genomic SV40 (BRL). Approximately 0.5 µl (1.5 units) HpaI (BMB) is mixed with 1µg SV40 DNA and a high salt buffer (BMB) followed by incubation overnight in a 37°C waterbath

for the purpose of isolating and purifying a 1kb DNA fragment composed of a portion of the large-T coding sequence. The DNA fragment is stored at 0°C until further use.

C. Agarose gel electrophoresis- DNA gel electrophoresis.

SV40 DNA, previously restricted with HpaI, was isolated using agarose gel electrophoresis. Powdered agarose (BRL) is placed in 0.5x TBE (10X TBE stock solution: 108 g Tris, 55 g boric acid, 4 ml 0.5M EDTA; brought to 1 liter with distilled water). The final concentration is 1.0%. This solution is heated to boiling and poured into a gel mold followed immediately by placement of a comb in the gel. DNA samples are incubated at 58-60°C for ten minutes and placed on ice for five minutes. To each sample is added 1.0 µl Bromophenol blue (40% sucrose, 0.25% bromophenol blue). The voltage is set at 70V; running time is approximately two hours. Upon completion of the run, the gel is stained via incubation for 30 minutes in 150 ml distilled water containing 0.5 ml 0.5 µg/ml EtBr (final concentration - 1.6×10^{-3} ug/ml). The appropriate-sized band is excised using a Geneclean purification kit (Geneclean). An additional electrophoresis is done as a means of insuring integrity of the recovered fragment.

D. Agarose gel electrophoresis-RNA gel electrophoresis

RNA extracted from cells is subjected to agarose gel electrophoresis, the purpose of which to check for DNA contamination as well as to insure integrity of the RNA as shown by the presence of 18S and 28S bands. The protocol is the same as the one described for DNA gel electrophoresis, although in this case a 0.8% gel is prepared.

E. Northern Analysis

The method used is described is outlined in Current Protocols in Molecular Biology (1987). A 1.2% formaldehyde agarose gel is prepared by dissolving 0.84 g agarose in 60.9 ml distilled water followed by boiling and cooling to 60°C, at which time 7 ml 10X MOPS (dilution to 1X) is added, followed immediately by 2.1 ml 37% formaldehyde (to 1%). The solution is poured into a gel mold containing a comb, and further cooled to solidification (30-45 minutes).

Each sample is prepared as follows: 1 µl 10X MOPS (0.83x), 5 µl Formamide, 1.75 µl 37% formaldehyde (5.38%) and RNA sample. The mixture is heated in a 60°C water bath for 10 minutes followed by the addition of 1 µl loading buffer containing bromophenol blue. The final volume of each sample is 12 µl. The gel is run at 50 volts (5 V/cm) for approximately 2.5-3.0 hours. At the conclusion of the run, the gel is washed with distilled water and placed in a

solution of 10X SSC for 45 minutes-1hr.

RNA in the gel is transferred to a nitrocellulose membrane (BioRad) according to the method outlined in Current Protocols in Molecular Biology (1987). Transfer is overnight. The membrane is baked for 2 hours at 80°C and either stored for further use or immediately placed in a sealed plastic bag containing prehybridization solution composed of 5 ml formamide (41.6%) , 2.5 ml 20X SSPE (4X), 100 µl 20% SDS (to 0.16%), 0.2 ml 50x Denhardt's solution (8.3x), 150 µl salmon sperm DNA to 10 mg/ml (Sigma) (sheared prior to use), and 4 ml distilled water for a final volume of 12 ml. In prehybridizations, 8 ml is used, with the remainder serving as hybridization solution.

Following prehybridization, which is of 1-2 hours duration, the liquid is removed from the bag; 4 ml of the remaining solution, containing the radiolabelleled probe, is added. During prehybridization incubation, the probe is labeled either by nick translation or random priming methods as described in a kit purchased from Boehringer Mannheim. Northern analysis involving hsp70 utilized the nick translation protocol, whereas random priming was used for labeling of the SV40 probe. In either case, the probe, once labeled, is denatured by boiling for 10 minutes, then placed on ice for 5 minutes. It is in this form that the probe is added to the hybridization solution. Incubation is overnight in a 42°C water bath.

After hybridization, the membrane is washed twice at room temperature in a solution of 0.1% SDS and 2x SSC for periods of 5 and 30 minutes, respectively. This is followed by a wash of 2 hours length, at 58-60°C in a solution of 0.5% SDS and 0.1x SSC. The radioactive filter is air-dried and subjected to autoradiography using Kodak X-ray film (Carlick). Exposure is overnight at -70°C. The Hsp70 DNA probe was commercially purchased (Stressgene, Vancouver British Columbia).

D. Dot blot Analysis

The method used is a modification of the one developed by Sambrook *et al.* (1989). RNA is diluted in distilled water to a total volume of 10 µl, to which is added 10 µl 20X SSC to a final concentration of 8x SSC and 5 µl 37% formaldehyde (7.4%). The mixture is incubated at 58-60°C for 10 minutes. During the incubation, a strip of nitrocellulose paper (BioRad) is soaked in a glass dish containing 10X SSC, subsequently removed from the dish and drained of excess fluid. Linear amounts of RNA are dotted onto the damp filter, e.g., 1 µg, 2.0 µg, 4 µg, *etc.*

The filter is baked in an 80°C vacuum oven for 2 hours, hybridized to a DNA probe, washed and air-dried. Results are visualized via autoradiography; these procedures are identical to those described above for Northern analysis.

Following autoradiography, each radioactive dot is quantitated by cutting it out of the filter and placing it in a plastic vial containing scintillation fluid (Ecoscint). Counting is accomplished using a Packard Tri-Carb 4530 scintillation counter as described by Goodman *et al.* (1989). Background levels are determined by cutting out and measuring random areas of the filter which do not contain dots. Standard error between experiments averaged 5-10% based on measured variations in different RNA control preparations using the SV40 early region DNA as a probe.

E. RT-PCR Amplification

HAL cells are heat shocked using as an initial guideline exposure parameters established by Bukh *et al.* (1990). The RT-PCR method (Figure 4) is utilized for transcript measurements. Experiments are conducted according to the protocol established by Broude *et al.* (1994). Two micrograms of experimental (from exposed cells) or control RNA are mixed with 4 µl 5X RT buffer to a final concentration of 1.25 x), 4 µl dNTPs (0.04 mM final concentration per nucleotide) (Sigma), 1 µl random primer (no. 6 tube from a random primer kit purchased from BMB), and 1 µl RNasin (Promega). The volume is brought to 19 µl using distilled water. To promote cDNA synthesis, 1 µl reverse transcriptase (8 U/ul) (Promega) is added to the mixture followed by incubation in a 42°C water bath for 45

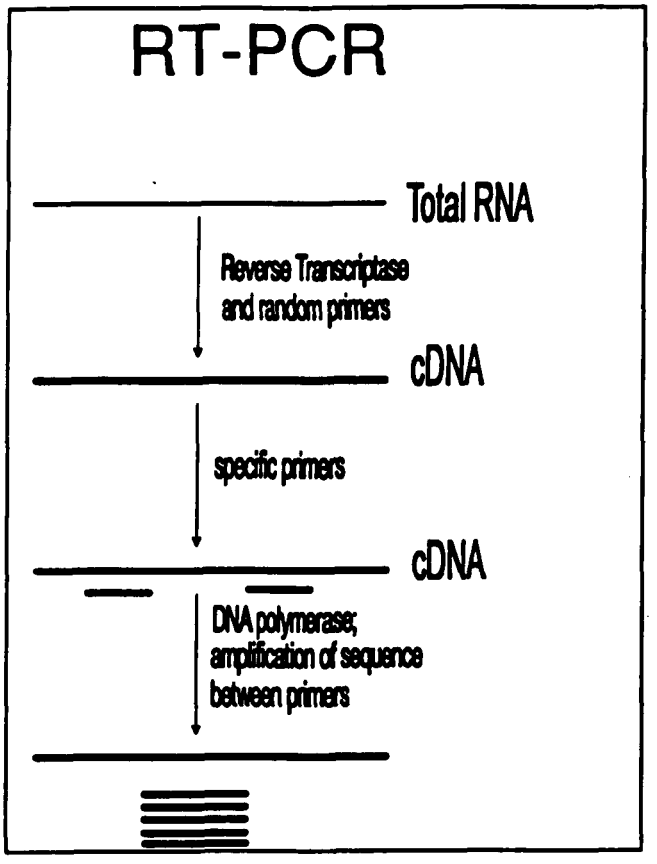


Figure 4. Schematic diagram of RT-PCR protocol. RNA is extracted and reverse transcribed into cDNA. A discrete region of the cDNA, representing a portion of the coding sequence of the gene of interest, is subsequently amplified using specific primers. The region is generally between 150-500bp in length.

minutes to an hour.

One to two micrograms of the cDNA solution are removed and placed in a separate tube containing 5 µl 10X PCR buffer, 5 µl dNTPs (0.04 mM final concentration per nucleotide) and 1-2 µl oligonucleotide primers (initial concentration 0.2 µM, prior to purification). Each oligonucleotide primer was constructed in the Sequencing and Synthesis laboratory of Hunter College. Purification was facilitated using a 10 ml borosilicate pipet containing 10 ml Sephadex G-50 (Sigma). The column is pre-washed with distilled water. The primer solution is passed through the column and collected in six fractions. Fractions of pH 7.0 are pooled. Primers of approximately 20 bases are used for PCR amplifications. Primer concentrations are measured using a Bausch and Lomb Spectronic 1001 spectrophotometer. In cases where newly chosen primers repeatedly failed to result in amplification of cDNA, different sequences were selected as primers. The primers for the amplification step are listed below:

for c-*fos* specific:

5'-GGCTTCAACGCAGAGTACGAGGCGT-3' Verma *et al.* (1986)

5'-CCTCCTGCAATGCTCTGCGCTCG-3'

for c-*jun* specific:

5'-TGAAGTGACGGACTGTTCTATG-3' Hattori *et al.* (1988)

5'-TTCTTGGCGCGGAGGTGCGGCTTCA-3'

for c-*myc* specific:

5'-ACTGCGACGAGGAGGAGAAC-3' Gazin *et al.* (1984)

5'-TACAAGCTGGAGGTGGAGCAG-3'

for Hsp70 specific:

5'-TTCGCCGACCCGGTGGTGTCC-3' Hunt and Morimoto (1985)

5'-CACACCCGCATCCTTGGT-3'

for p53 specific:

5'-ATGGAGGAGCCGCAGTCAGAT-3' Lamb and Crawford (1986)

5'-CTCACAACCTCCGTCATGTGCCT-3'

Distilled water is added so that the final volume (including DNA polymerase) is 50 µl. The mixture is denatured in water (94°C) for 5 minutes and placed on ice for another 5 minutes. This is followed by addition of 1.0-1.5 µl (1:5) DNA polymerase or 8units/ml (Biolabs). The mixture is overlayed with a

single drop of mineral oil (Perkin Elmer) as a means of preventing evaporation during the amplification process.

The sample is placed in a Savant temperature cycler and subjected to 35 amplification cycles. Each cycle consists of 60 seconds at 94°C, 60 seconds at 60°C and 60 seconds at 72°C. At the conclusion of amplification, the products are analyzed by 2% agarose gel electrophoresis containing 0.05% mg/ml ethidium bromide. The gels are then photographed in UV-light (Polaroid 665 film) and the negatives are scanned for quantitative purposes using a computerized laser scanning system (Zeineh scanning densitometer, model SL-DNA, Biomed Instruments, Inc., Ca). A tracing of each band is generated and is subsequently weighed on a Mettler H78AR microbalance device for the purpose of quantitation.

Exposure Equipment and Parameters

The highly complex nature of the pulsing electromagnetic field (PEMF) used in treating bone non-unions signal poses a problem for the researcher in delineating a mechanism(s) of action of exposure for several reasons. It is difficult to correlate a specific portion of this asymmetric signal with observed changes in biologic behavior. The use of a symmetric signal, which correlates more closely with the environmental fields with which most people are in contact, is therefore more suitable from the standpoint of interpretation of a cause-effect relationship.

A sinusoid signal (60 Hz) is used in all experiments described.

For experiments involving large-T antigen transcription, two exposure systems are used. One is a pair of Helmholtz-aiding coils, composed of two circular, parallel wire copper wire bundles (164 turns, 19 gauge magnet wire) which produces an E-field of 7.5 μV/m (Figure 5a and 5b). The coils are 13 cm in length and 14 cm in width and are separated from each other by 8 cm. T-25 flasks containing HAL cells are placed between the coils. This device has in the past been shown to elicit transcriptional modulation in HL-60 cells (Goodman *et al.*, 1989). Experiments investigating large-T antigen protein production utilized this apparatus as well. The second apparatus was a cylindrical, solenoid-type apparatus (Figure 6), composed of a wire copper bundle 1.2 cm in diameter (38 μV/m E-field). The structure is 26 cm in length and 17 cm in width. Flasks are placed within its interior and subjected to exposure.

To minimize the effect of extraneous EM fields, the exposure apparatus (be it Helmholtz-aiding or solenoid) is placed in a μ metal container (Figure 7). Control flasks are placed in a separate μ metal box during exposure. Both control and exposed cells are situated in the same incubator during an experiment. The sine wave generator (Wavetek) is located outside the incubator. For solenoid experiments, an 80 watt amplifier (Realistic) and accompanying control unit (manufactured by ERM) are utilized for generation of the signal.

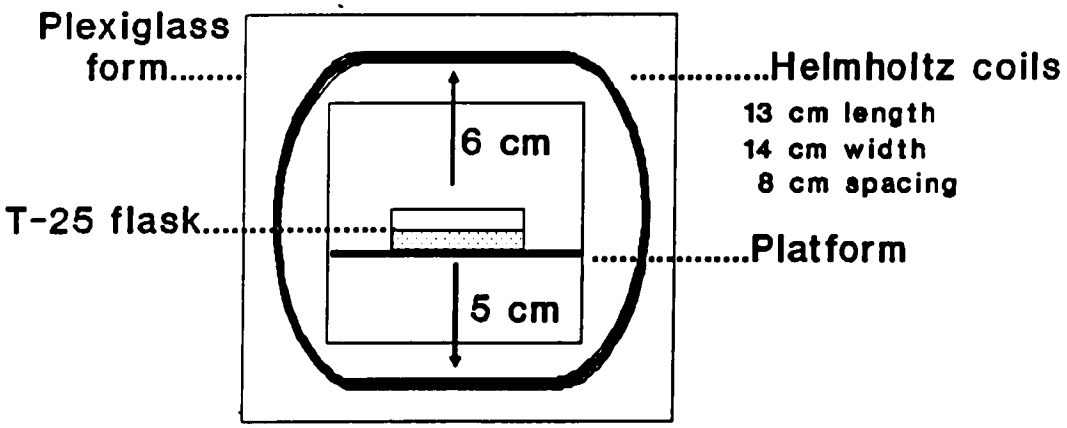


Figure 5a. **Helmholtz coils.** See appendix for description of the device.

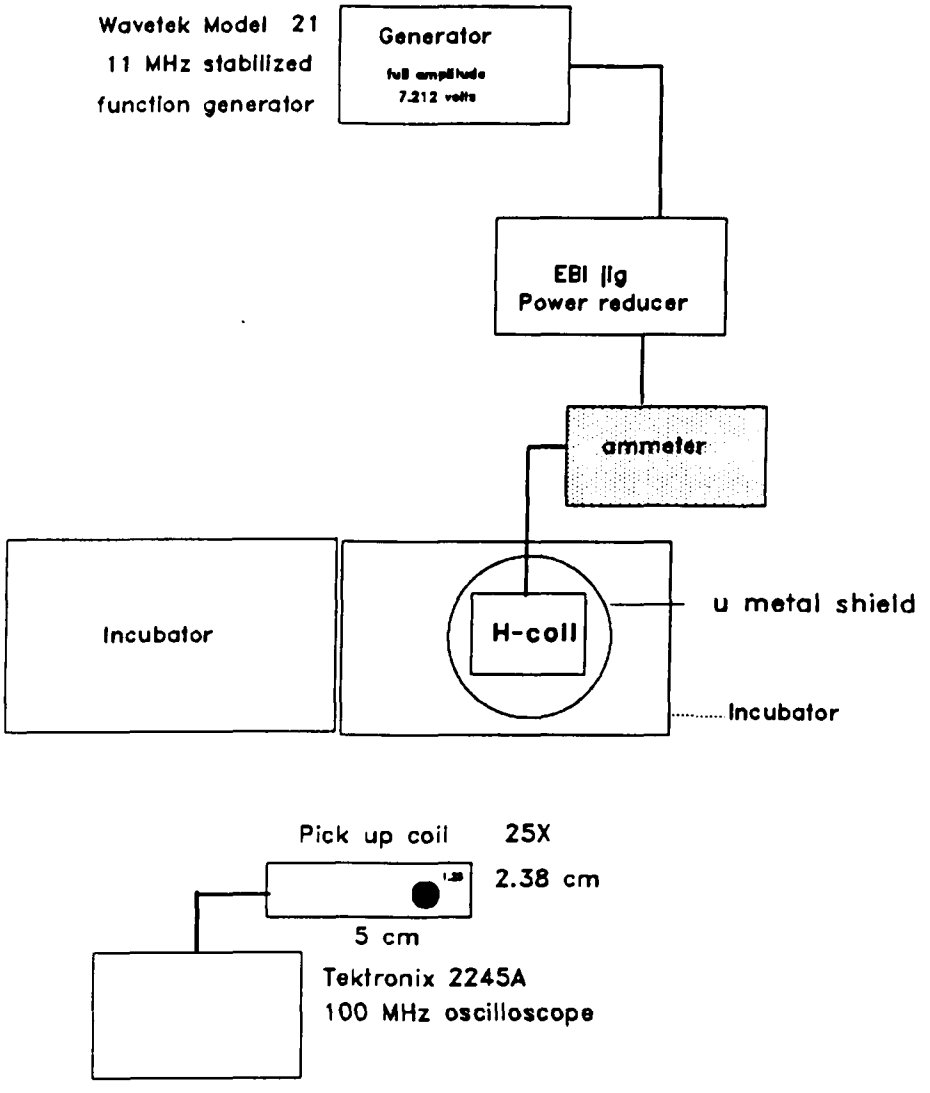


Figure 5b. Helmholtz exposure system.

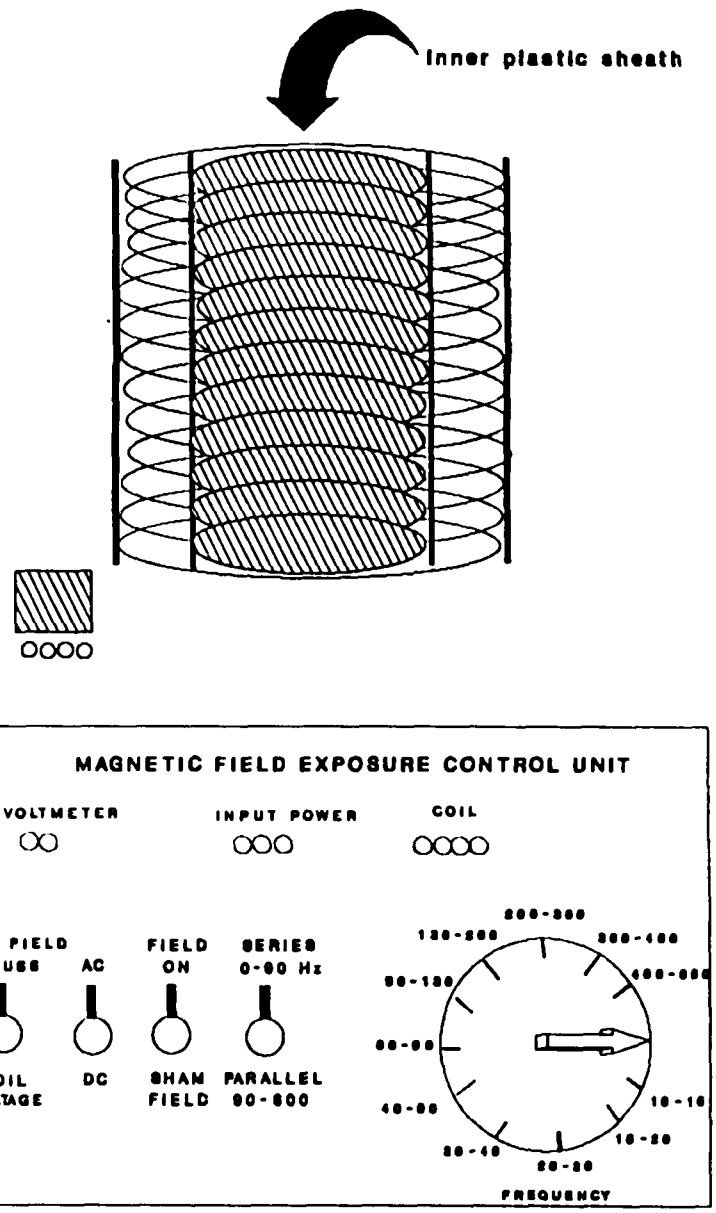
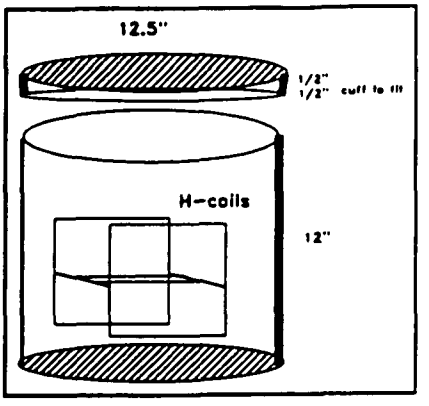


Figure 6. Solenoid exposure system and control panel.

Side view



View from the top of the shield with coils in place.

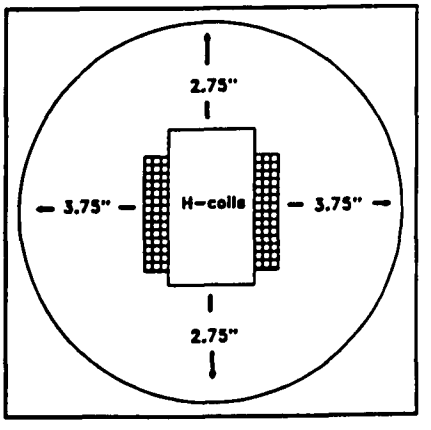


Figure 7. **Use of the u metal box.** A u metal box is used to prevent the effect of stray fields in the exposure system, as well as prevent possibly exposing cells in the adjacent incubator. The seam of the u metal box is always positioned in the same orientation, as are the coils.

Analysis of T-Antigen Protein

Protein analysis uses the Western blot protocol as described by Radna *et al.* (1989). T-25 flasks (Fisher) are plated at 3.0-4.0 $x10^5$ cells per flask one day prior to exposure. Following exposure, cells are scraped, washed twice with cold PBS and suspended in 0.3-0.5 ml lysis buffer (20 mM Tris pH 8.0, 80 mM NaCl, 20 mM EDTA) supplemented with the protease inhibitors DTT ($5x10^{-4}$M final concentration) and PMSF ($2x10^{-3}$M final concentration). The lysate is subjected to three freeze-thaw cycles, at room temperature and -70°C, respectively, in order to maximize cell lysis. The mixture is centrifuged and the resulting supernatant removed for measurement of total protein concentration using a commercially obtained protein assay kit (Bio Rad). Samples were stored at -70°C.

Western blots. As a first step, a polyacrylamide gel electrophoresis sandwich, composed of glass plates, is constructed. The separating gel solution is then prepared using the following ingredients: 6.7 ml H_2O, 5 ml Tris (4% w/v 10% SDS, 1.5M tris, pH 8.8), 8.3 ml 30% acrylamide/bisacrylamide and 60 µl 10% ammonium persulfate. The solution is placed in a vacuum for approximately 45 minutes to remove air bubbles, followed by addition of 9 µl TEMED. The mixture is poured into the interior of the glass sandwich and allowed to solidify for 1 hour.

The stacking solution is prepared as follows: 6.5 ml H_2O, 2.5 ml Tris (0.5M tris pH 6.8, 4%w/v 10% SDS), 1.0 ml 30% acrylamide/bisacrylamide and 60 µl 10% ammonium persulfate. Following 45-minute incubation in a vacuum, 20 µl TEMED is added to the solution, which is then poured into the glass sandwich up to the brim. A plastic comb is inserted in the top of the sandwich, and solidification of the gel is complete within an hour.

The sandwich is placed in a chamber containing running buffer [100 ml 10X Tris-glycine (0.25 M Tris, 1.9 M glycine), 10 ml 10% SDS, brought to 1 liter with H_2O]. Protein samples are prepared by adding loading buffer in a 10:1 ratio (loading buffer stock solution: 1.52 g Tris, 20 ml glycerol, 2.0 g SDS, 1 mg bromophenol blue; pH to 6.8 with 1N HCl and bring to 100 ml with H_2O). Samples are boiled for 5 minutes and placed in the wells of the gel. A molecular weight marker (BRL) is placed in a separate well. The gel is run for 2.5 hours at 110 volts.

The gel is removed from the glass and placed in a foam rubber-lined electroblot sandwich with a strip of nitrocellulose paper (BioRad) of equal size to the gel. The sandwich is submerged in a chamber filled with running buffer (200 ml methanol and 100 ml 10X Tris-glycine brought to 1 liter with H_2O). A current of 0.1A is applied overnight to transfer the proteins in the gel to the nitrocellulose.

The nitrocellulose filter is removed from the electroblot sandwich and

briefly washed with 1X TBS (5X TBS stock solution: 100 mM Tris pH 7.5, 250 mM NaCl). The filter is then transferred to a plastic "seal-a-meal" bag to which is added 10 ml blocking buffer (2% Carnation dry fat milk powder). The bag is sealed and placed on a shaker for an hour of gentle rocking at room temperature. The filter is transferred to a new seal-a-meal bag to which is added a solution composed of the following ingredients: 0.2 ml H_2O, 0.5 ml 5X TBS (to a final concentration of 0.625X), 1.3 ml blocking buffer and 2 ml polyclonal antibody (assayed by experimental effect) specific for SV40 large-T antigen (supplied by H.L. Ozer). The bag is sealed and gently shaken at room temperature for 1 hour.

The filter is removed from the bag and washed three times for 5 minutes in 1X TBS. The filter is transferred to another plastic bag containing the following: 1.44 ml blocking buffer, 2.66 ml 1X TBS, 8 µl goat anti-mouse secondary antibody (> 10,000 units/ml) (BMB), and incubated for one hour at room temperature.

Following incubation, the filter is removed from the bag and washed 3 times at room temperature with 1XTBS. This is followed by placement in a combined solution of cold 100 ml 1XTBS (containing 60 µl H_2O_2) and 20 ml cold methanol [containing 60 mg 4-chloro-1-napthol (Sigma)] to result in visualization of the protein band. The filter is stored in a dark area for future densitometric measurement.

Results

The purpose of these experiments was to investigate selected transcriptional and translational responses of the HAL cell line to a 20 minute, 60 **Hz** sinusoid exposure field. Our goal was to learn whether a tumor virus, SV40, is modulated both in terms of gene expression and protein and the degree to which the heat shock and EM response patterns converge (or diverge) in HAL cells, based on a comparison of expression of several genes, *e.g.*, c-*fos*, c-*jun* and c-*myc* which are involved in signal transduction pathways. The underlying goal of these experiments was to obtain information that would help define the mechanism of action.

A. Large-T antigen transcription. The effect of EM exposure on the expression of large-T antigen, a product of SV40, was examined using dot blot analysis. Large-T antigen mRNA was monitored using a fragment from a HpaI digest of total genomic SV40 DNA. This sequence, approximately 1 Kb in length, encompasses a portion of the viral genome between bases 2666 and 3733. The region is composed of part of the large-T coding region (Figure 3). Figure 8 shows an autoradiogram of dot blot hybridization of RNA from HAL cells exposed to a Helmholtz coil which generated a 60 **Hz** signal. Exposure time was 20 minutes. Figure 9 shows an autoradiogram comparing RNA from exposed and

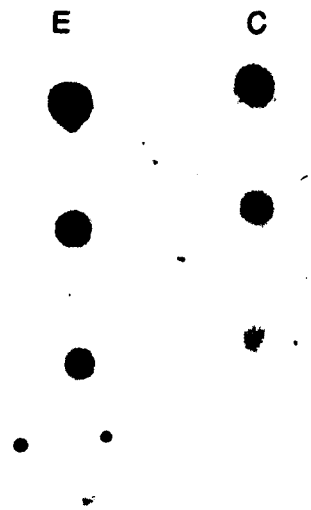


Figure 8. RNA from Helmholtz-exposed cells. Dilutions were as follows: 0.3ug, 0.6ug, 1.2ug and 2.4ug. The autoradiogram was obtained following 2hr exposure with intensifying screen. (E) exposed cells (C) control cells. Two experiments were performed using the Helmholtz apparatus.

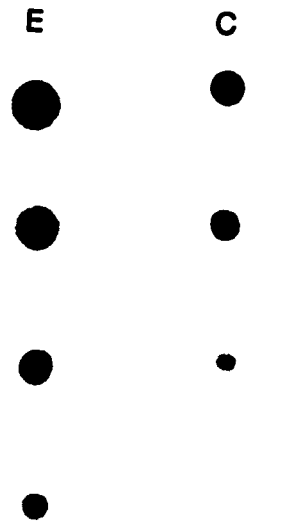


Figure 9. RNA from solenoid-exposed cells. Dilutions were as follows: 0.25ug, 0.5ug, 1.0ug and 2ug. The autoradiogram was obtained after 2.5 hrs exposure with an intensifying screen. Three experiments were performed using the solenoid apparatus.

control cells. Exposure parameters were the same, although in this case a solenoid apparatus served as the exposure system. Quantitation of dot blots for cells exposed to either Helmholtz coils or the solenoid is given in Table 1. To confirm specificity of the probe, Northern analysis was employed using the same 1 Kb probe utilized for dot blot analysis (Figure 10). A single band of approximately 2.4 Kb in size was seen, corresponding to the early region of the SV40 genome. This sequence, which represents a pre-mRNA, is composed of both the large-T and the small-t coding sequences, is subsequently processed into separate mRNA species. Thus the probe measured levels of combined large-T/small-t mRNA species, as opposed to large-T antigen specifically.

B. Large-T antigen translation. Large-T antigen protein in HAL cells was measured using Western blot analysis (Figure 11). An extract of the mouse polyoma cell line PyT was included as a negative control. Scanning densitometry revealed an average increase of 40-50% in protein content of the experimental over the control (Figure 12). The elevated levels of large-T protein observed could either be indicative of increased production, or alternatively, increased half-life within the cell.

EXPOSURE CONDITIONS

HELMHOLTZ COIL		SOLENOID	
EXPOSED	CONTROL	EXPOSED	CONTROL
649	327	185	119
1134	533	304	187
1992	928	512	295
3275	1522	959	509

Table 1. Radioactive quantitation of dot blot analyses (cpm / spot)

Figure 10. Northern hybridization of HAL RNA (4ug). The RNA was hybridized to a DNA probe composed of a 1kb HpaI restriction fragment. Overnight exposure.

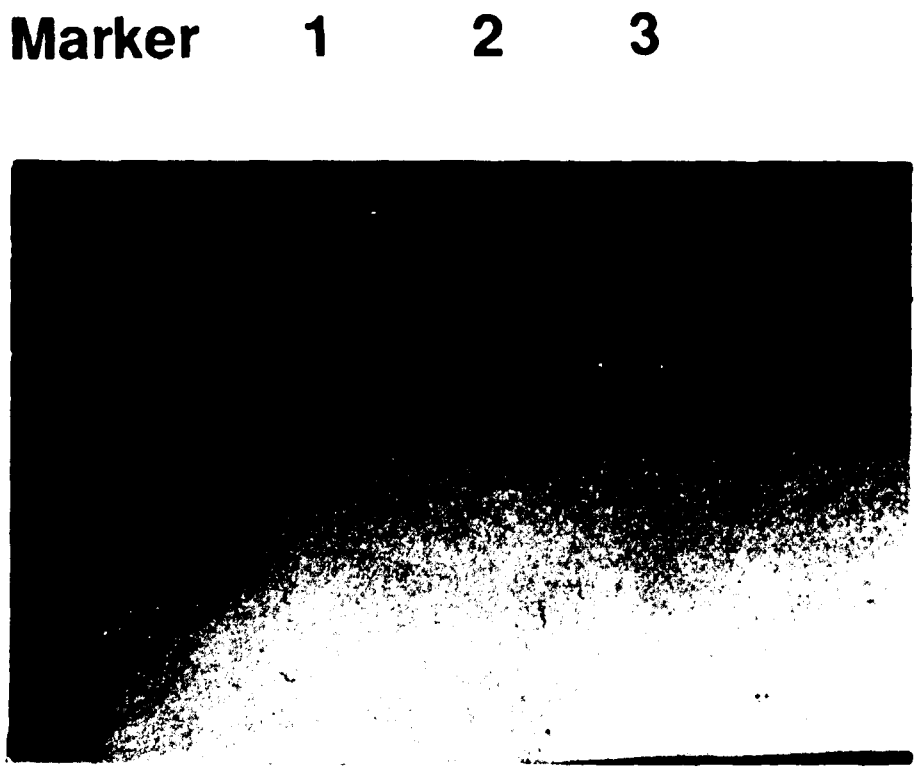


Figure 11. Western blot of HAL protein. The cells were exposed to the Helnholtz coil for 20 minutes (60Hz, 8uT). Each lane contains 42ug total protein.

lane 1: Protein derived from exposed cells.

lane 2: Control using PyT protein

lane 3: Protein from unexposed cells.

M: Molecular weight marker

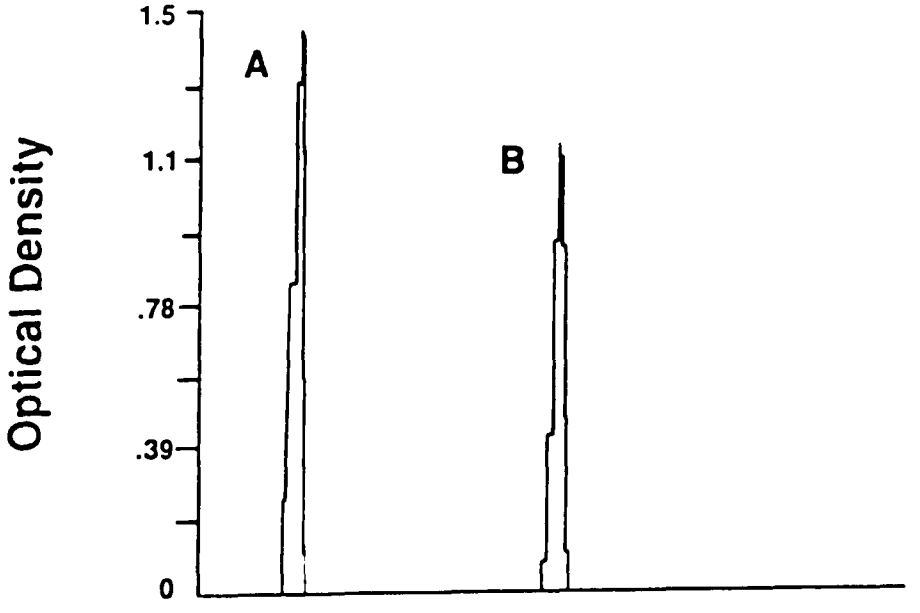


Figure 12. Scanning densitometry of protein bands given in Figure 11. Values were standardized prior to scanning. Four experiments were performed.

(12A) Scan of the band from protein obtained from exposed cells.

(12B) Scan of band from protein of unexposed cells.

C. Qualitative analysis of heat shock response in HAL Cells. We wished to learn if the HAL cell line, a temperature-sensitive mutant, exhibits heat shock response according to parameters established by Bukh *et al.* (1990) with thymocytes, Daudi (B cells) and Hyon (pre-B cells). Northern analysis showed a band of approximately 2.6 Kb (Figure 13) following hybridization with a DNA probe representing the inducible form of Hsp 70.

Comparing electromagnetic field and heat shock respose: RT-PCR amplification analysis

Our purpose was to compare electromagnetic and heat shock transcriptional response in HAL cells. We reverse transcribed RNA and amplified the resulting cDNA using primers specific for each gene. Prominence was given to those genes known to be associated with signal transduction pathways, the goal being the gathering of mechanistic information concerning EM exposure and HAL cells.

A. Effect of EM exposure on transcription in HAL cells

These experiments tested the effect of EM exposures as generated by a Helmholtz coil (60 **Hz;** 8 μT). All exposures were for 20 minutes. Transcripts were by measured by RT-PCR. The results showed that steady state levels of a series

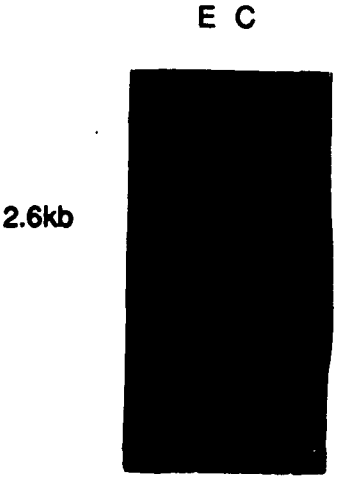


Figure 13. Northern hybridization of RNA from heat-shocked (E) and control (C) cells. Nine micrograms in each lane. Incubation parameters were 42°C, 4hrs. A 2.6kb band represents hsp70 mRNA. Overnight exposure with screen.

of transcripts were increased about 40% over that of control values. For example, transcripts for the oncogene c-*myc* were increased an average of 42% (Figure 14a); transcript levels of the anti-oncogene, p53, were increased to an average of 40% (Figure 14b) and an average increase of 41% in exposed cells over control levels was observed for c-*fos* (Figure 14d).

The response was not as pronouced for other transcripts measured. Steady state transcript levels of c-*jun* were only increased an average of 15% in exposed cells as compared to controls (Figure 14c). Nine amplifications covering 6 exposures were carried out were carried out to confirm this result. Critical to the hypothesis of the research, however, was that Hsp70 transcripts were only increased an average of 8% (Figure 14e).

Figure 15 and Table 2 summarize these findings. Although we found increases of varying degree in mRNA levels of several oncogenes, we also saw an elevation in p53 transcript levels as well; thus the cell appears to be "pulled" in two different directions in terms of its proliferative tendencies. In addition, we did not observe any change in hsp70 mRNA levels, unlike the situation in HL-60 cells. This may be indicative of a cell type- or cell line-specific response.

B. Effect of heat shock on transcription in HAL cells.

Results of Northern hybridization, using hsp70 as a probe, showed that a 4 hr incubation at 42°C caused a heat shock response in HAL. We used this

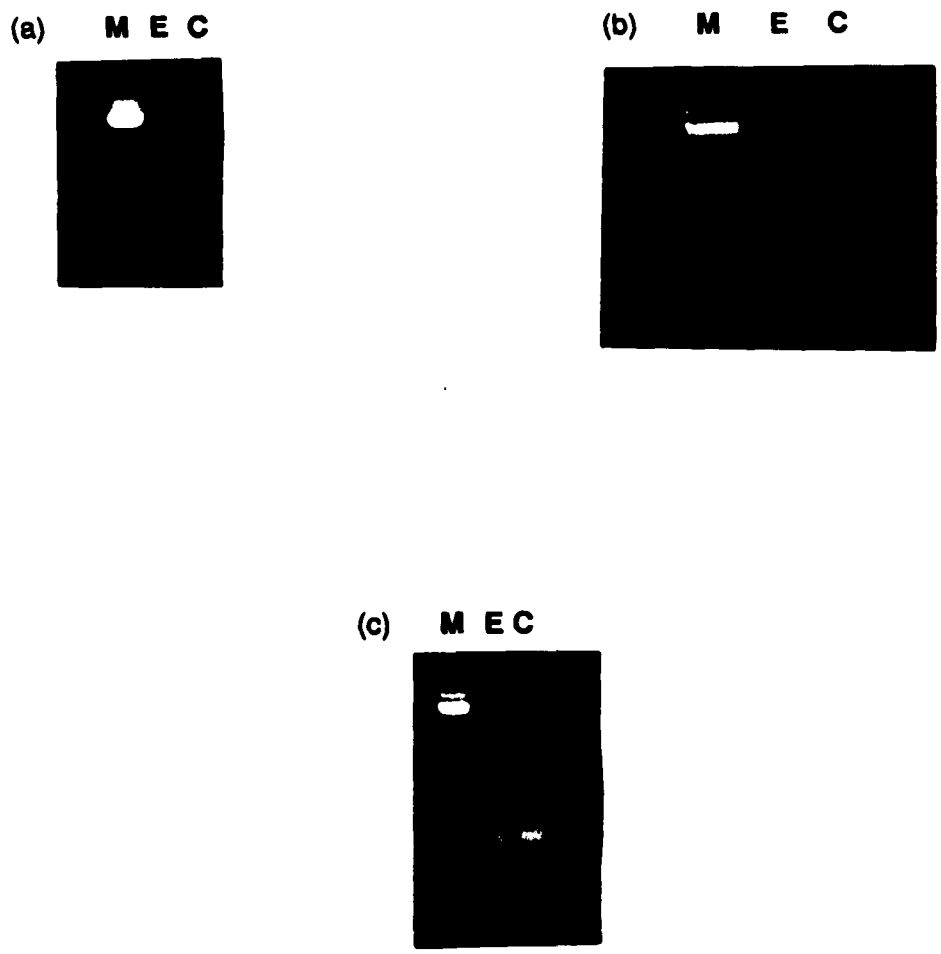
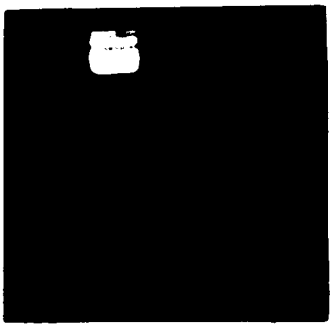


Figure 14. RT-PCR amplification of (a) c-myc (b) p53 (c) c-fos (d) c-jun (e) hsp70. (E) Exposed (C) control (M) molecular weight marker. 20 minute exposure at 60 Hz

(d) M E C



(e) M E C

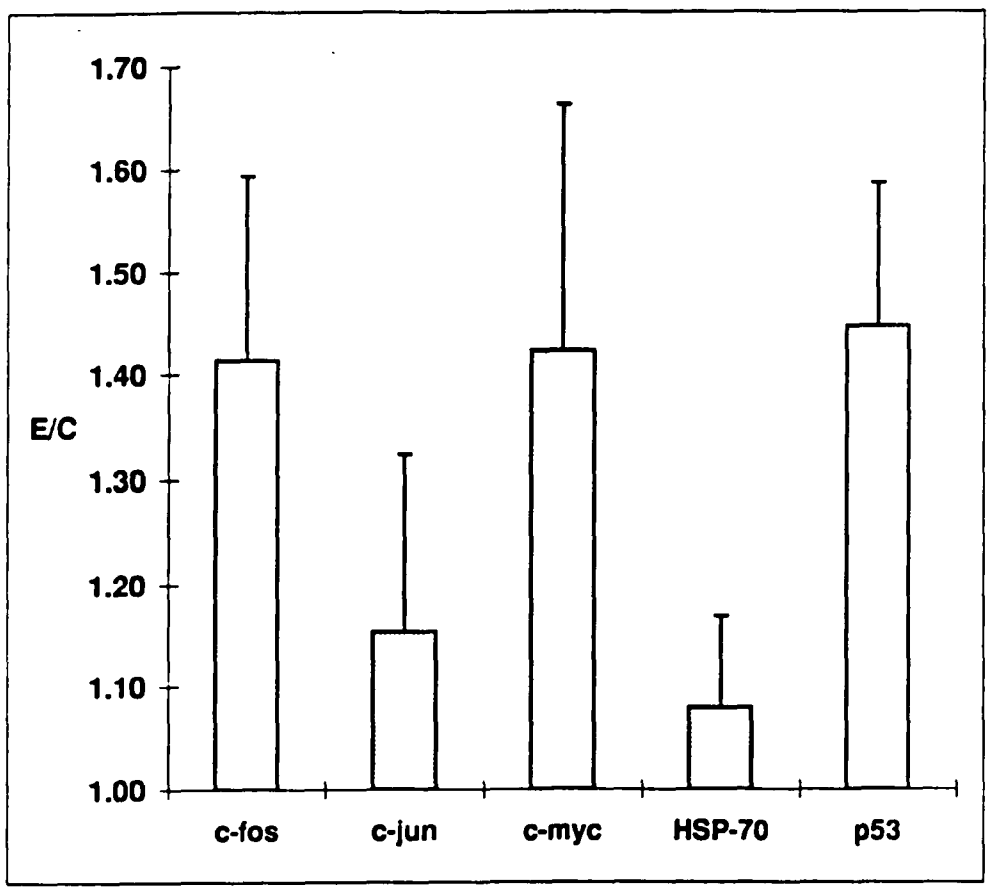


Figure 15. Bar graph summary of RT-PCR experiments from cells which were exposed in Helmhotz device (20 minutes, 60 Hz)

Exp #	c-fos			c-jun			c-myc			HSP-70			p53		
	Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C
1	715.5	393	1.82	987	590.5	1.67									
	911	839.5	1.09												
	962	384	2.51												
Mean	862.83	538.83	1.80	987	590.5	1.67									
2				549	968	0.57									
				403	686	0.59									
Mean				476	827	0.58									
3	938	713	1.32							841	1014	0.83			
	865	749	1.15	827	413	2.00									
Mean	901.5	731	1.24	827	413	2.00				841	1014	0.83			
4				1385	861	1.61	1025	810	1.27	1123	969	1.16	941	780	1.21
	913	706	1.29	976	1172	0.83				976	1134	0.86	955	709	1.35
Mean	913	706	1.29	1180.5	1016.5	1.22	1025	810	1.27	1049.5	1052	1.01	948	745	1.28
5							1012	389	2.60	1011	601	1.68	774	425	1.82
	957	1082	0.88	1029	1173	0.88	944	865	1.09	1143	1012	1.13	791	404	1.96
Mean	957	1082	0.88	1029	1173	0.88	978	627	1.85	1077	807	1.41	783	415	1.89
6	1183	945	1.25	904	889	1.02	1200	901	1.33	868	880	0.99			
							1137	972	1.17	1517	1356	1.12	1272	1076	1.18
				1432	1177	1.22	1454	1353	1.07	1609	1451	1.11	1048	902	1.16
Mean	1183	945	1.25	1168	1033	1.12	1263.67	1075.33	1.19	1331.33	1229	1.07	1160	989	1.17
Total Mean	930.56	726.44	1.41	943.56	881.06	1.15	1128.67	881.67	1.42	1103.22	1048	1.08	964	716	1.45
S.E.			0.18			0.17			0.24			0.09			0.14

Table 2. Quantitative summary of RT-PCR experiments performed with HAL cells exposed to EM field (20 minutes, 60 Hz)

parameter in our RT-PCR experiments in order to measure and subsequently compare the pattern of transcriptional response to both heat shock and EM exposure. The results from the 4 hour heat shock were as follows: Steady state levels of c-*jun* were increase about 20% in heat shocked cells as compared to controls (Figure 16a). C-*myc* transcripts were also increased (32%) in heat shocked cells (Figure 16b). C-*fos* was increased only slightly under heat shock conditions (Figure 16c). The heat shock conditions were effective as shown by the 53% average increase in HSP70 over control levels (Figure 16d).

The experiments are summarized in Figure 17 and Table 3. These results differ somewhat from those obtained by Bukh's group, which involved human lymphoid cells; they saw a decrease in c-*myc* expression using the same exposure parameters, a reflection perhaps of a cell type-specific response.

These experiments were also carried out under consitions of heat shock for 20 minutes. Since experiments involving EM exposure were of 20 minutes duration, this provided a more direct means of comparison between the two stimuli. This was an effective means of producing a heat shock reaction since Hsp70 was increased about 34% exposed cells compared to controls (Figure 18d). The increases in other transcripts measured, however, were low under these conditions. C-*jun* and c-jun transcripts showed an average 12% increase in exposed cells (Figures 18a and 18c), but c-*myc* transcript levels were not

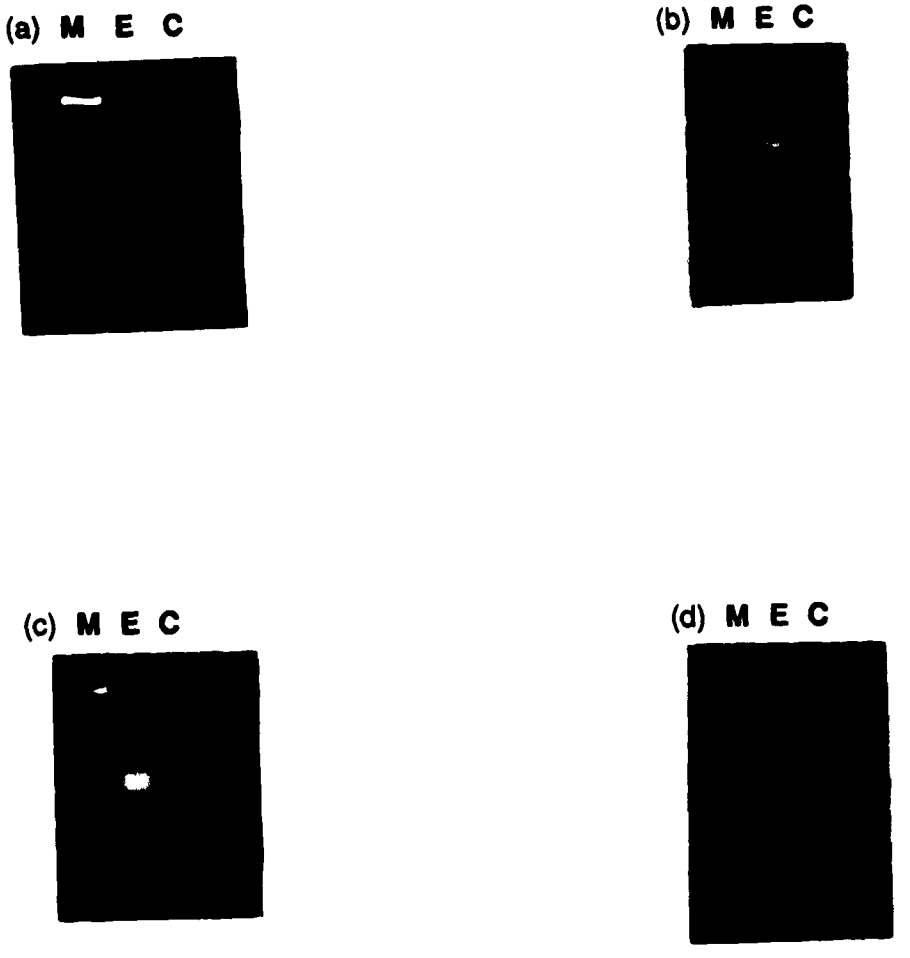


Figure 16a-d. RT-PCR amplification of (a) c-jun (b) c-myc (c) c-fos (d) hsp70
(M) molecular wight marker (E) experimental (C) control
Cells were incubated at 42°C for 4hrs.

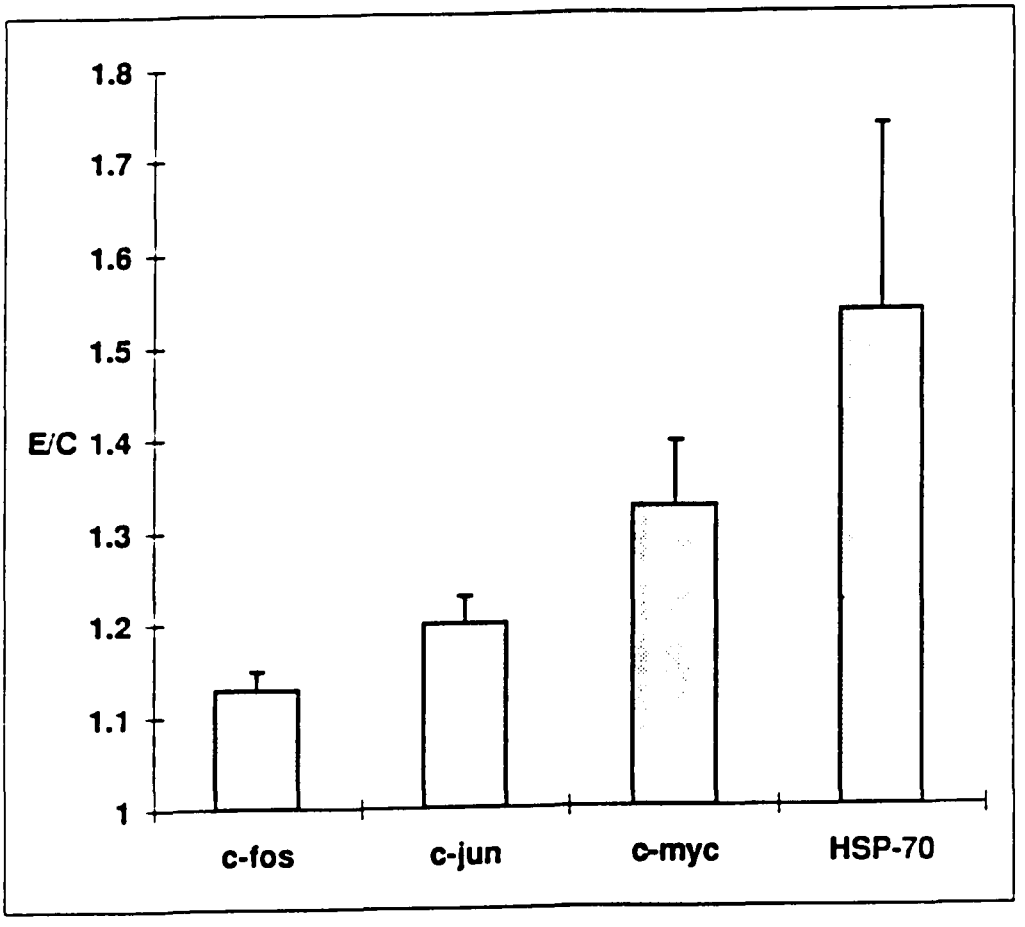


Figure 17. Bar graph summary of RT-PCR experiments from cells which were incubated at 42°C for 4 hrs.

# of exp		c-fos			c-jun			c-myc			HSP-70		
		Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C
1					652	602	1.08	827	667	1.24	1561	1245	1.25
					796	645	1.23	905	589	1.54	1543	1081	1.43
	Mean				724	623.5	1.16	866	628	1.39	1552	1163	1.34
2		704	603	1.17	724	575	1.26	857	601	1.43	1301	901	1.44
		617	576	1.07	947	856	1.11				1426	714	2.00
	Mean	660.5	589.5	1.12	835.5	715.5	1.18	857	601	1.43	1363.5	807.5	1.72
3		614	557	1.10	813	659	1.23	889	689	1.29	1617	584	2.77
					798	625	1.28	666	589	1.13	1312	1186	1.11
		763	671	1.14							1519	1379	1.10
		981	841	1.17							1596	1358	1.18
	Mean	786	689.67	1.14	805.5	642	1.26	777.5	639	1.21	1511	1127	1.54
	Total Mean	735.8	649.6	1.13	788.33	660.33	1.20	828.8	627	1.32	1484.38	1056	1.53
	S.E.			0.02			0.03			0.07			0.20

Table 3. Quantitative summary of RT-PCR experiments performed with HAL cells incubated at 42°C for 4 hrs.

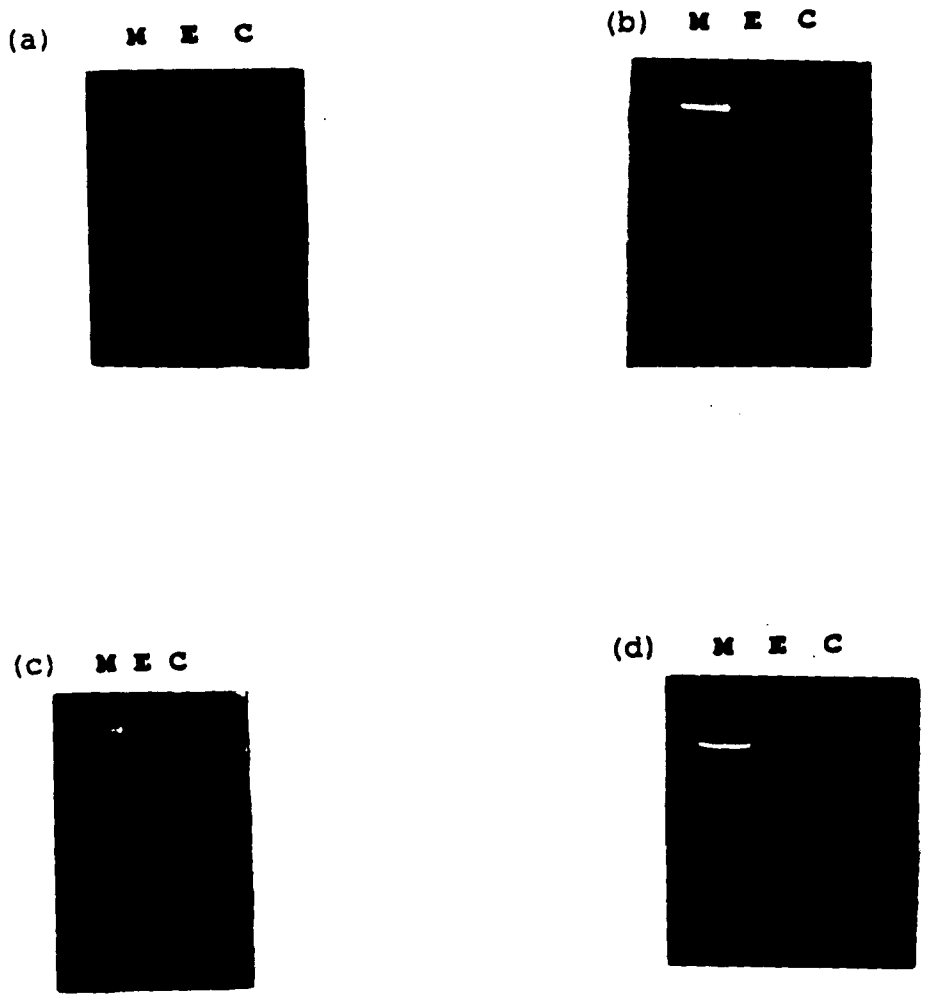


Figure 18. RT-PCR amplification of (a) hsp70 (b) c-jun (d) c-myc
(E) experimental (C) control (M) molecular weight marker
Cells were incubated at 42°C for 20 minutes

increased (Figure 18b). Figure 19 and Table 4 summarize the results of these experiments. Only hsp70 increased significantly, although the increase was smaller than that seen following a 4-hr incubation; control levels of c-*fos* were higher than those of experimental levels. This may be indicative of a transient adaptive mode on the part of HAL cells. Thus in comparing gene expression in both EM-exposed and heat-shocked HAL cells, we observed the following: 1) notable elevations in c-*myc* expression (42% in EM-exposed cells); and in cells heat-shocked for 4 hrs (33% average increase), but no change seen in 20-minute heat-shocked cells (1% increase); 2) a large increase in c-*fos* expression in EM-exposed cells (40%) and a smaller increase in 4-hr heat-shocked cells (12%) and a 12% decrease in 20-minute heat shocked cells; 3) a small increase in c-*jun* expression in EM-exposed (15%), as well as in cells heat shocked for 4 hrs (20%) and 20 minutes (12%) and 4) Essentially no change in Hsp70 levels in EM-exposed (+8%) were observed, but a considerable increase was noted in cells that were heat-shocked cells for 4 hrs (53%) or 20 minutes (33%). Our interpretation of RT-PCR investigations is that EM field exposure is most likely a form of stress response in HAL cells since, among other results, c-*fos* and c-*myc* transcript levels were found to be increased. This is in agreement with results of prior work done in our lab with HL-60 cells (Goodman *et al.*, 1989; Broude *et al.*, 1994) obtained using dot blot analysis. Whether this represents a

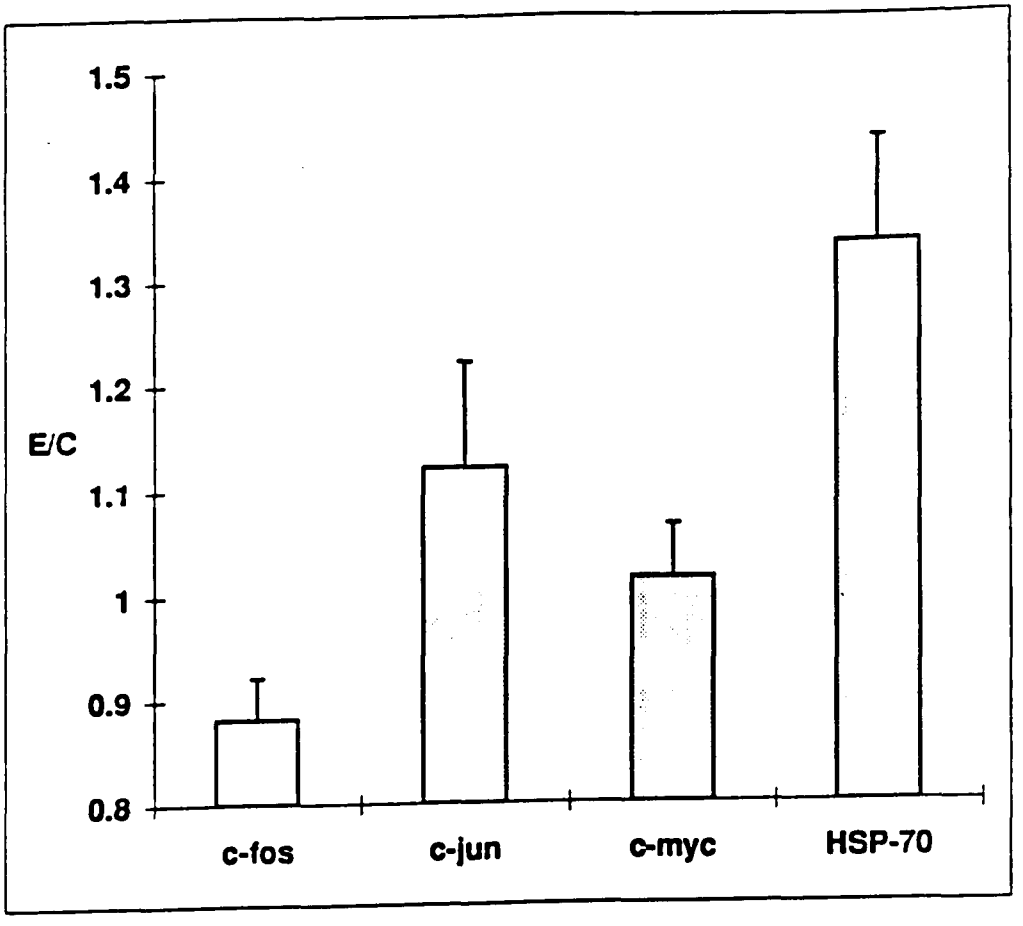


Figure 19. Bar graph summary of RT-PCR experiments from cells which were incubated at 42°C for 20 minutes.

# of exp		c-fos			c-jun			c-myc			HSP-70		
		Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C	Exp	Cont	E/C
1		462	467	0.99	459	353	1.30	649	648	1.00	779	683	1.14
		325	388	0.84	422	329	1.28	722	784	0.92	1041	767	1.36
	MEAN	393.5	427.5	0.91	440.5	341	1.29	685.5	716	0.96	910	725	1.25
2					395	453	0.87	354	365	0.97	588	472	1.25
		288	353	0.82	292	284	1.03	504	431	1.17	869	544	1.60
	MEAN	288	353	0.82	343.5	368.5	0.95	429	398	1.07	728.5	508	1.42
	Total Mean	358.33	402.67	0.88	392	354.75	1.12	557.25	557	1.02	819.25	616.5	1.34
	S.E.			0.04			0.10			0.05			0.10

Table 4. Quantitative summary of RT-PCR experiments performed with HAL cells incubated at 42°C for 20 minutes.

pathologic development in the case of HAL cells is unclear because we also saw a significant increase in transcript levels of the anti-oncogene p53.

We did not detect any noticeable increase in hsp70 mRNA levels in EM-exposed cells. This observation is not the result of an atypical pattern of transcription in this temperature-sensitive cell line as hsp70 transcript levels are markedly elevated following either 4-hr or 20-minute heat shock incubations. Therefore, we believe HAL cells experience a stress response when placed in an EM field, though it is probably not of the heat shock mode.

Discussion

Overview: An investigation of selected transcriptional and translational responses of a human fibroblastic cell line (HAL) to EM fields was conducted. The use of HAL affords the researcher several advantages. For example, although there have been studies examining DNA synthesis in exposed fibroblasts, there exists little information concerning the molecular biology of EM exposure, particularly as it relates to specific gene expression. Further, HAL contains a stably integrated copy of the DNA virus SV40. Viral behavior in EM fields has yet to be investigated; a demonstration of quantitative changes in the activity of this tumor virus may nonetheless help to narrow the gap separating epidemiological and cellular perspectives. Further, SV40 is known to affect a number of host cellular processes. For our purposes, the most relevant of these processes is the increase in heat shock 70 transcription caused by the viral large-T antigen. For this reason, levels of hsp70 were measured in HAL cells exposed to a 60 **Hz** field. These experiments formed the basis of a subsequent study which directly compared the heat shock and EM responses in HAL. This was done because prior work by Goodman and Henderson (1988) implicated EM exposure as a heat shock-type response.

The current study focused mainly on expression of those genes known to

be involved in signal transduction pathways. Again, this choice was made based on earlier work of Goodman *et al.* (1989, 1992), which were conducted using a lymphocyte cell line and which showed EM field modulation of several of these genes. Thus the purpose of this set of experiments was a comparative study in order to determine the degree of similarity between a known stress response, heat shock, and a putative one, elf EM field exposure, in HAL cells. The goal, therefore, was to shine a mechanistic light upon EM field interactions at the molecular level.

A. Effect of Exposure on a DNA tumor virus in HAL cells

HAL cells were exposed to a 20-minute, 60 Hz field in order to measure the effect, if any, on transcription of large-T mRNA. The most direct way of assaying mRNA levels is the dot blot hybridization protocol, which our laboratory has been utilizing in recent years to study transcript levels of genes including c-*fos*, c-*myc* and histone 2B (Goodman *et al.*, 1989). Many of these experiments were conducted using Helmholtz coils as the exposure system. This system was used to examine SV40 early region mRNA levels in HAL cells. An average increase of 50% was observed in exposed cells compared to controls. In an effort to strengthen the validity of these data, HAL cells were exposed in another apparatus, a solenoid-type structure, using the same exposure parameters as before. Again, an average increase of 50% in exposed cells compared to controls

was observed.

The SV40 probe for dot blot experiments was a 1 Kb fragment consisting of a portion of the large-T antigen coding region. Strictly speaking, the probe measured transcription of the entire early region of SV40, of which the large-T coding open reading frame is a part, as opposed to the specific large-T region. This is because the virus transcribes the early region as a single entity which is processed into two main products, large-T and small-t mRNAs (reviewed in Livingston and Bikel, 1986). If early region transcription of SV40 is modulated by the 60 Hz field, then by inference both large-T and small-t mRNAs would be affected. What this means from the cell's perspective is unclear. In fact, the function of small-t antigen in SV40 viral synthesis is unknown and it is not required for transformation of the host cell (Stahl and Knippers, 1987).

The probe that was utilized for these experiments most likely measured what it was intended to measure, namely, the early coding region of SV40 including large-T antigen: we observed the expected 2.4-2.5 Kb fragment as a result of Northern hybridization. In addition, when the probe was hybridized to RNA from a cell line uninfected with SV40, the band did not appear (K. McDowell, personal communication). Thus the appearance of the band is contingent upon SV40 presence in the cell.

Although large-T is primarily a nuclear protein, there is a subpopulation

associated with the plasma membrane (Klockmann and Deppert, 1983), the significance of which remains unknown. The extent to which it is affected by the elf EM field is also unclear. For this reason, a direct numerical comparison of RNA and protein cannot be made. Data presented suggest a coupling of the viral transcriptional and translational responses to a 60 Hz sinusoidal field. *In vivo* protein synthesis, however, has been shown without detection of transcriptional activation (Kim and Novak, 1990)

An increase in intracellular large-T antigen does not necessarily lead to changes in cellular processes normally affected by its presence. For example, it follows that Hsp70 mRNA levels would be elevated due to increased amounts of large-T available for binding to its promoter as a result of exposure. No noticeable difference in Hsp70 mRNA levels was observed. It is possible that saturation levels of large-T antigen are already attained regardless of any increase attributed to exposure.

The laboratory techniques utilized for measuring levels of large-T transcription products do not provide information concerning rates of synthesis; nor do they answer the question of whether transcription itself is directly affected by the exposure field. Goodman and Henderson (1983) have nonetheless provided evidence for such a scenario based on results of *in situ* transcription autoradiography involving insect salivary gland chromosomes. Phillips *et al.*

(1991a) confirmed these conclusions by performing nuclear run-on experiments with human lymphoid cells. Thus SV40 transcription may be directly influenced as well by the presence of the field.

The site of SV40 integration is random (reviewed in Tooze, 1981), but the suggestion that viral response to EM exposure is related to the integration site cannot be discounted. For example, viral insertion could occur immediately downstream of a host promoter which is strongly induced by the EM field. If the virus is under transcriptional control of a host promoter, then its transcriptional apparatus could likewise be altered. Conversely, virus transcription which is under control of a host promoter normally unaffected by an exposure field could also be unresponsive. One hypothesis, as yet unproven, is that specific promoters respond to exposure in specific ways. Transcription studies of SV40 utilized two different exposure systems: Helmholtz-aiding coils and a solenoid system. The magnetic fields in each system was the same at 8μT, but the electric fields were different. The solenoid generated a 38 μV/m field, whereas the Helmholtz coils had an E-field of about 10 μV/m. The SV40 transcription was modulated to the same degree irrespective of the E-field, which implies that the magnetic field component is the principal arbiter of the transcription response.

Located between the early and late transcription regions of the SV40 genome are two 72-base pair sequences, known as enhancers, which are crucial

for SV40 early region transcription (Moreau *et al.*, 1981). Contained within each enhancer, as well as the promoters of the host genes *fos* and *jun*, are copies of the consensus sequence TGACTA (Lee *et al.*, 1987). The cellular protein AP-1 binds to the consensus sequence to affect transcription in a positive manner. It has been shown to bind the SV40 enhancer *in vitro* (Lee *et al.*, 1987). It is unknown whether AP-1 binding occurs *in vivo*, but it is reasonable to assume such an event takes place since transcriptional activation of the related human papilloma 16 (HPV16) by AP1 protein has been observed (Chong *et al.*, 1990). In this way, AP-1 sequences may provide a molecular sign post in the cells's response to EM signals.

B. Use of RT-PCR to investigate mechanistic approaches to EM field interactions with HAL cells.

The expression of a particular gene under study must be of sufficiently high level for detection if the dot blot protocol is to be of value. Many transcripts do not meet this requirement. This is a reflection of the overall rate of biosynthesis which varies depending on the cell line.

In order to bypass the problem of low-level expression, especially as it pertains to oncogene expression in HAL cells, we have employed a reverse transcription-polymerase chain reaction (RT-PCR) protocol as an investigatory

tool. This method allows for the study of vanishingly small amounts of transcription product. The degree of consistency obtained is similar that of dot blots.

The effect of a 20-minute, 60 Hz field on c-*myc* expression in HAL cells was measured and found to result in an average increase of greater than 40% in exposed cells compared to controls. This result is similar to that obtained in HL-60 cells using dot blot analysis (Goodman *et al.*, 1989). The c-*myc* gene was among the first to be associated in its mutated form with malignant transformation (Hayward *et al.*, 1981). The protein product of c-*myc* functions as a transcription factor which binds *in vitro* the sequence CACGTC (Blackwell *et al.*, 1990). Initial experiments provided evidence for a c-*myc* role in DNA synthesis as antibodies against c-*myc* protein reversibly inhibited this process in HL-60 cells (Studzinski *et al.*, 1986). Later, treatment of cells with mitogenic agents resulted in rapid induction of c-myc expression (Murphy *et al.*, 1988; Patel *et al.*, 1992). Inhibition of c-*myc* mRNA with antisense nucleotides slows proliferation of HL-60 cells, as demonstrated by Wickstrom *et al.* (1988). Superficially, an increase in c-*myc* expression in HL-60 and HAL cells under exposure conditions would support the notion that EM fields exert an accelerating influence on the cell's proliferative capacities and, by extension, may be deleterious to it. A 40% increase in expression of the tumor suppressor p53 in exposed HAL cells was also found.

Like c-*myc*, p53 is a transcription factor with sequence-specific DNA-binding capacity (Bargonetti et al., 1991; Scharer and Iggo, 1992). It has recently been shown to induce expression of WAF1, which in turn suppresses growth of human brain, lung and tumor cells *in vitro* (El-Deiry et al., 1993). Thus on one hand, the machinery of cell proliferation may be promoted by elf EM fields; on the other, its tumor-suppressor properties may be enhanced. One can envision a genetic "tug-of-war" between these countervailing tendencies which in the non-malignant cell may not be sufficient to change the status quo; but might in the malignant cell expedite growth and perhaps other events characteristic of the malignant state, a state established prior to and independent of EM exposure.

A series of RT-PCR experiments was conducted in order to directly compare the patterns of heat shock and EM response within the same cell line, HAL. Such a study had not been previously done. More importantly, however, is the fact that these experiments represent a follow-up, from a transcriptional standpoint, to the work of Goodman and Henderson (1988), who observed heat shock protein induction in EM-exposed insect salivary gland cells. The goal of the current study was to gain insight into the mechanism of action of EM exposure.

Since HAL cells are temperature-sensitive mutants, it was necessary to insure that they are heat shock-responsive as defined by induction of hsp70 mRNA. The heat shock 70 family of genes code for proteins that function in the

transport of other proteins across intracellular membranes (Murakami *et al.*, 1988). They are thought to play a role in guaranteeing proper folding of certain proteins as well (Pelham, 1986; Pelham, 1989). Both the constitutive (hsp73) and inducible (hsp73) forms are sequestered in the centrosome during mitosis in mammalian cells (Rattner, 1991). Rattner proposes a role for hsp70 in microtubule nucleation during cell division.

A 4-hour, 42° incubation of HAL cells elicited the heat shock response in HAL cells. These parameters were previously demonstrated to result in the heat shock effect in human lymphoid cells (Bukh *et al.*, 1990), and were chosen on that basis. In lymphoid cells, heat shock results in a decrease in c-*myc* mRNA levels and an increase in c-*fos* and c-*jun* mRNA levels, although values were not quantified. The oncogenes c-*fos* and c-*jun* code for proteins which form the AP-1 complex, which in turn bind to the DNA sequence TGACTCA to affect cellular transcription (Lee *et al.*, 1987) and possibly SV40 transcription as well.

HAL cells were heat-shocked for 4 hours and assayed for mRNA levels of c-*myc*, c-*fos*, c-*jun* and hsp70. (see Table 3). It is of interest to note that heat shock results differ somewhat from those in lymphoid cells in that c-*myc* mRNA levels decreased and c-*jun* and c-*fos* increased; this may be a reflection of the temperature-sensitive phenotype of HAL, or it may be indicative of a cell type-specific reaction to heat-shock stress. This may well be the case as it relates to

elf EM expsures of various cell types. There was little or no change in hsp70 (+8%) transcript levels in exposed cells compared to controls.

To some extent, these results confirm studies by others which show theses fields to be capable of causing a pathogenic state *de novo*. If they elicit such a response, it is probably in the augmentation of an already pathogenic state. The lack of any notble increase in HSP70 transciption in response to EM field exposure was uinexpected given earlier results reported from the laboratory, which suggested a heat-shock-like behavior in insect cells. It is difficult to interpret the data obtained using HAL cells since a number of conflicting factors come into play. In addition to the positive influence of large T-antigen onm Hsp70 transcription, p53 has been shown to inhibit Hsp70 transcript levels. It is possible that p53 does not play a significant role in this ystem since it is essentially inactivated by the presence of the large T-antogen. On the other hand, c-*myc* protein is known to elevate HSP 70 transcription (Klingston *et al.*, 1984). This leads to a number of scenarios that are at play in the microenvironment of HAL cells. It is not clear which of these interactions takes precedence as it relates to low energy field exposure.

Our overall interpretation of these data is that short-term, 60 **Hz** EM field exposure represents a stress event in HAL cells, but one which is ultimately exclusive of the heat shock response in the same cell line. Hsp70 mRNA levels

are greatly elevated in heat shocked cells, as expected, but not to any large degree in EM-exposed cells. In addition, c-*fos* expression is not stimulated in heat-shocked HAL cells as it is in response to electromagnetic exposure. Increases in c-*myc* and c-*jun* were noted in both heat-shocked and EM-exposed HAL cells.

Although results of the 4-hr heat shock exposure show this temperature-sensitive cell line to be capable of such a response, a 20-minute exposure would allow for direct comparison with a 20-minute EM exposure. Results of a 20-minute heat shock exposure of HAL cells, with subsequent measurement of c-*myc*, c-*jun*, c-*fos* and hsp70 mRNA levels, showed only hsp70 to be modulated, averaging 33% increase over controls; c-*jun* and c-*myc* show little or no increase; c-*fos* levels actually decreased. As stated earlier, this may represent an initial form of adaptation by HAL cells.

We have, in a sense, used the RT-PCR results of hsp70 mRNA measurements from heat-shocked HAL cells as a yardstick in helping to interpret data concerning measurements of other transcripts, whether obtained from EM-exposed or heat-shocked cells. This was done as a way to gauge the suitability of the protocol since inducibility of hsp70 transcripts under heat shock conditions is essentially a universal occurence. Had the RT-PCR protocol not detected a notable elevation in hsp70 transcripts, especially in light of results of Northern

hybridization using hsp70 DNA as a probe, then RT-PCR would not have been considered a viable means of measurement, at least for the purpose delineated above. That hsp70 transcript levels were seen to increase in heat-shocked HAL cells provides evidence of the suitability of the RT-PCR method.

The likelihood that heat shock stress and elf EM field exposure are indistinguishable to the cell is, in retrospect, small. There are aspects of these two forms of stimuli, however, that elicit similar biological responses, at least at the transcriptional level. The physiological state of the cell at the moment of exposure is probably a deciding factor and may in fact determine its "competence".

The possibility exists that different cell types, or even different species, respond to the same stimulus in a cell-specific or species-specific manner. If such is the case, this would complicate the situation as it relates to formulation of an all-encompassing theory explaining empirically derived results. Alternatively, exposure conditions may change imperceptibly, but sufficiently to elicit a varied biological response. Cell concentration, as well as the volume of liquid in which the cells are placed, may also be important factors in promoting the EM and/or heat shock responses, although these parameters would be expected to have greater impact on EM-exposed cells by virtue of the resulting change in electric field gradients. In any event, both the volume of liquid and the cell concentration

of HAL cells used in these studies differed from those of HL-60 experiments in previous studies. An increasing amount of evidence has been collected, both in our lab and in others, that signal transductions are responsive to EM fields. This is based on observed changes in expression of genes, *e.g.*, c-*fos*, c-*myc*, etc., which are known to be associated with these pathways. But it is still not clear whether this represents a primary response or merely an offshoot of a separate event occurring elsewhere in the cell or at its surface.

The findings reported here provide evidence that EM fields represent a form of cellular stress. But would such stress necessarily cause harm to the cell's inherent biosynthetic machinery? Most likely not. Data collected from p53 and c-*myc* experiments, for example, suggest the cell's proliferative and non-proliferative tendencies may cancel each other out, with no net change in the microenvironment. On the other hand, results of studies concerning an exogenously derived entity, SV40, raise the question of whether other tumor viruses are also affected by EM fields; or whether SV40 is modulated in cell lines other than HAL. The answers to these questions may have epidemiological implications.

The belief here is that specific types of sequences are more responsive to EM exposure than others. One way to test this hypothesis would be to measure and compare transcript levels of artificially constructed GC- or AT-rich sequences,

or repeat sequences such as the type found in rDNA, and measure their behavior when placed in an EM field.

SUMMARY

The overall strategy employed in these experiments is given in Figure 20. The experimental results are summarized in Figure 21. The experiments used the cell line HAL. There are two principal advantages to using the HAL cell line: 1) It is immortalized, allowing for a series of extended experiments and 2) the agent of immortalization, SV40, represents in essense a genome within a genome and provides an ideal system for studying the effects of electromagnetic exposure. Large-T antigen is a distinct and readily measurable entity within the cell. This is notable since viral behavior in a low energy field had yet to be investigated.

We found evidence that large-T antigen is modulated at the transcriptional and translational levels. This observation, along with the fact that large-T enhances transcription of heat shock 70 (hsp70), led us to extend our studies in that a transcriptional comparison was made between a known stress response, heat shock, and a putative stress respnse, low energy field exposure. Thus, large-T antigen provides the linchpin which connects the two approaches outlined in Figure 20.

The identity of a governing principle(s) which would explain the observed

OBJECTIVE: TO DETERMINE A MECHANISM(S) FOR A CELL'S INTERACTION WITH EM FIELDS

APPROACH 1: TO USE EXPRESSION OF AN INTEGRATED SV40 VIRUS AS A SIMPLIFIED MODEL SYSTEM

APPROACH 2: IF APPROACH 1 IS SUCCESSFUL, TO TEST THE HYPOTHESIS THAT EM FIELDS ARE A FORM OF CELLULAR STRESS.

RATIONALE: T-ANTIGEN AFFECTS HSP-70 EXPRESSION

Figure 20

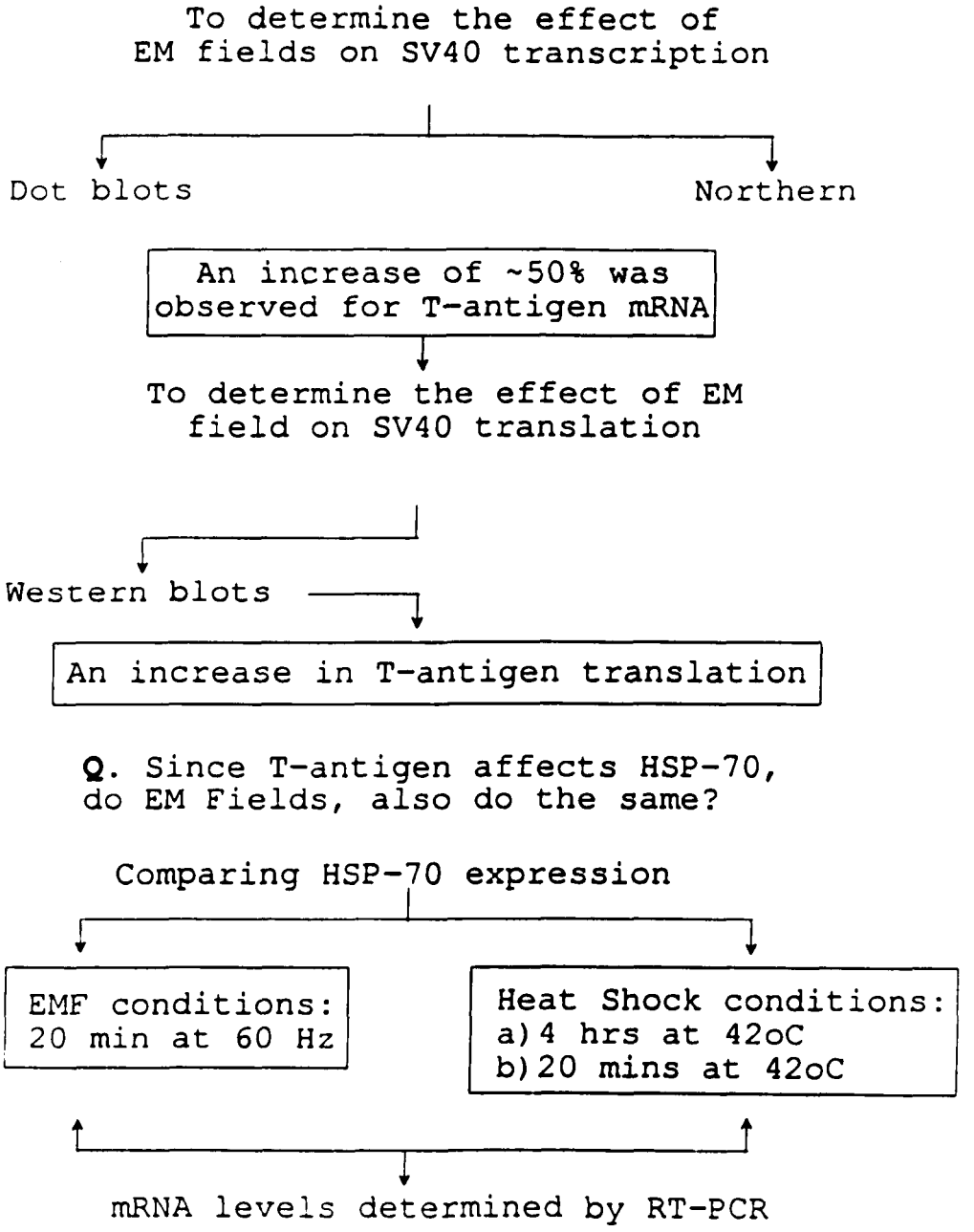

To determine the effect of EM fields on SV40 transcription
Dot blots
Northern
An increase of ~50% was observed for T-antigen mRNA
To determine the effect of EM field on SV40 translation
Western blots
An increase in T-antigen translation
Q. Since T-antigen affects HSP-70, do EM Fields, also do the same?
Comparing HSP-70 expression
EMF conditions: 20 min at 60 Hz
Heat Shock conditions: a) 4 hrs at 42oC b) 20 mins at 42oC
mRNA levels determined by RT-PCR

Figure 21

effects of EM exposure on living organisms is currently unknown. Indeed, given the byzantine nature of the cell interior, it is possible no such principle(s) exists. A more likely scenario argues in favor of a defined set of guidelines, the elucidation of which may await the development of more sensitive analytical methods or a careful examination of the effects.

APPENDIX

Signal generating equipment

1. Helmholtz coils

Helmholtz-aiding coils were constructed using 50 turn rectangular windings of gauge 14 magnet wire measuring approximately 13cm X 14cm with 8cm spacing. The coils are 1cm in diameter wound around an approximate square form. They are positioned with their central axis horizontal; samples are placed on a plexiglass stand in the horizontal plane in an area shown to have a uniform magnetic field and maximum field strength. The bottom of the flask is 2 cm below the axis level. The height of the liquid is approximately 0.5 cm.

A sine wave generator is used with variable frequency control. An ammeter is placed between the "jig" and the Helmholt coils on the recommendation of Fred Dietrich. Flasks are place vertically oriented to the field.

Calibration of Search Coil (measurements by Dr. Martin Masakian-NIST). This probe was constructed at EBI as the "special" search coil; it is ~25x more sensitive than the standard (1x) used at EBI. Dr. Masakian made 4 to 6 measurements for frequencies we have used from which he derived mean values relating the search coil output in peak-to-peak volts to his measured B-field in Gauss. (These were standardized against a similar probe constructed at NIST.)

Frequency	Calibration Factor for search coil-peak-to-peak volts/ 10^{-4} peak teslas	Equivalent to: Teslas (peak)/mV (p-p) (x 10^{-4})
45	0.00197	0.51
60	0.00264	0.38
72	0.00317	0.32
90	0.00394	0.25

Charactistics of the signal generated by Helmholtz coils. The exposure parameter utilized for all experiments using the Helmholtz coils were 5μV, 60 Hz (20 minutes duration). The characteristics of the 60 Hz signals using a power regulator ("jig setting")of 5 μV are as follows:

GAUSS (RMS)	0.057
PEAK B FIELD [Teslas]	8×10^{-6}
E FIELD[a] [volts/meter]	3×10^{-6}
E FIELD[b] [volts/meter]	15×10^{-6}
E FIELD[c] [volts/meter]	11×10^{-6}
μamp/cm^2 [d]	2.6×10^{-3}
dB/dT [Tesla/sec]	3×10^{-3}

Parameters of the 60 Hz fields. [a]In H_2O at 2mm radius and circular path as estimate of magnitude (by Dr. John Moore, EBI); [b]independent measurement in laboratory of Dr. T. Litowitz using a dipole antenna and the calculations of McLeod *et al.*; the field was measured at the bottom of a Petri dish where the height of the buffer was 1.1 cm.; [c]from calculations of Dr. H. Bassen; [d]estimated from E-field measurements (see b); from $J = \sigma \times E$, where the conductivity is 1.7 S/m (balanced culture media with 10% fetal calf serum) at 37° C. The rate of change was measured directly by a Hall effect probe or by a calibrated inductive test coil at EBI. The B-field was calculated from the measurements of Moore at 60 Hz, 3×10^{-3} Teslas/sec, using the formula for peak B-field: dB/dT Teslas/sec/ 2π 60.

2. Solenoid system

A solenoid system, constructed by Fred Dietrich (Electric Research and Management, Inc.), was used. An amplifier was employed in order to generate the exposure signal. The magnetic field was measured to be 8 μT; the E-field was 38 μV. Ambient field strength was 0.8 mG. The flasks are placed horizontally oriented to the field during exposure. dB/dT is 3×10^{-3}T.

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