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Evaluation of *In Vitro* Effects of 50 and 60 Hz Magnetic Fields in Regional EMF Exposure Facilities

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A weak association between magnetic-field exposure and increased incidences of cancer has been reported. While alterations in cellular processes after *in vitro* magnetic-field exposures have also been reported to provide plausibility for this association, other laboratories have been unable to repeat the findings. As part of an accelerated electric- and magnetic-field (EMF) research program, the National Institute of Environmental Health Sciences with the Department of Energy identified the replication of the published positive effects as a priority. Regional EMF exposure facilities were established to investigate major *in vitro* effects from the literature. These included effects on gene expression, intracellular calcium, colony growth in soft agar, and ornithine decarboxylase activity. The laboratories that first reported these effects provided experimental protocols, cell lines, and other relevant experiment details. Regional facility studies included sham/sham exposures (no applied field in either chamber) and were done in a blinded fashion to minimize investigator bias. In nearly all experiments, no effects of magnetic-field exposure were found. The effort provided insight into dealing with the difficulty of replication of subtle effects in complex biological systems. Experimental techniques provided some clues for the differences in experimental results between the regional facility and the original investigator. Studies of subtle effects require extraordinary efforts to confirm that the effect can be attributed to the applied exposure. © 2000 by Radiation Research Society

INTRODUCTION

Electric and magnetic fields (EMFs) associated with the production, transmission and use of electricity are ubiquitous in industrialized society. The U.S. Congress, prompted by concerns about the possible increased incidence of childhood leukemia in homes predicted to have high magnetic fields (*1, 2*), authorized an accelerated 5-year EMF research program. The Department of Energy (DOE) managed the program. The National Institute of Environmental Health Sciences (NIEHS) was responsible for the health effects research. Since it was unlikely that epidemiology studies could be designed, conducted and reported within the 5 years, the NIEHS decided to focus on *in vitro* and *in vivo* findings that might support the epidemiology studies. The main emphasis of the EMF program was on extramural grants to university-based investigators.

For many of the EMF *in vitro* findings, the reported effect was observed in a single laboratory, and in many instances other laboratories could not repeat the finding. Therefore, NIEHS and DOE decided to also concentrate on reproducing some of the more fundamental magnetic-field effects reported in the literature. Regional EMF exposure facilities were established at the Food and Drug Administration (FDA) in Rockville, MD, the National Institute for Occupational Safety and Health (NIOSH) in Cincinnati, OH, and DOE laboratories in Oak Ridge, TN and Richland, WA. The goal of this program was to replicate some of the controversial *in vitro* effects of EMFs that have been reported in the literature under carefully controlled experimental conditions. A second goal was to provide a regional EMF exposure facility for investigators who did not have access to such facilities at their own institutes. The DOE constructed similar exposure systems for each facility using a design that provided the capability of blocking the Earth's static fields and recreating alternating and static field conditions similar to those at the laboratory that had reported an effect. Once an effect was replicated in a regional exposure facility, it could be verified quickly in a second or third regional exposure facility with a matched exposure system. After an effect was clearly demonstrated and replicated, there could be rapid progress in determining what parameters of exposure (e.g. frequency, intensity) were most effective in causing the effect. This would provide a basis for DOE to develop a mitigation strategy.

The four reported magnetic-field effects that were se-

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lected for study have frequently been cited to support the plausibility of weak associations between magnetic-field exposure and increased rates of childhood leukemia. These *in vitro* effects were increased *MYC* expression in HL60 cells (*3–5*), increased intracellular calcium (Ca^{2+}) in human leukemia (Jurkat) cells (*6–11*), transformation and colony proliferation in JB6 cells (*12–15*), and increased ornithine decarboxylase (ODC) activity in L929 cells (*16–18*). Of the four effects, increased ODC activity was considered to be the one reproducible *in vitro* magnetic-field effect by the National Research Council EMF Committee (*19*). Increased gene expression and increased intracellular Ca^{2+} had been prominently featured in the EMF literature to support the epidemiological associations with childhood cancer. Finally, transformation of JB6 cells was considered to be a cellular change more closely related to the cancer process than other reported *in vitro* effects. While the relevance of the four *in vitro* effects to childhood leukemia is not always clear, the demonstration of any robust *in vitro* effect would increase the plausibility of adverse health effects from magnetic-field exposure. Further, an easily replicated *in vitro* effect could be used as a biomarker of exposure for additional research investigating the exposure parameters critical to eliciting a biological effect. The purpose of this paper is to provide a description of the attempts to replicate these four EMF effects frequently cited and the lessons learned in this effort. The results of the four individual studies have been reported or are in press (*20–23*).

MATERIALS AND METHODS

Approach

The goal of the regional EMF exposure facilities program was to provide sufficient documentation to facilitate replication of effects by other regional EMF facilities using the same magnetic-field exposure systems. In addition, the replication effort needed to be extensive and detailed to identify the reasons for the different results if the reported biological effect could not be demonstrated. Therefore, several aspects of the experimental approach were common to all regional facility studies. The first was to obtain detailed protocols from the investigator reporting the effect and to develop very detailed protocols for the EMF regional facilities including source and lot numbers for the biological reagents. The studies were to be conducted in a blind fashion with internal positive and negative controls, except for the Ca^{2+} study, where this was precluded by the microscope apparatus. Exposures could occur in either chamber (selected randomly), and in some cases, no exposure occurred in either chamber (sham/sham controls). A quality assurance unit reviewed the protocols, data analysis and study reports. In addition, the DOE and the National Institute of Standards and Technology (NIST) conducted site visits to evaluate exposure systems, document exposures, record ambient magnetic fields and electric fields, record temperatures in chambers and exposure systems, and document stray fields. These visits sometimes identified problems that were then resolved. The regional exposure facilities used the first year for baseline studies for the various experimental conditions to determine background levels, establish variability in assays, and identify critical factors that could affect the biological end point of interest. Positive controls were routinely included to demonstrate the responsiveness of the assay system to experimental manipulations and to determine the magnitude of effect that was detectable.

Regional Facility Exposure System

The exposure system for the regional exposure facilities (model 2XC) provided by DOE was manufactured by Columbia Magnetics Inc. (Kennewick, WA) and has been described (*24*). The regional exposure system consisted of two coil systems energized by a function generator/power amplifier combination under computer control (Fig. 1). Uniform temperature, humidity and CO_2 were maintained by a modified commercial incubator that was isolated from the two exposure chambers. The incubator supplied atmosphere to the exposure chambers through insulated pipes. The exposure chambers had inner coils to apply the magnetic-field exposure under study while the outer coils could limit ambient fields. For a sham exposure, the double-wound coils were energized with opposing current flow so that the net applied magnetic field was zero. Computer-controlled random determination selected which side was the exposure chamber or selected sham/sham exposures (no applied field in either chamber) in a blind fashion. All data including flux density and temperature were saved to a disk. Field intensities within the chamber did not vary by more than 1.4% from the chamber's central measurement. Temperature stability in the exposure chambers was within ± 0.1°C. Alternating magnetic-field intensity was determined by NIST (Dr. Martin Misakian) to be within 5% of the targeted values at both exposure facilities. This exposure system was used for *MYC* expression, ornithine decarboxylase (ODC) activity, and JB6 cell colony growth studies. The regional exposure facilities at FDA and NIOSH also had other magnetic-field exposure systems (*21, 25*) that were used to supplement studies conducted using the regional exposure system. A comparison of the exposure systems is shown in Table 1.

Exposure System for Calcium Studies

Studies of intracellular calcium required exposure of cells while they were being viewed under a microscope. This could not be done in the regional facility exposure system, and a special exposure system for calcium studies was constructed at NIOSH (*21*). The magnetic-field exposure chamber was custom-machined from an aluminum block. The exposure chamber was open in the center and secured the cover slip holder in place over the microscope objective. The coil was designed to operate at 50 Hz and produce a 0.15-mT field over an exposure volume of 1 cm^3. The coil contained bifilar magnet wire wound to allow either active field cancellation (sham exposure) or applied field generation. A crossover switch with positions labeled ''A'' and ''B'' was used to mask these two magnetic-field conditions. In addition, a second switch labeled ''C'' and ''D'' was used for no current flow (no field condition) or current flow for applied-field or sham-field conditions. The switches controlling the exposures were in a box hidden from the experimenter. Experiments were performed in a randomized block design; thus all exposure conditions were performed each day in a random fashion. The extensive temperature control conditions needed to maintain cells within a small tolerance (0.2°C maximum fluctuation) have been described (*21*).

Exposure System for Gene Expression and ODC Activity

In addition to the regional magnetic-field exposure system, the FDA exposure facility contained a magnetic-field exposure system that consists of a custom-built incubator with two mu-metal exposure chambers stacked vertically within a common incubator chamber (*25*). The chambers are equipped with fans to circulate air through and around the mu-metal enclosures. Temperature and a.c. and d.c. magnetic fields in each of the exposure chambers were continuously monitored and controlled by computer.

The investigators who reported an effect of EMF exposure on ODC activity used an exposure system consisting of two similar mu-metal chambers in separate incubators (*17, 18*). This exposure system was reproduced at FDA and used the same coils, amplifier and mu-metal enclosures that the original investigators used in their earlier ODC experiments (*22*).

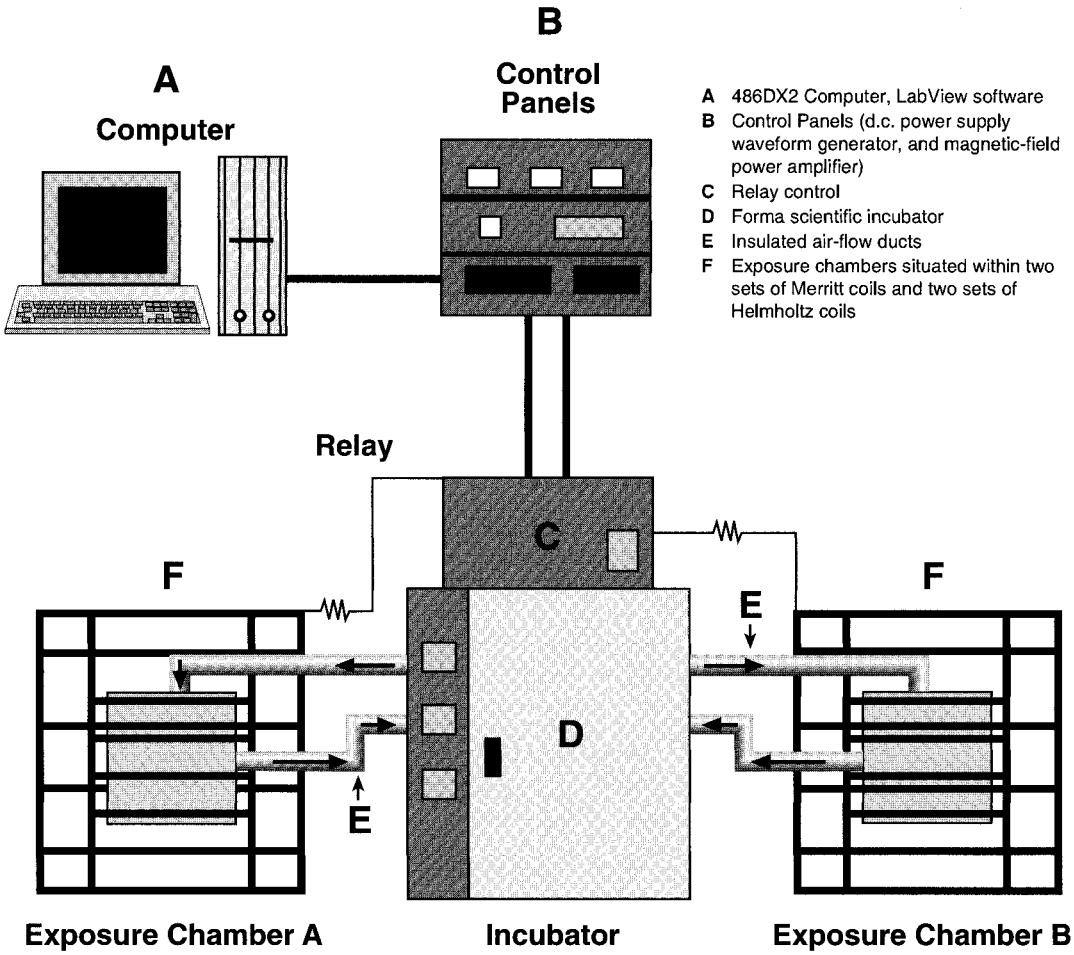

FIG. 1. Regional exposure system. The system consists of an incubator and two exposure chambers. Two concentric sets of Merritt coils with a common vertical axis surround each exposure chamber. Each chamber also has two Helmholtz coils with horizontally directed orthogonal axes. A computer controls field variables and records exposure conditions (see text for details).

Gene Expression Procedures

A draft protocol of all procedures was prepared from a review of the original studies. This protocol was revised in a page-by-page review by a panel including the original investigators, the FDA investigators, and two independent cell biologists. All experiments were done in a blind fashion. The positive control for gene induction in HL60 cells was treatment with 5 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) with 0.1% DMSO as the solvent control. TPA was added at the start of the incubation to demonstrate the responsiveness of the cells compared to nonstimulated cells that were incubated simultaneously. The details of the methods have been published (*23*). HL60 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) as well as from the original investigator at Columbia University who had reported increased *MYC* expression with EMF exposure. Serum lot, culture medium, and plastic ware were identical or equivalent to those used in the laboratory at Columbia. Upon receipt, cells were grown to provide a large supply of cryogenically preserved aliquots. This allowed fresh cells in the same range of passage numbers to be used in all experiments. Minimal handling of the cells was performed as specified by the original investigator and the protocol reviewers to reduce the possibility of gene induction unrelated to magnetic-field exposure. A greater number of flasks than needed were prepared and the flasks were randomized with respect to the order of preparation. The cell density was carefully controlled both for cell maintenance and for cells used in the experiments. Flasks were coded and sealed before placement in the exposure system. To decrease variability due to temperature changes, cells were rapidly transported from the culture incubator to the exposure chambers using a heated insulated enclosure.

RNA was isolated using the method of Case and Daneholt (*26*) as modified by the original investigators (*3, 27, 28*). Northern blots were hybridized to radiolabeled probes and subjected to storage phosphor autoradiography (*23*).

Procedures for Measuring Intracellular Calcium Concentration

The original investigator reported that 50 Hz magnetic fields induced real-time changes in intracellular calcium (Ca^{2+}) as measured by the Ca^{2+} probe fura-2 using fluorescence microscopy (*6*). Investigators at NIOSH attempted to replicate this finding using an exposure system similar to that used by the original investigators (*21*). Cells of the Jurkat cell line (clone E6-1), derived from a human T-cell leukemia, were obtained from the original investigators. Details of the cell cultures and imaging of cells have been reported (*21*). Intracellular Ca^{2+} was determined through changes in fluorescence of the radiometric dye fura-2 using a fluorescence microscopy imaging system. It was noted that the process of mounting the culture caused changes in Ca^{2+}, but most cells returned to normal after 20 min. Thus cells were kept in the dark for approximately 20 min prior to starting the experiment.

The original investigation used morphology to select individual cells,

TABLE 1
Magnetic-Field Exposure Systems at the Food and Drug Administration and the National Institute for Occupational Safety and Health

Exposure system used	Coil type	Shielding	Applied field orientation	Coil radius	Studies performed
FDA	Helmholtz	Mu-metal	Horizontal	30 cm	Gene transcription (ODC, *MYC*)
FDA regional exposure facility	Merritt	Energized coils	Vertical or horizontal	30 cm	Gene transcription (ODC, *MYC*)
FDA/Catholic University of America	Helmholtz	Mu-metal	Horizontal	11 cm	Enzyme activity (ODC)
NIOSH	Single soil	None	Vertical	3.2 cm	Intracellular Ca^{2+} concentration
NIOSH regional exposure facility	Merritt	Energized coils	Vertical	30 cm	Anchorage independence (JB6 cells)

and the original report is based on the fura-2 response in 20 cells (*6*). The fluorescence data were collected for 16 min; in the NIOSH experiments, an additional 6 min was included for adding a positive control (anti-CD3 antibody). Thus each NIOSH experiment was 8 min baseline, followed by magnetic-field exposure, sham exposure, or no-field conditions for 8 min, followed by anti-CD3 antibody for 6 min. In the studies of the original investigators, the method of fluorescence detection was with a photomultiplier tube, dictating collection from a single cell at a time. Thus the cell had to be selected prior to exposure. In the NIOSH experiments, the use of fluorescence imaging enabled data collection from a field of cells simultaneously and eliminated the need to impose selection criteria prior to exposure. It was possible to observe 20 to 40 cells per field at the regional facility, and thus it was not necessary to select a cell for observation. Cells that did not exhibit rapid intracellular Ca^{2+} changes during the control period and did respond to a positive stimulus after the exposure/sham exposure were selected for analysis. The response of the cells during each period was determined as a yes or no for the presence of Ca^{2+} transients. Ca^{2+} transients were identified as the F_{340}/F_{380} ratio exceeding the baseline by two standard deviations for five consecutive data points (10 s). In this manner, more than 1000 cells were examined for a response to magnetic-field exposure. The incidence of responders in the presence of magnetic fields was compared to the incidence of responders in the absence of magnetic fields (sham and no-field conditions) by analysis of variance using a randomized block design.

Growth of JB6 Colonies on Soft Agar

Plastic ware, media, serum and all other reagents were obtained from the commercial sources used by the original investigator (*12*). Prior to the beginning of the experiments at NIOSH, the principal investigator at NIOSH visited the laboratory of the investigator reporting increased growth of JB6 cells after magnetic-field exposure. Vials of JB6 cells from this laboratory were cryopreserved to provide sufficient quantities of cells of similar passage to last for the entire series of experiments. The same serum lot as used by the original investigator was used. Another lot of serum from the same source that met specific requirements for growth of JB6 cells was also used. In addition, promotion-sensitive cells (clone 41) were obtained from the ATCC to examine differences in transformation frequency between cells from different sources. Three or more replications per treatment were used in each assay. The exposure conditions of both chambers were randomized by the computer system and were unknown to scientists conducting the experiments and counting colonies (*20*). The positive controls for the JB6 cells included adding from 0.01 to 1.0 ng/ml of TPA at the beginning of the culture period.

TABLE 2
Variation in Cell Response with Microscope Objective

Microscope objective	Number of experiments	Number of cells	Percentage responders[a]
100×	7	61	11.6 ± 13.5
40×	3	103	18.2 ± 1.0
20×	3	296	3.4 ± 1.6

[a] Ca^{2+} responses were identified as the F_{340}/F_{380} ratio exceeding the baseline by two standard deviations for five consecutive data points.

Procedures for the Determination of ODC Activity

L929 cells were obtained from the original investigators reporting the effect (Catholic University of America, Washington, DC) and from the ATCC. Details for the experimental methods have been published (*22*). Cells were grown in an open-front mu-metal box for consistency with the methods of the original investigators.

ODC activity was determined using the methods of Seeley and Pegg (*29*) as modified for cell culture by the original investigators (*18*). Positive controls for the ODC assays included exposure of L929 cells to various factors known to stimulate ODC (*30*). Cells that had been serum-deprived for 24 h were treated for 4 h with fresh Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. Other positive controls included treatment with 100 ng/ml of fibroblast growth factor, 125 ng/ml of platelet-derived growth factor, or 2.5 μM insulin-like growth factor. Cells that were not serum-deprived were kept in normal Eagle's MEM to allow comparison between serum-deprived cells and cells under normal growth conditions.

For each flask, the ODC activities calculated from duplicate assays were averaged. These values from the three independent flasks in each exposure condition were used to calculate both the average cellular ODC activity and the standard deviation of that value for a given exposure condition. For each experiment, the average and the standard deviation for each of the exposure conditions were used to calculate the ODC activity ratio. This ratio is expressed as exposed/control for experimental treatments, and as chamber A/chamber B for sham/sham experiments. The ODC activity ratio is used to account for variations between experiments in control ODC activity, and this facilitated comparisons among the experiments (*18*).

RESULTS

Gene Expression

A series of studies demonstrated the variability in the *MYC* expression assays for both cells from the laboratory at Columbia University and those from ATCC (Fig. 2). A

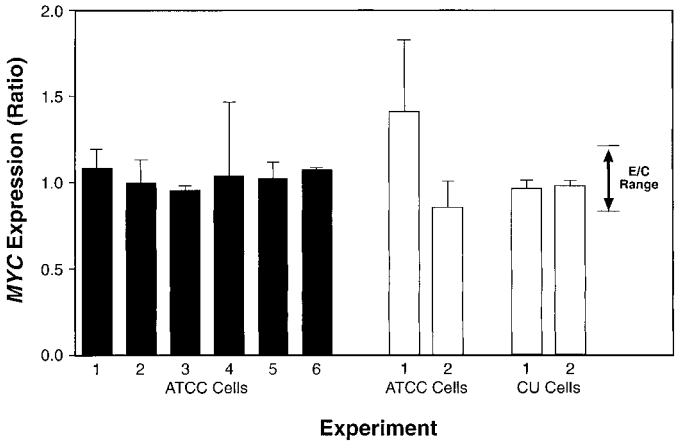

FIG. 2. Inherent variability of the *MYC* assay. RNA was subjected to Northern blot analysis after sham/sham incubation (S/S; no applied magnetic field) of cells. Each bar represents the average ratio of cellular *MYC* transcript levels between two chambers for an independent experiment. Data are shown for a series of six S/S experiments with cells from the ATCC (solid bars) and for four S/S experiments performed in the midst of a series of EMF exposure experiments (cells from ATCC and Columbia University, i.e. CU cells, open bars). Error bars represent ±1 SD. The range of ratios obtained from EMF exposure experiments is bracketed.

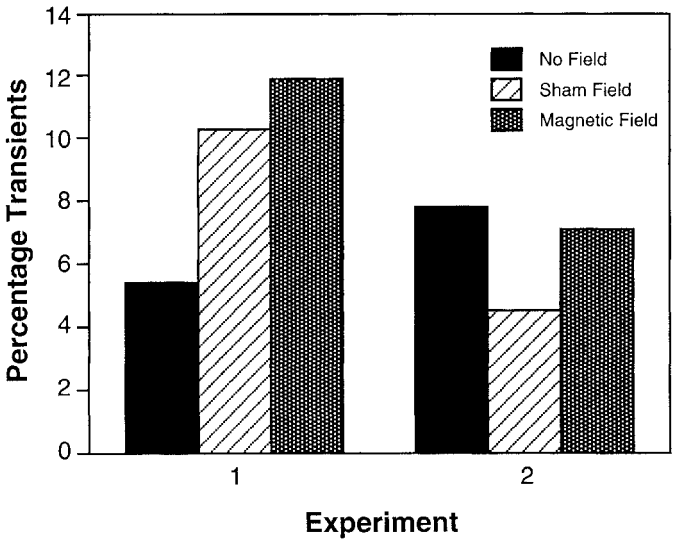

FIG. 3. The percentage of qualified Jurkat cells that exhibited an intracellular calcium transient during the 8 min of application of field conditions (no field, sham-field exposure, or magnetic-field exposure). A qualified cell had no transients during the pre-exposure period and responded to the positive control stimulus in the postexposure period, as noted in the text. Details for Experiments 1 and 2, each using a 40× objective, are also given in the text.

60 Hz magnetic-field effect was not found. Increased expression was occasionally seen in one of the two chambers, but this was not a consistent effect. Magnetic-field exposure did not affect *MYC* gene expression (see E/C range in Fig. 2) as has been reported (*23*). Positive controls showed a mean induction ratio of 83%, demonstrating that the cells responded to 5 μ*M* TPA with *MYC* induction, and that the experimental assay system had sufficiently low inherent variability to allow detection of that response (*23*).

Intracellular Calcium Concentration

Jurkat cells may display rapid changes in intracellular Ca^{2+} concentrations or ''spontaneous transients''. Therefore, it was critical to distinguish these changes in intracellular Ca^{2+} from those that may have been stimulated by magnetic-field exposures. Thus cells were selected that did not show rapid changes in intracellular Ca^{2+} concentrations in the 8 min prior to exposure. Further, the cells had to be capable of responding to a positive stimulus during a 6-min postexposure period. Using fluorescence imaging, data could be collected from a field of cells. Typically there were 25 to 50 cells in the field of view, and approximately 50% of these exhibited no rapid changes in Ca^{2+} during the baseline period and subsequently responded to the anti-CD3 antibody with rapid changes in intracellular Ca^{2+} (*21*).

Experiment 1 was a series of eight studies with 319 to 333 total cells analyzed per group. The lowest number of rapid changes in intracellular Ca^{2+} concentrations was seen in cells exposed to no fields (Fig. 3), with essentially similar numbers of changes in Ca^{2+} in the sham-field and magnetic-field groups. Experiment 2 was a series of seven studies with 324 to 363 total cells analyzed per group. The lowest number of rapid changes in intracellular Ca^{2+} concentrations was seen in cells exposed to sham fields (Fig. 3), with essentially similar numbers of changes in Ca^{2+} in the no-field and magnetic-field exposure groups. It was also observed that the number of cells with rapid changes in intracellular Ca^{2+} varied depending on the microscope objective used (Table 2). The metal objective would alter the shape and intensity of the magnetic field to some extent. In addition, the objective power alters the intensity of the UV illumination, and UV illumination has been reported to stimulate Ca^{2+} responses in Jurkat cells (*31, 32*).

TABLE 3
Colony Counts of JB6 Cells after Sham/Sham Exposure

Experiment	TPA (ng/ml)	Chamber A	Chamber B
1	0.00	40 ± 6	49 ± 7
1	0.01	82 ± 4	64 ± 4
1	0.10	134 ± 30	122 ± 5
1	1.00	369 ± 7	375 ± 9
2	0.00	19 ± 1	16 ± 1
2	0.01	63 ± 5	75 ± 7
2	0.10	210 ± 15	205 ± 14
2	1.00	533 ± 35	597 ± 22
3	0.00	13 ± 1	14 ± 4
3	0.01	20 ± [a]	18 ± 4
3	0.10	136 ± 8	153 ± 60
3	1.00	313 ± 17	356 ± 9

[a] Several samples lost.

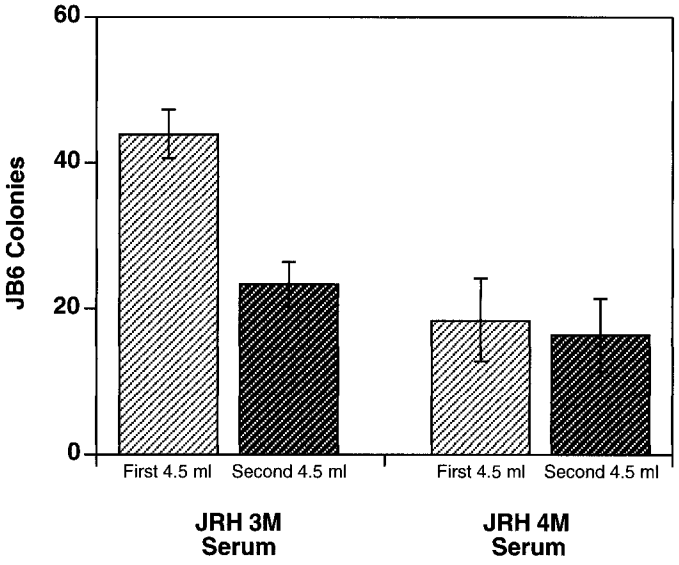

FIG. 4. Effect of serum lot (JRH 3M or JRH 4M) and plating order on anchorage-independent growth of JB6 cells treated with TPA (0.01 ng/ml). Values are number of colonies per dish (mean ± SD). The difference in the first and second 4.5 ml pipetted with the JRH 3M serum was statistically significant ($P < 0.05$).

Growth of JB6 Colonies on Soft Agar

The growth of JB6 colonies in soft agar showed a similar dose response to that after treatment with TPA when cells were grown in the other incubator used for cell culture or in the regional exposure system. The JB6 cells responded to as little as 0.01 ng TPA/ml of medium with an approximate doubling of colonies (Table 3). When 1.0 ng TPA was added, a 10- to 20-fold increase in the number of colonies occurred, suggesting that this assay is quite sensitive to a positive stimulus. The magnitude of the dose response was consistent across three experiments.

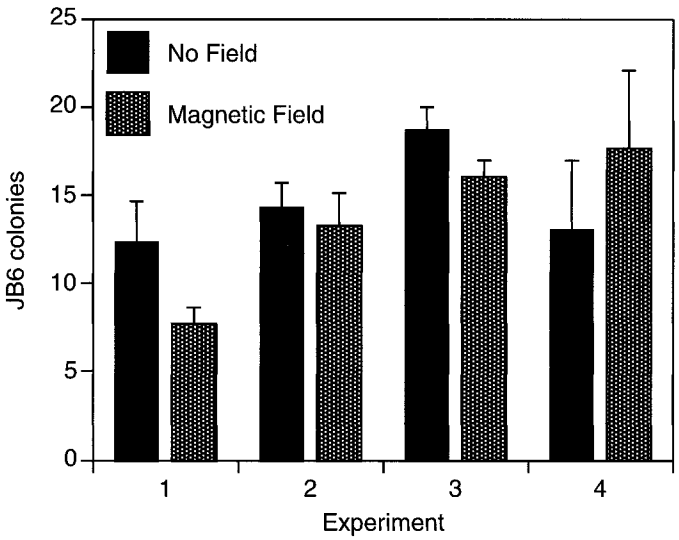

FIG. 5. Effect of a 60 Hz magnetic-field exposure (0.96 mT) on anchorage-independent growth of JB6 mouse epidermal cells on day 14. Two experiments used chamber A as the exposed side, while two experiments used chamber B as the exposed side, with the investigator blinded to the exposure condition until colony counts were completed. Data shown are mean number of colonies per plate ± standard deviation, $n = 3$ or 4 plates/treatment.

TABLE 4
Ornithine Decarboxylase Activity with 60 Hz 10-μT Magnetic Fields

Exposure time	ODC ratio exposed/control
1 h	1.05 ± 0.06
2 h	1.03 ± 0.18
4 h	1.00 ± 0.07
6 h	1.05 ± 0.05

The serum used in the original studies caused a slight clumping of the cells; another lot of serum from the same source did not. Since the original investigator used a 10-ml pipette to plate 1.5-ml aliquots, an experiment was conducted to determine whether the order of plating played a significant role in colony-forming efficiency. Nearly twice the number of colonies were found from the first 4.5 ml compared to the second 4.5 ml using the 3M but not the 4M serum (Fig. 4). When the plating order was randomized for the magnetic-field and control chambers, no effect of the magnetic field on the number of JB6 colonies was found (Fig. 5). The results of this series of experiments have been reported in detail (*20*).

ODC Activity

For a series of 10 experiments conducted over several weeks, the variability of the baseline ODC activity was 36% and 42% for the cells from ATCC and those from the laboratory at Catholic University of America, respectively (*22*). This variability compares favorably with that reported by the original investigator for experiments conducted under comparable conditions (*33*). Serum-deprived cells showed a 19-fold increase in ODC activity when fresh medium containing serum was added, and a more than 4-fold increase with either fibroblast growth factor, platelet-derived growth factor, or insulin-like growth factor (*22*). Positive controls for a doubling of ODC activity were included

TABLE 5
Effect of Magnetic Fields on Intracellular Calcium in the First 4 min[a]

Experiment	Exposure	Total responding cells	Responding during exposure
1	No field	167	9 (5.4%)
1	Sham field	174	18 (10.3%)
1	Magnetic field	177	21 (11.9%)
2	No field	232	18 (7.8%)
2	Sham field	200	9 (4.5%)
2	Magnetic field	212	15 (7%)

[a] Microscope objective was 40× for all experiments.

TABLE 6
Summary of Study Conditions and Results

Laboratory	Effect	Result (reference)	Comments
Columbia University, New York	*MYC* expression in HL60 cells	Increase (*3, 4*)	No sham exposures
FDA, Rockville, MD	*MYC* expression in HL60 cells	No effect (*23*)	Positive controls, sham exposures, temperature ± 0.1°C
University of Umeå, Sweden	Intracellular Ca^{2+} in human Jurkat cells	85–92% had increased Ca^{2+} (*6–8*)	Small number of cells studied after preselection, positive controls, no sham exposures
NIOSH, Cincinnati, OH	Intracellular Ca^{2+} in human Jurkat cells	Response rate same with or without MF (*21*)	Greater number of cells observed (no preselection), positive controls, sham exposures
NCTR, Jefferson, AR	Transformation and colony cell growth in JB6 cells	Increase (*12, 13*)	Growth differences similar at each MF intensity
NIOSH, Cincinnati, OH	Transformation and colony cell growth in JB6 cells	No effect (*20*)	Identified growth variation with serum lot and plating order, positive controls
Catholic University of America, (CUA) Washington, DC	Ornithine decarboxylase activity in L929 cells	Increase (*17, 18*)	No sham exposures, all exposures in one chamber; controls in different chamber
FDA, Rockville, MD	Ornithine decarboxylase activity in L929 cells	No effect (*22*)	Positive controls, sham exposures, temperature ± 0.1°C

in most of the experiments on the possible effects of EMF exposure (*22*).

In a series of experiments performed at the laboratory in which the effect was first found, sham/sham exposures comparing two chambers (A and B) to a third control chamber, the ODC activity ratio varied from less than 1 to 1.56 (Fig. 6). Similarly, using exposure coils from that laboratory in separate incubators at the FDA, the ODC ratio varied from 0.58 to 1.09. In a series of four experiments using the original laboratory coils in their mu-metal enclosures to conduct sham/sham experiments, cells in one chamber had consistently higher ODC activity (ODC ratios of 1.18 to 1.4, mean 1.29). Using the original laboratory coils in the FDA exposure system without their mu-metal boxes, no consistent exposure effect could be demonstrated. The two mu-metal enclosures from the original laboratory were not identical; one was lined with felt. Whether this caused a temperature differential between the two chambers was not determined. For sham/sham experiments performed with the FDA exposure system or the regional exposure system, the ODC activity ratio varied from 0.82 to 1.14 (Fig. 6). Experiments comparing magnetic-field exposure with sham exposures demonstrated no effect on ODC activity (Table 4, ref. *22*).

DISCUSSION

Many of the difficulties in replication of studies and many of the required control procedures were similar among the four effects studied. Controls were included to ensure that the experimental model exhibited both positive and negative responses.

Need for Adequate Exposure System

A multidisciplinary approach was necessary to create both the cellular conditions and the magnetic-field exposure systems to conduct experiments with limited bias. The design of the regional exposure system eliminated many experimental variables. Magnetic-field exposures may create heat, vibration and electric currents in the medium, altering cellular processes (*34*). Under some conditions, a 0.3-mT field may increase the temperature of the cell medium by 0.6°C (*35*). In the series of studies at the regional exposure facilities, it was found that it was critical that the temperature be kept within 0.1°C (*25*).

It was critical to have exposure systems that allow for randomization of the exposure conditions. The ability to run exposures in either of two chambers or no exposure (sham/sham) in both chambers in a blinded fashion was a great asset to conducting these studies, because it facilitated efforts to establish whether consistent differences between chambers existed and what degree of variability could be expected from an assay in the absence of an EMF exposure. The selection of either exposure chamber or the sham/sham exposures was coded to eliminate observer bias. In the case of Ca^{2+} studies that did not use chambers, a switching apparatus randomized the exposure conditions; after the assays were completed, the code was broken. In spite of ex-

TABLE 6
Extended

Applied fields	Assay methods	Cells	Exposure system
60 Hz; 6 μT; 20 min	RNA isolation, modification of ref. (*26*), Northern blots	HL60 cells	Helmholtz coils, mu-metal enclosures
60 Hz; 6 μT; 20 min	RNA isolation, modification of ref. (*26*), Northern blots	HL60 from ATCC and original investigator	Helmholtz coils, mu-metal enclosures (*23*)
50 Hz; 100 or 150 μT; 8 min	Ca^{2+} probe fura-2 with fluorescence microscopy, photomultiplier tube (single cell)	Jurkat (clone E6-1) from human T-cell leukemia	Helmholtz coils with microscope system for real-time viewing
50 Hz; 150 μT; 8 min	Ca^{2+} probe fura-2 with fluorescence microscopy, fluorescence imaging from field of cells simultaneously	Jurkat (clone E6-1) from original investigator	Custom single-coil with microscope system for real-time viewing
60 Hz; 1, 100, 1000 and 1100 μT; 14 days	Colony growth in soft agar	JB6 cells (clone 41)	Custom Helmholtz coil system in incubator
60 Hz; 100 or 960 μT; 10–14 days	Colony growth in soft agar	JB6 cells (clone 41) from original investigator	Regional Exposure System (Columbia Magnetics), with modifications (*20*)
55, 60, 65 Hz; 1, 10, 100 μT; 1–8 h	Seeley and Pegg (*29*) modified for cell culture (*18*)	L929 cells	Helmholtz coils, mu-metal enclosures
60 Hz; 5, 10, 20 μT; 1, 2, 4, 6 h	Seeley and Pegg (*29*) modified for cell culture (*18*)	L929 cells from ATCC and original investigator	Helmholtz coils, mu-metal enclosures; CUA system; Regional Exposure System (*22*)

tensive efforts, the exposures may have been only a close approximation of the exposures in the original laboratories.

Baseline Testing and Variability

All data from the regional facility studies were archived and kept. Any exclusion of data from analyses was done using carefully defined criteria established prior to the conduct of the studies. An EMF Regional Facility steering committee with members from various facilities provided oversight.

It was necessary to run an extensive series of sham/sham experiments to establish the inherent variability for each experimental assay system. Establishing the level of variability helps determine what level of difference between exposed and control cells may be meaningful. Further, all four experimental assay systems responded to positive control treatment, and for most experiments positive controls were routinely included. All studies were analyzed for a chamber effect; a consistent chamber effect across experiments from the regional facilities was not found for any of the end points measured. Most studies reporting subtle effects of EMF exposure on cellular processes lack sufficient data to demonstrate the baseline variability of the assay system used. Further, many do not demonstrate that the EMF effects are sufficiently robust to justify the conclusions that such effects have been demonstrated.

For the gene expression studies described herein, the average *MYC* expression ratio for sham/sham experiments ranged from a 40% increase to a 15% decrease, though six of the ten experiments gave ratios within 5% of unity (Fig. 2). In the earlier gene expression studies showing *MYC* expression in HL60 cells (*3–5*, *36*), sham exposures were not included in the experimental protocols.

Cells responding to the positive control (anti-CD3 antibody) and exhibiting stable Ca^{2+} levels during the baseline period were considered as potential responders to magnetic fields. The proportion of cells with intracellular Ca^{2+} changes averaged between 4 and 12% during the period after the baseline for no-field, sham-field and magnetic-field exposure (Table 5). Sham exposures were not performed in the original studies that reported increased Ca^{2+} transients in human leukemia (Jurkat) cells (*6–8*). One possibility for the lack of confirmation of the effect at regional facilities was that the original investigators observed cells for only 30 to 180 s at the start of the magnetic-field exposure while the cells exposed in the regional facility were evaluated over a longer period. To examine the possibility of a difference in the initial response of cells to magnetic fields, Ca^{2+} transients were separated into those that occurred in the first 4 min (Table 5). Again, no difference in Ca^{2+} transients with magnetic-field exposure was found.

In the growth of JB6 cells, the background number of colonies (no TPA) varied from 13 to 49 colonies per milliliter after 7 days of growth (Table 3). This series of studies demonstrated that significant differences in colony growth occurred between experiments and that the difference be-

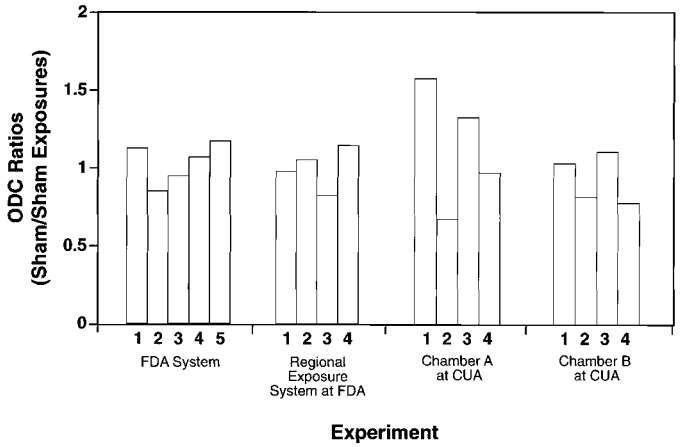

FIG. 6. Inherent variability of the ODC assay. ODC activity was measured after sham/sham incubation (S/S = no applied magnetic field) of cells in different exposure systems. Each bar represents the average ratio of cellular ODC activity between two chambers for an independent experiment. S/S experiments were performed in the midst of a series of EMF exposure experiments. Exposure system identities and experiment numbers are indicated. CUA, Catholic University of America.

tween the sham-exposed chambers was nearly 20%. The series of studies showing increased colony proliferation in JB6 cells (*12–15*) often did include sham controls.

A number of experimental variables were identified in the study of JB6 cells. For example, one serum lot caused clumping of the cells. Plating sequence was an important variable when 1.5-ml aliquots of soft agar were plated from a 10-ml pipette in five or six plates. When the order of plating was analyzed, a significant increase in the number of colonies from the first few plates plated was found (Fig. 4). No difference between the exposure and control groups occurred when the cells in each experimental group were plated similarly. Using serum that did not cause clumping, the original investigator did not find magnetic-field exposure effects. The clumping of cells and order of plating may explain the differences between the reported results and those at the regional facility.

Evaluation of the ODC assay showed moderate variability from a 56% increase to a 34% decrease when sham exposures were compared to control chambers. To address the possibility that different subcultures of L929 cells might differ with respect to ODC response to magnetic-field exposure, cells obtained from the ATCC were compared to those obtained from the laboratory where the ODC effect had been reported. The results of experiments using these two subcultures were not significantly different. Similar findings were obtained in experiments comparing the source of cell culture medium.

In a series of experiments at Catholic University of America, there was some evidence to suggest that there was a subtle difference between the exposure chambers (*22*). Mu-metal boxes used to block ambient fields may create temperature differentials unless the exposures are very carefully controlled. A series of experiments was performed without the mu-metal enclosures used in the laboratory at Catholic University to address the possibility of temperature differences between exposed and control samples due to the use of small mu-metal boxes. A magnetic-field effect on ODC activity was not found. For studies involving increased ODC activity (*17, 18*), sham exposures were not done, and, perhaps more significantly, it appears that all exposures were done in one chamber while controls were done in another chamber. Temperature variability between chambers might contribute to the reported differences between the two laboratories.

Synchronization of Methods among Laboratories

All studies included visits to the laboratories of the investigators reporting the original results. In addition, in three of the four cases, the original investigators also visited the regional facility at the initiation of the study. The visits to the original laboratories were possibly viewed at times as intrusive to the host investigators. The need to establish as precisely as possible all aspects of the experimental protocol meant tracking down serum lots and sources of plastic ware, establishing generation and cell density at the time of plating, and determining the methods of analysis of end points. Since research is not static, protocols had subtle to substantial changes from the published techniques. In some cases, original materials and supplies were not available. A protocol acceptable to the EMF regional facility staff and to the original investigator was established. As the baseline studies were being performed at the regional facility, the interaction and questions continued. When the first replication results were negative, an attempt was made to further clarify possible differences.

Investigators at the regional EMF facilities were unable to replicate four widely cited magnetic-field exposure effects (Table 6). However, the effort brought greater focus and clarity to EMF studies, and it offers lessons for those trying to reproduce subtle biological effects of societal concern.

First was the recognition of the essential need for the use of sham/sham controls. In many of the regional facility studies reported herein, the largest differences between chambers were seen during the sham/sham exposures. The ability to completely blind the studies, to conduct exposures in either exposure chamber, and to conduct sham/sham exposures was critical.

Another lesson was that data should be discarded only by following rigorous criteria that have been determined prior to the experiment and are included in the protocol. All data must be saved so additional analyses can be performed. Selective use of data can easily create misleading results. Positive controls are useful in setting the criteria for data inclusion. This is especially important when exposure-related effects are not found, as was the case for the magnetic-field exposures described herein.

With these complex cell systems, an exact replication of all of the experimental conditions is impossible. A team

with outside reviewers was helpful in defining the critical parameters for confirming the finding. We feel that subtle effects that cannot be confirmed under carefully controlled conditions may have little application for determining potential adverse human health effects, especially for results that fall within the variability of the assay.

The investigators whose effects were being studied showed constraint and patience with the regional facilities staff and were open with their data and techniques. In future studies, where resolution of an effect is imperative, support for both the original investigators and scientists attempting to confirm the finding would be helpful.

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REFERENCES

1. N. Wertheimer and E. Leeper, Electrical wiring configurations and childhood cancer. *Am. J. Epidemiol.* **109**, 273–284 (1979).
2. D. A. Savitz, H. Wachtel, F. A. Barnes, E. M. John and J. G. Tvrdik, Case–control study of childhood cancer and exposure to 60-Hz magnetic fields. *Am. J. Epidemiol.* **128**, 21–38 (1988).
3. H. Lin, R. Goodman and A. Shirley-Henderson, Specific region of the c-myc promoter is responsive to electric and magnetic fields. *J. Cell. Biochem.* **54**, 281–288 (1994).
4. R. Goodman, Y. Chizmadzhev and A. Shirley-Henderson, Electromagnetic fields and cells. *J. Cell. Biochem.* **51**, 436–441 (1993).
5. J. L. Phillips, Effects of electromagnetic field exposure on gene transcription. *J. Cell. Biochem.* **51**, 381–386 (1993).
6. E. Lindstrom, P. Lindstrom, A. Berglund, K. H. Mild and E. Lundgren, Intracellular calcium oscillations induced in a T cell line by a weak 50 Hz magnetic field. *J. Cell. Physiol.* **156**, 395–398 (1993).
7. E. Lindstrom, P. Lindstrom, A. Berglund, E. Lundgren and K. H. Mild, Intracellular calcium oscillations induced in a T cell line after exposure to ELF magnetic fields with variable frequencies and flux densities. *Bioelectromagnetics* **16**, 41–47 (1995).
8. E. Lindstrom, A. Berglund, K. H. Mild, P. Lindstrom and E. Lundgren, CD45 phosphatase in Jurkat cells is necessary for response to applied ELF magnetic fields. *FEBS Lett.* **370**, 118–122 (1995).
9. D. B. Lyle, X. Wang, R. D. Ayotte, A. R. Sheppart and W. R. Adey, Calcium uptake by leukemic and normal T-lymphocytes exposed to low frequency magnetic fields. *Bioelectromagnetics* **12**, 145–156 (1991).
10. J. Walleczek and R. P. Liburdy, Nonthermal 60 Hz sinusoidal magnetic-field exposure enhances $^{45}Ca^{2+}$ uptake in rat thymocytes: Dependence on mitogen activation. *FEBS Lett.* **271**, 157–160 (1990).
11. J. Walleczek, Electromagnetic field effects on cells on the immune system: the role of calcium signaling. *FASEB J.* **6**, 3177–3185 (1992).
12. R. W. West, D. B. Hinson, M. L. Lyle and M. L. Swicord, Enhancement of anchorage-independent growth in JB6 cells exposed to 60 Hz magnetic fields. *Bioelectrochem. Bioenerget.* **34**, 39–43 (1994).
13. R. W. West, D. B. Hinson and M. L. Swicord, Anchorage-independent growth with JB6 cells exposed to 60 Hz magnetic fields at several flux densities. *Bioelectrochem. Bioenerget.* **39**, 175–179 (1996).
14. J. Schimmelpfeng and H. Dertinger, The action of 50 Hz magnetic and electric fields upon cell proliferation and cyclic AMP content of cultured mammalian cells. *Bioelectrochem. Bioenerget.* **16**, 143–150 (1993).
15. S. M. Ross, Combined DC and ELF magnetic fields can alter cell proliferation. *Bioelectromagnetics* **11**, 27–36 (1990).
16. C. V. Byus, S. E. Pieper and W. R. Adey, The effects of low-energy 60-Hz environmental electromagnetic fields upon the growth-related enzyme ornithine decarboxylase. *Carcinogenesis* **8**, 1385–1389 (1987).
17. T. A. Litovitz, D. Krause and J. M. Mullins, Effect of coherence time of the applied magnetic field on ornithine decarboxylase activity. *Biochem. Biophys. Res. Commun.* **178**, 862–865 (1991).
18. T. A. Litovitz, D. Krause, C. J. Montrose and J. M. Mullins, Temporally incoherent magnetic fields mitigate the response of biological systems to temporally coherent magnetic fields. *Bioelectromagnetics* **15**, 399–409 (1995).
19. National Research Council, *Possible Health Effects of Exposure to Residential Electric and Magnetic Fields.* National Academy Press, Washington, DC, 1997.
20. J. E. Snawder, R. M. Edwards, D. L. Conover and W. G. Lotz, Effect of magnetic field exposure on anchorage-independent growth of a promoter-sensitive mouse epidermal cell line (JB6). *Environ. Health Perspect.* **107**, 195–198 (1999).
21. H. E. Wey, D. L. Conover, P. Mathias, M. A. Toraason and W. G. Lotz, Studies of magnetic field effects on intracellular calcium in Jurkat cells. *Environ. Health Perspect.*, in press.
22. L. W. Cress, R. D. Owen and A. B. Desta, Ornithine decarboxylase activity in L929 cells following exposure to 60 Hertz magnetic fields. *Carcinogenesis* **20**, 1025–1030 (1999).
23. R. D. Owen, *MYC* mRNA abundance is unchanged in subcultures of HL60 cells exposed to power-line frequency magnetic fields. *Radiat. Res.* **150**, 23–30 (1998).
24. B. W. Wilson, K. Caputa, M. A. Stuchly, J. D. Saffer, K. Davis, C. E. Washam, L. G. Washam, G. R. Washam and M. A. Wilson, Design and fabrication of well confined, uniform magnetic fields exposure system. *Bioelectromagnetics* **15**, 563–577 (1994).
25. C. C. Davis, I. Barber and M. L. Swicord, Food and Drug Administration low-level extremely-low-frequency magnetic field exposure facility. *Bioelectromagnetics* **20**, 203–215 (1999).
26. S. Case and B. Daneholt, A simple method of isolation of undegraded giant RNA molecules. *Anal. Biochem.* **74**, 198–206 (1976).
27. R. Goodman, J. Bumann, L. X. Wei and A. Shirley-Henderson, Exposure of human cells to electromagnetic fields: Effect of time and field strength on transcript levels. *Electro. Magnetobiol.* **11**, 19–28 (1992).
28. H. Lin and R. Goodman, Electric and magnetic noise blocks the 60 Hz magnetic field enhancement of steady state *c-myc* transcript levels in human leukemia cells. *Bioelectrochem. Bioenerget.* **36**, 33–37 (1995).
29. J. E. Seeley and A. E. Pegg, Ornithine decarboxylase. *Methods Enzymol.* **94**, 158–161 (1983).
30. J. G. Hovis, D. J. Stumpo, D. L. Halsey and P. J. Blackshear, Effects of mitogens on ornithine decarboxylase activity and messenger RNA levels in normal and protein kinase C-deficient NIH-3T3 fibroblasts. *J. Biol. Chem.* **22**, 10380–10386 (1986).
31. G. L. Schieven, J. M. Kirihara, L. K. Gilliland, F. M. Uckun and J. A. Ledbetter, Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signaling in lymphocytes. *Mol. Biol. Cell* **4**, 523–530 (1993).
32. I. Ihrig, C. Heese and R. Glaser, Alterations of intracellular calcium concentration in mice neuroblastoma cells by electrical field and UVA. *Bioelectromagnetics* **18**, 595–597 (1997).
33. L. M. Penafiel, T. A. Litovitz, D. Krause, A. B. Desta and J. M. Mullins, Role of modulation on the effect of microwaves on ornithine decarboxylase activity in L929 cells. *Bioelectromagnetics* **18**, 132–141 (1997).
34. M. Misakian, A. R. Sheppard, D. Krause, M. E. Frazier and D. L. Miller, Biological, physical, and electrical parameters for in vitro studies with ELF magnetic and electric fields: A primer. *Bioelectromagnetics* **2** (Suppl.), 1–73 (1993).
35. Y. L. Zhao, P. G. Johnson, G. P. Jahreis and S. W. Hui, Increasing DNA synthesis in INIT/10T1/2 cells after exposure to a 60 Hz magnetic field: A magnetic-field or a thermal effect? *Radiat. Res.* **151**, 201–208 (1999).
36. R. Goodman, L. X. Wei, J. C. Xu and A. Henderson, Exposure of human cells to low-frequency electromagnetic fields results in quantitative changes in transcripts. *Biochim. Biophys. Acta* **1009**, 216–220 (1989).