

Rodent Cell Transformation and Immediate Early Gene Expression Following 60-Hz Magnetic Field Exposure

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Some epidemiological studies suggest that exposure to power frequency magnetic fields (MFs) may be associated with an elevated risk of human cancer, but the experimental database remains limited and controversial. We investigated the hypothesis that 60-Hz MF action at the cellular level produces changes in gene expression that can result in neoplastic transformation. Twenty-four hour 200 μ T continuous MF exposure produced negative results in two standard transformation systems (Syrian hamster embryo cells and C3H/10T_{1/2} murine fibroblasts) with or without postexposure to a chemical promoter. This prompted a reexamination of previously reported MF-induced changes in gene expression in human HL60 cells. Extensive testing using both coded and uncoded analyses was negative for an MF effect. Using the same exposure conditions as in the transformation studies, no MF-induced changes in ornithine decarboxylase expression were observed in C3H/10T_{1/2} cells, casting doubt on a promotional role of MF for the tested cells and experimental conditions. *Key words:* β -actin, C3H/10T_{1/2} cells, *c-fos*, *c-myc*, heat, 60-Hz magnetic field, neoplastic transformation, ODC, ribosomal genes, SHE cells, TPA, X rays. *Environ Health Perspect* 104:1188–1198 (1996)

Adverse health effects, including cancer, have been positively associated with human exposures to power frequency (50 or 60 Hz) magnetic fields (MF) in numerous epidemiological studies. Childhood leukemia, brain cancer, breast cancer, and certain neurological and reproductive effects among residentially exposed children and among occupationally exposed adults were reported in some studies; other studies were negative for these effects (1,2). The possible scientific foundation for these effects is uncertain. Results of animal and cellular studies that investigated MFs for possible cancer-related activity were either contradictory or not replicated, as were results concerning effects on cell growth and differentiation and modulation of gene transcription and/or translation (3–14).

In the experiments reported here, we tested the hypothesis that changes in gene expression occurring under MF exposure may result in neoplastic transformation. *In vitro* studies of neoplastic transformation are very relevant to the purposes of carcinogenic risk assessment (15–17). They permit investigation of the mechanisms of cancer initiation, promotion, and progression without interference from other *in vivo* factors at the tissue and organ-system levels. Available *in vitro* assays are more practical and precise than are studies on exposed animals. Most importantly, *in vitro* neoplastic transformation correlates well with cancer induction in humans in the sense that few, if any, tested agents have yielded false positive results (18).

For the purposes of the current study, we

used two well-characterized embryonic cell models, based on murine C3H/10T_{1/2} cells or Syrian hamster embryo (SHE) cells (19–22), to examine the transforming potential of 60-Hz MFs. The C3H/10T_{1/2} system is suitable for initiation–promotion protocols (16,17,23–28); we previously described two-stage protocol for investigating 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promotion of 2.45-GHz microwave- and X ray-induced transformation (24–27). In addition to our long established experience with the C3H/10T_{1/2} assay, this assay has been chosen in particular because it is widely used and exhibits a low spontaneous background (19) and no sensitivity to heat (29–31); in the case of other endpoints, such as enzymatic or gene activity or proliferation, thermal effects might confuse a possible MF effect. The SHE system is suitable for studying diverse agents (chemical, viral, hormonal, and physical irritants), including weak and epigenetic carcinogens (15–22). It should be noted that there are no analogous assays based on human cell lines. Our transformation studies were performed with long continuous exposures at 200 μ T (equal to 2 G), a field level in the typical upper range of power line and household exposure (11). To our knowledge, only one other study of neoplastic transformation *in vitro* has been reported, and this mainly concerns the effects of intermittent coadministration of TPA and MF on preinitiated C3H/10T_{1/2} cells (8).

Because our working hypothesis is based on changes in gene expression, we

needed to address contradictory reports concerning the ability of MFs to increase levels of a number of gene transcripts (32–43). Our second series of studies was designed to confirm with solid data whether MFs induce significant changes in transcription of seven genes. Studies of *c-myc*, *c-fos*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, and two ribosomal genes were performed using HL60 cells (44,45), short exposure times, and several flux densities matching the exposure conditions of Goodman et al. (34). Three replication experiments have recently been reported (12–14), but they were more limited in terms of both the number of genes and MF conditions studied. Our ornithine decarboxylase (ODC) transcription studies using C3H/10T_{1/2} cells were performed at 200 μ T to elaborate the C3H/10T_{1/2} transformation results.

The *c-myc*, *c-fos*, GAPDH, β -actin, ODC, and ribosomal genes play an important role in normal cell physiology, including metabolism, protein synthesis, growth, apoptosis, and differentiation. Aberrant activity of *c-myc*, *c-fos*, and ODC is associated with neoplastic transformation. For example, cell lines stably expressing transfected *c-myc*, *c-fos*, and ODC readily convert to a tumorigenic phenotype (46–50). Another common characteristic of *c-myc*, *c-fos*, and ODC is their ability to be induced by potent tumor promoters such as TPA

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(51–56). Furthermore, both *c-fos* and *c-myc* have been implicated in the regulation of *ODC* transcription induced by TPA (56–59). In the context of cancer promotion by TPA, the induction of these genes is seen as an indicator of a global stress response that leads to genetic instability (60–62). Based specifically on Goodman's data on gene transcription (32–35), it has recently been proposed that the deleterious effects of MFs are also connected with similar TPA-like indirect genotoxic effects and epigenetic changes (63). These implications add to the need to confirm the original findings on gene expression.

Materials and Methods

60-Hz magnetic field exposure system. Cells were exposed in tissue culture flasks placed in the central planes of long solenoids. The growing cells formed a thin layer (HL60 cells in suspension) or a monolayer (attached SHE or C3H/10T1/2 cells) parallel to the field lines. The exposure system, as described previously (28), was recently modified and characterized as required by the NIEHS Electric and Magnetic Fields Research and Public Information Dissemination (EMF RAPID) program (64). The solenoids (38.7 cm long, 12 cm in diameter, 199 turns, 1.59 mH, 1.76 Ω) were wound on thin-walled plexiglas cylinders; the internal diameter was sufficient to accommodate 75-cm² tissue culture flasks. A sham-control solenoid was bifilar-wound and energized at the same level as the exposure solenoids. Consequently, control cells experienced zero magnetic field, but were subject to the same level of possible disturbances, including local acoustic noise and convective heating as field-exposed cells. A system schematic is shown in Figure 1. Power to the coil was line derived, with added surge protection. To eliminate the geomagnetic and other background DC/AC fields inside the solenoids, they were enclosed in individual magnetic shield cylinders with a diameter of 22.9 cm, fabricated from 0.13-cm thick Amumetal (Amunel Manufacturing Corp., Philadelphia, PA). These cylinders were constructed with two removable friction-fit end caps. Both the cylinders and end caps were fabricated with 4-mm holes for water circulation, with the holes covering 10% of the surface area of the bottom halves of their circumference.

The control and up to two exposure solenoid assemblies were placed parallel to each other in a circulating temperature-controlled water bath (1.2 m long, 0.7 m wide, 11 cm deep). The total water volume of approximately 100 liters was circulated through an external reservoir with heaters and pumps, with temperature control referenced to thermistors immersed in the exposure bath. This

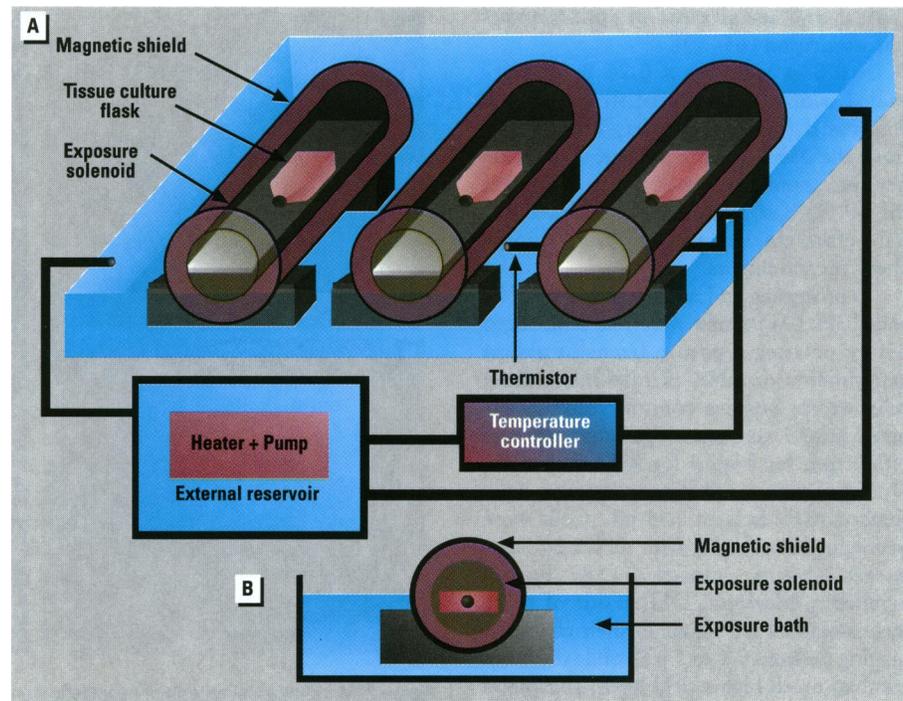


Figure 1. 60-Hz magnetic field exposure system (A), with a cross-section of one solenoid (B). Elements include solenoid coils, μ -metal shields, and water bath system for temperature control.

arrangement was used to minimize extraneous fields in the exposure bath, notwithstanding the effective shielding already provided for the solenoids. The exposure bath was insulated with styrofoam to reduce heat loss, and the temperature of the bath was maintained at $37.0 \pm 0.1^\circ\text{C}$. This temperature was realized over the central region of the bath, including the location of the solenoids. Although several hours were required to equilibrate this large thermal mass, the target temperature was then very stable over long-duration exposures. Exposure flasks were placed horizontally on plexiglas platforms in the solenoid so that they were half submerged in water. Flask contact with circulating liquid ensured good heat transfer and clamped the cell temperature at the desired level. According to direct measurement of culture medium temperature within flasks, temperature equilibrium was reached within 3 min for flasks with 15 ml of medium or within 20 min for medium-filled flasks.

Magnetic field measurements. Measurements of the fields within the exposure coils were made with a three-axis detector (Bartington model MAG-03MC; Bartington Instruments, Oxford, U.K.) and agreed within 5% to calculated solenoid fields values as well as with measurements by National Institute of Science and Technology (NIST) personnel and equipment during an EMF RAPID-sponsored site visit. NIST measurements also indicated that the field in the energized sham exposure coil did

not exceed $0.02 \mu\text{T}$. Field uniformity was $\pm 2\%$ along the 18-cm-long central plane inside exposure coils where exposure flasks were positioned. The voltage across each exposure coil was monitored continuously during experimental runs to document temporal stability. Mean, minimum, and maximum levels were logged and indicated stability to $\pm 3\%$ over 24 hr. Occasional transient power losses were noted in a few runs. No distortion of the 60-Hz magnetic field wave form was evident in oscilloscope displays; measurements of the power spectrum (Hewlett-Packard model 3562A; Hewlett-Packard, Palo Alto, CA) of exposure and background fields revealed a third-harmonic component 20 to 40 dB down from the 60-Hz fundamental, according to spot measurements on three separate occasions.

The presence of the expected magnetic fields in each coil containing treatment flasks was verified before each experiment using a small field sensor (Bell model 4060; F.W. Bell, Orlando, FL). Exposure flasks were manually introduced into energized solenoids and removed prior to turning them off in order to minimize transient fields.

Concerning possible accessory field exposure, the maximum 60-Hz background field in our exposure facility obtained by activating all the power devices was $0.1 \mu\text{T}$, but typical levels were less than $0.01 \mu\text{T}$. In previous 2.45-GHz microwave exposures of C3H/10T1/2 cells at the same location (26–28) extensive controls gave

typical and usual rates of spontaneous transformation (65–69).

A 0.5 m × 0.5 m × 0.25 m double-walled container fabricated from 0.13-cm Amumetal was used to carry cells to and from the MF exposure facility. This journey was approximately 200 m, over which the 60-Hz background did not exceed 0.2 μ T before shielding, according to spot-check measurements.

In processing and maintaining cells, SHE and C3H/10T1/2 cells were not centrifuged before or after exposure (transformation experiments or RNA extractions). HL60 cells (except positive control treatments as noted below) were centrifuged after exposure (1000 rpm for 8 min) for RNA extraction. During this time, cells from all groups experienced MF levels of 1–3 μ T. Cells were maintained in a facility with six similar incubators (Forma model 3158; Forma Scientific, Marietta, OH). Four of these incubators had MF levels in their chambers ranging between 0.1 to 1 μ T, while the other two had much higher field levels, apparently emanating from the fan motor; motor replacement has solved this problem.

Positive control treatments. We defined positive controls as agents other than MF previously known to induce neoplastic transformation or to modify the abundance of a specific mRNA. Sham-exposed cells were subjected to appropriate positive control treatments immediately following MF exposure.

We used 1.5 Gy of X rays to elicit a positive transformation response in SHE or C3H/10T1/2 cells (16,20,24–26,64–68) and as a negative control for *c-fos* expression in HL60 cells (55). Hyperthermia at 45.5°C for 9 min (53) and TPA at 0.3 μ g/ml for 30 min or 50 ng/ml for 3 hr (51–55) were used as treatments modifying the expression of *c-myc* and *c-fos* in HL60 cells and the expression of ODC in C3H/10T1/2 cells. TPA was obtained from Chemsyn Science Laboratories (Lenexa, KS). The TPA solvent acetone (purity >99.9%) was obtained from Sigma Chemical Company (St. Louis, MO). For heat treatments, glass test tubes, each containing 15 ml of HL60 cell suspension at 5×10^5 cells/ml, were submerged in a 45.5 \pm 0.1°C temperature-controlled water bath for 9 min (70). TPA treatment and X irradiation of C3H/10T1/2 cells were provided as previously described (24–26).

C3H/10T1/2 transformation assay. C3H/10T1/2 cells established from clone 8 of this lineage (15) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in basal medium, Eagle (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (HyClone, Provo, UT) (24–26,67,69) without antibiotics, in conformity with published guidelines for this

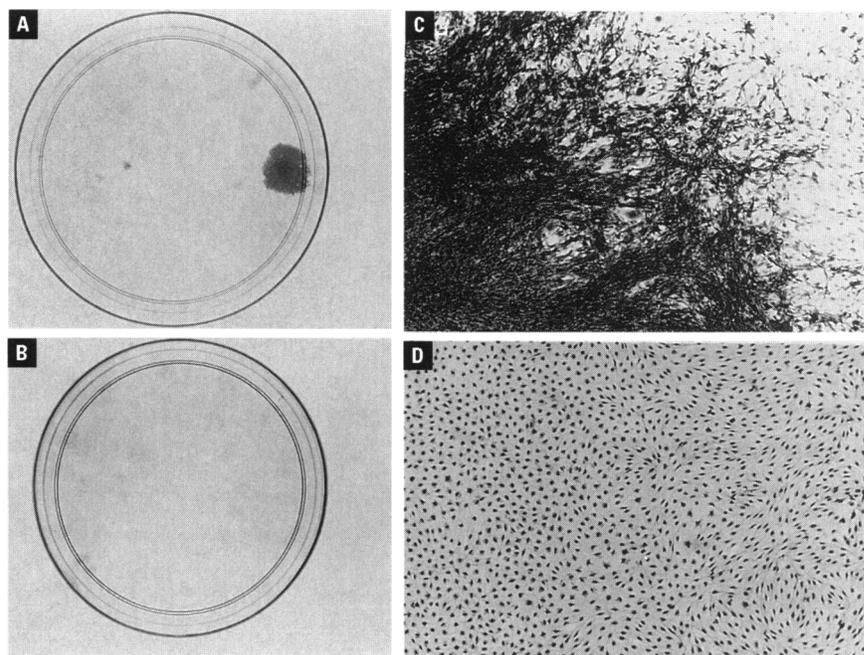


Figure 2. Morphological criteria of neoplastic transformation in C3H/10T1/2 cells. Gross morphology of (A) positive and (B) negative cultures; (C) the edge of focus of neoplastically transformed cells; (D) the area outside the transformed focus on the same positive dish.

assay (65); our quality control of experiments with C3H/10T1/2 cells is also described in these reports. A single lot of serum [selected according to the criteria described previously (65,69)] was used in all the experiments reported here. Actively growing cells in passage 10 were used for experimentation.

Each experiment included one 200- μ T MF group and two control groups. Immediately following a 24-hr exposure to MF, cells were transported in a μ -metal container to our X-ray exposure facility and one sham group was exposed to 1.5 Gy of X rays. Then, the groups were blinded using an alphanumeric code according to a computer-generated sequence of random numbers and cells plated into correspondingly coded 100-mm dishes containing culture medium with 0.1 μ g TPA/ml or TPA solvent (acetone at 0.05%) for cell survival and transformation determination (24–26). As in our previous experiments, the growth medium was renewed at weekly intervals (2 weeks for survival assay, 8 weeks for transformation assay) (24–26,67,69). Cell survival and plating efficiency were determined by colony formation, while neoplastically transformed foci (Fig. 2) were identified according to published criteria (19,65). The endpoint of transformation per surviving cell was calculated by the null method (66), with uncertainties determined according to our analysis (67).

SHE (pH 6.7) transformation assay. Several aspects of the SHE transformation assay (16,20) have recently been modified

(21,22). This now-standardized assay was used in our present study. Cryopreserved stocks of SHE cells, culture media samples, detailed laboratory protocols, and references of morphological phenotypes were kindly provided by G.A. Kerckaert. Cells were cultured in Dulbecco's LeBoeuf Modified Eagle's medium, pH 6.7 (Quality Biological, Gaithersburg, MD), containing 20% fetal bovine serum (HyClone) at 37°C in 10% CO₂ in air (21,22).

Following thawing of frozen stock SHE cells and culturing cells for 2 days, feeder layers were prepared by plating 5×10^4 X-irradiated (60 Gy) SHE cells in 2 ml of full medium into up to 80 60-mm tissue culture dishes. One day prior to feeder layer preparation, cells from another vial of frozen stock were used to establish cultures for exposure to MF by plating 5×10^4 cells into several 25-cm² flasks containing 5 ml of medium. Flasks containing 1-day-old SHE cell cultures were filled with medium as described previously for experiments with C3H/10T1/2 cells (24–27,67,69). Following 24-hr MF exposure, cells were plated onto the feeder layer at 80 cells per plate in 3 ml of fresh medium for survival and transformation assessment (21,22). The cultures were grown for 8 days without medium change. Other methods used were as in our present experiments with C3H/10T1/2 described above. Morphologically normal and transformed colonies in Figure 3 were identified using published criteria (16,20–22). Transformation frequency is the number of

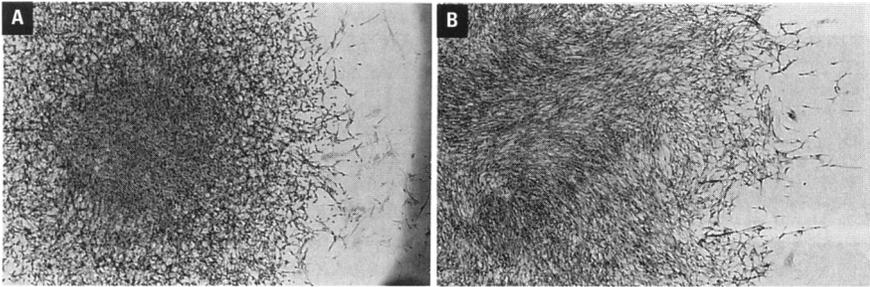


Figure 3. Morphological criteria of neoplastic transformation in SHE cells cultured at pH 6.7. (A) Transformed colony with disorganized cellular growth pattern including multilayered, criss-crossed cell distribution; (B) colony of normal cells that retained the ability to divide after treatment, exhibiting typical organized streaming pattern with uniform cellular separation.

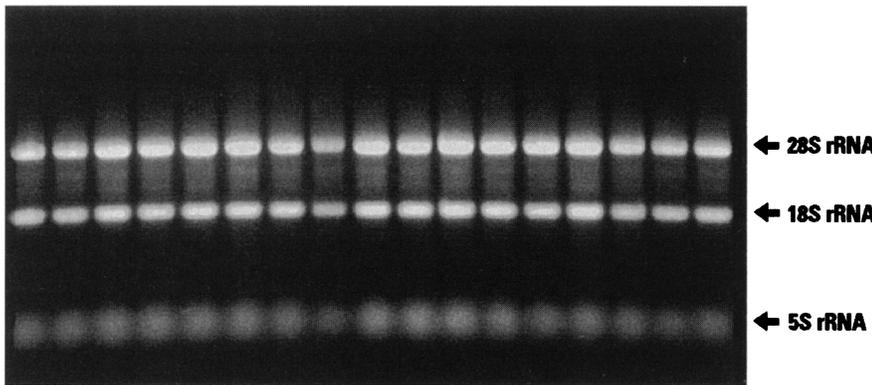


Figure 4. Ethidium bromide-stained coded RNA samples in a non-denaturing 1% agarose gel, with total RNA extracted from control or field-exposed HL60 cells. Cells were lysed immediately (less than 10 min) after 10, 20, or 40-min exposures; there were two flux density groups: in the first run, 0, 5.7, and 570 μ T and in the second run 0, 57, and 570 μ T. All the samples were obtained in a single experiment. See Figures 5 and 6 for Northern analysis results performed using these RNA samples.

positive colonies divided by the total number of colonies; uncertainty was calculated by assuming that transformants are Poisson-distributed with the mean equal to the number of positive colonies (68).

ODC expression in C3H/10T_{1/2} cells. To determine the levels of ODC mRNA, C3H/10T_{1/2} cells at passage 10 were plated into several 75-cm² flasks at 2000 cells/cm² 3 days prior to MF exposure (200 μ T, 24 hr) and cultured as for transformation experiments. Following MF exposure, the spent medium was removed and flasks were refilled with 15 ml of fresh medium containing 0.3 μ g TPA/ml; a final acetone concentration in the culture medium was 0.015%. Following the 30-min treatment, medium was removed and cells were washed three times with phosphate buffered saline. The cells were immediately lysed *in situ* using 4 ml of TRIzol (Gibco BRL) and stored at -70°C. Three independent experiments were performed, each consisting of the four groups described above.

c-myc, c-fos, GAPDH, β -actin, and ribosomal gene expression in HL60 cells. HL60 cells (44,45), the same clone as used by the Goodman group (32–35), were maintained

in Roswell Park Medium Memorial Institute 1640 medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (HyClone) without antibiotics. Stock cultures were maintained by passage at 1:20 dilution every 3 days (44,51,52,54,55). Two days before MF exposure, cells were seeded into several 150-cm² flasks at 5×10^5 cells/ml. On the day of the experiment, those multiple cultures were pooled and distributed into the required number of 25-cm² flasks at 5×10^5 cells/ml in 15 ml of fresh medium (34). To ensure pH stability during exposures, these exposure flasks were conditioned by first flushing with CO₂; they were then filled with 20 ml of growth medium and placed inside a CO₂ incubator overnight. This medium was removed before seeding flasks for exposure.

Each of three experiments consisted of three MF flux densities (5.7, 57, and 570 μ T), three MF exposure durations (10, 20, and 40 min), and corresponding concurrent no-field controls, as described by Goodman et al. (34). However, in our replication experiments, exposures were completed in two consecutive runs, each run consisting of nine field/time combinations (two flux den-

sity groups, one sham-exposed control group, three exposure times), for a total of 18 groups per run [instead of one sham-exposed control, one field, and three exposure durations per experiment in experiments described in (34)].

Immediately following MF treatments, cells were concentrated by centrifugation (1000 rpm, 8 min at 4°C) and lysed using 1 ml of ice-cold TRIzol. Cell lysates were transferred into new coded 1.5-ml microfuge tubes and refrigerated at -70°C. For interlaboratory comparison, cell lysates were divided into two equal parts before freezing; two collaborating groups in the Department of Radiation Oncology and the Department of Medicine, University of Maryland School of Medicine, were involved in this study. Similar methods were used in processing HL60 cells subjected to positive treatments with X irradiation or hyperthermia. Modification of these methods was necessary to process HL60 cells following TPA treatments because TPA had induced the majority of HL60 cells to differentiate into adherent macrophages, as previously reported (44,45,51,52,54). In contrast, MF did not induce this differentiation. Following the induction period, nonadherent cells (less than 20%) in medium were pelleted as described above, while attached cells (more than 80%) were lysed *in situ* using 2 ml of ice-cold TRIzol; small volumes of lysate were used to dissociate pellets. Both fractions were combined for RNA extraction and analyses.

Isolation and analysis of total RNA from C3H/10T_{1/2} and HL60 cells. Total RNA was extracted from test and control cells following the manufacturer's protocols for RNA isolation from cells grown as suspension or monolayer cultures using TRIzol reagent (71). Other methods used were from our previously published procedures (72,73). RNA concentrations were determined spectrophotometrically. The A_{260}/A_{280} was greater than 1.8. In addition, the integrity of the RNA samples was determined by examination of the ethidium bromide-stained 1% agarose gels; Figure 4 shows one representative example of the RNA samples analyzed in Figures 5 and 6. Both the sharpness of the bands and the relative intensity of the 28S, 18S, and 5S rRNA were assessed. Samples were considered to be degraded if the 28S band was not more intense than the 18S band or if the fluorescence suggested low-molecular weight products. If any sample in a series was degraded, the experiment was repeated.

RNA samples (10–20 μ g) were size-fractionated by electrophoresis (35 V overnight) in 1% agarose gels containing 2.2 M (6.6%) formaldehyde and 1X MOPS (Quality Biologicals, Gaithersburg, MD), followed by

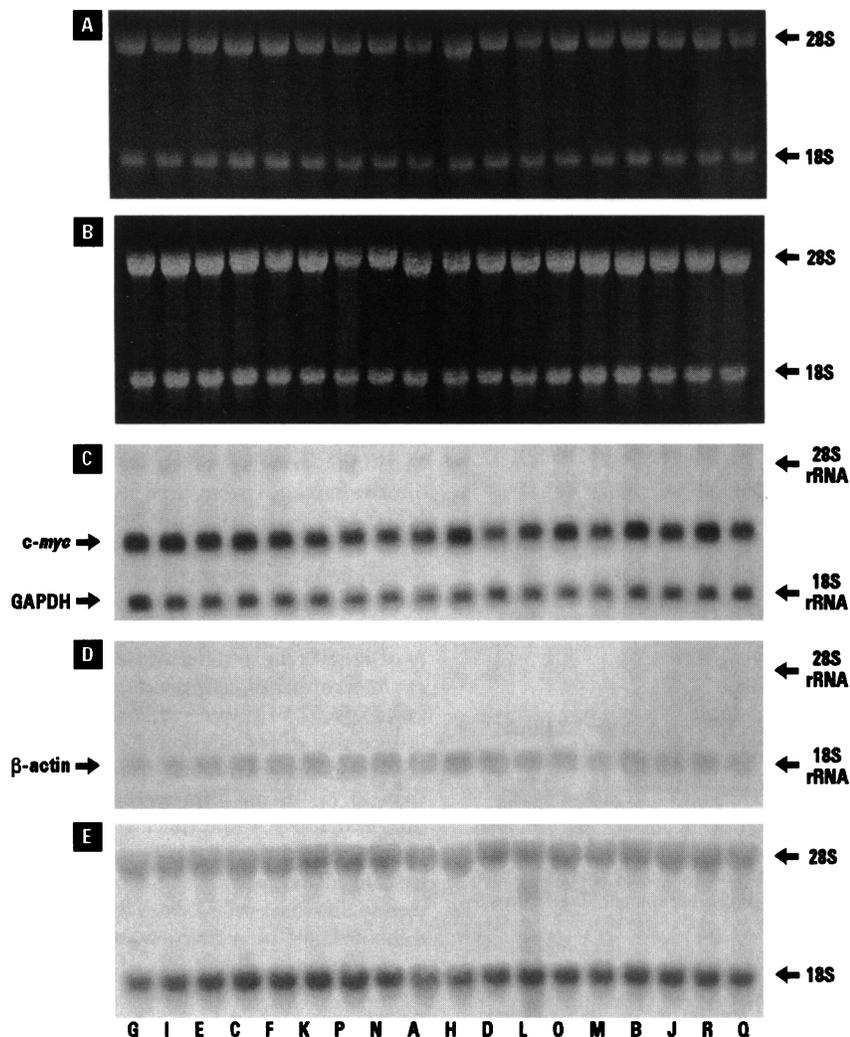


Figure 5. Expression of *c-myc*, GAPDH, and β -actin mRNA and 18S and 28S ribosomal gene transcripts in sham- or MF-exposed HL60 cells. The results shown are for the original coded RNA samples from one representative experiment. (A) Ethidium bromide-stained RNA samples (from Fig. 4) in a denaturing agarose/formaldehyde gel. (B) Ethidium bromide-stained RNA samples following transfer onto the membrane are shown as controls for the amounts of RNA loaded in each lane (10 μ g/lane). (C) Levels of *c-myc* mRNA and GAPDH mRNA in sham or magnetic field-exposed HL60 cells. Stripped membrane was sequentially hybridized with 32 P-labeled human probes for β -actin (D) and 18S and 28S ribosomal gene transcripts (E), as described in Materials and Methods.

capillary blotting from the formaldehyde gel to a Hybond-N 0.45- μ m membrane (Amersham Corporation, Arlington Heights, IL). Air-dried membranes were cross-linked by UV irradiation to immobilize the RNA using a UV Stratalinker (Stratagene, La Jolla, CA) on automatic setting. The membranes prehybridized in Hybrisol I (Oncor, Gaithersburg, MD) were hybridization at 45°C with DNA probes radiolabeled with [α - 32 P] dCTP (10⁵ decays/min; New England Nuclear Research Products, Boston MA) using a random-primers DNA-labeling kit (Multiprime DNA Labelling System, Amersham International, Amersham, UK). Conditions for hybridization and washing of Northern blots were as described previously

(72,73). After washing, membranes were exposed to X-Omat Kodak Scientific Imaging film (Eastman Kodak, Rochester, NY) at -70°C for 2 hr (ribosomal genes) to 2 days. To remove hybridized probes for reprobing, membranes were washed in 0.5X Denhardt's solution (Quality Biologicals), 0.1% sodium dodecyl sulfate, 25 mM Tris, pH 7.5, at 90°C for 1 hr, followed by another 30-min wash at 85°C.

Hybridization probes. The ribosomal cDNA probes for the 5-kb 28S rRNA and 1.9-kb 18S rRNA were generated from total RNA using SuperScript RNase-H Reverse Transcriptase (Gibco BRL). Other probes used in Northern analysis were the clone pODC 821 containing a 7.78-kb DNA

fragment of the rat *ODC* inserted into the pUC13 vector (American Type Culture Collection, Rockville, MD) for the 2.2-kb and 2.7-kb *ODC* transcripts; the 1.4-kb human *c-myc* (3rd exon) fragment (Oncor, Inc., Gaithersburg, MD) for the 2.3-kb *c-myc* transcript; the clone pc-*fbs*-1 containing a 9-kb fragment of the human *c-fos* inserted into the pBR322 vector (American Type Culture Collection for the 2.2-kb *c-fos* transcript); and fragments of human β -actin and GAPDH for the 2.2-kb β -actin transcript and the 1.1-kb GAPDH transcript was a gift from Kathleen Daher (University of California, Los Angeles, CA).

Evaluation of variation in relative mRNA content. We assumed that the transcripts of 18S rRNA, 28S rRNA, β -actin, GAPDH, *c-myc*, *c-fos*, and *ODC* may all be modulated by MF and by positive-control treatments. This approach was not adopted in related papers (12-14,32-43).

To determine the amounts of hybrids formed between the transcript of interest and an appropriate probe, autoradiograms were analyzed by densitometry using a ScanJet 4C (Hewlett Packard) and the Intelligent Qualifier, version 2.1 (BioImage, Ann Arbor, MI).

The coded autoradiographic data for 18S rRNA, 28S rRNA, β -actin, GAPDH, and *c-myc*, exemplified in Figure 5 C-E, were analyzed as previously described (74,75). Accordingly, principal component analysis was performed by transforming the raw densitometry data into their Z-scores (obtained by subtracting the mean and dividing by the standard deviation). Z-score distributions provide an objective measure of the variability of the transcripts that is independent from the mean and from the scale of the optical densities. Therefore, this method is well suited for comparing the coded data from different autoradiograms.

The Z-scores from three independent experiments were pooled for each of five genes (54 Z-scores for each gene) and organized as a 5 \times 54 analysis of variance (ANOVA) table. Statistical variation within groups and among five gene groups was tested by two-way ANOVA. In addition, Z-scores in each of the five gene groups were analyzed by the box-whisker plots. In Figure 7, *p* is the one-sample *t*-test two-tailed probability that the group mean is zero.

The uncoded radiographic data for 18S rRNA, 28S rRNA, β -actin, GAPDH, and *c-myc*, as shown in Figure 6, and the data for *ODC* (Fig. 8) were analyzed as follows. For different hybridizations, the ratio of optical densities of a band from an MF exposure group to the appropriate control group band was calculated. MF group means of replicate ratios and the coefficients of variation (i.e.,

the standard deviation/mean) were calculated. The coefficients of variations and visual examinations of autoradiograms provide similar information about heterogeneity within the data subset because both methods are sensitive to the mean optical density and the standard deviations of the mean. To minimize this source of error, the differences in the mean basal abundance among various transcripts were partially compensated by adjusting the amount of RNA to be examined and by differential autoradiography, so that in terms of optical density, the bands for

c-myc, GAPDH, 28S rRNA, and 18S rRNA were similar, and the β -actin bands were only twofold lighter (Figs. 5 and 6). In the case of *c-fos*, mRNA levels are usually not normalized to the control samples because basal levels of the *c-fos* mRNA in control cells are too low to estimate accurately (53–55). Therefore, results were quantified based on the absolute band optical densities.

Required calculations were performed using commercial statistical software (PSI-Plot, version 3, Poly Software International, Salt Lake City, UT).

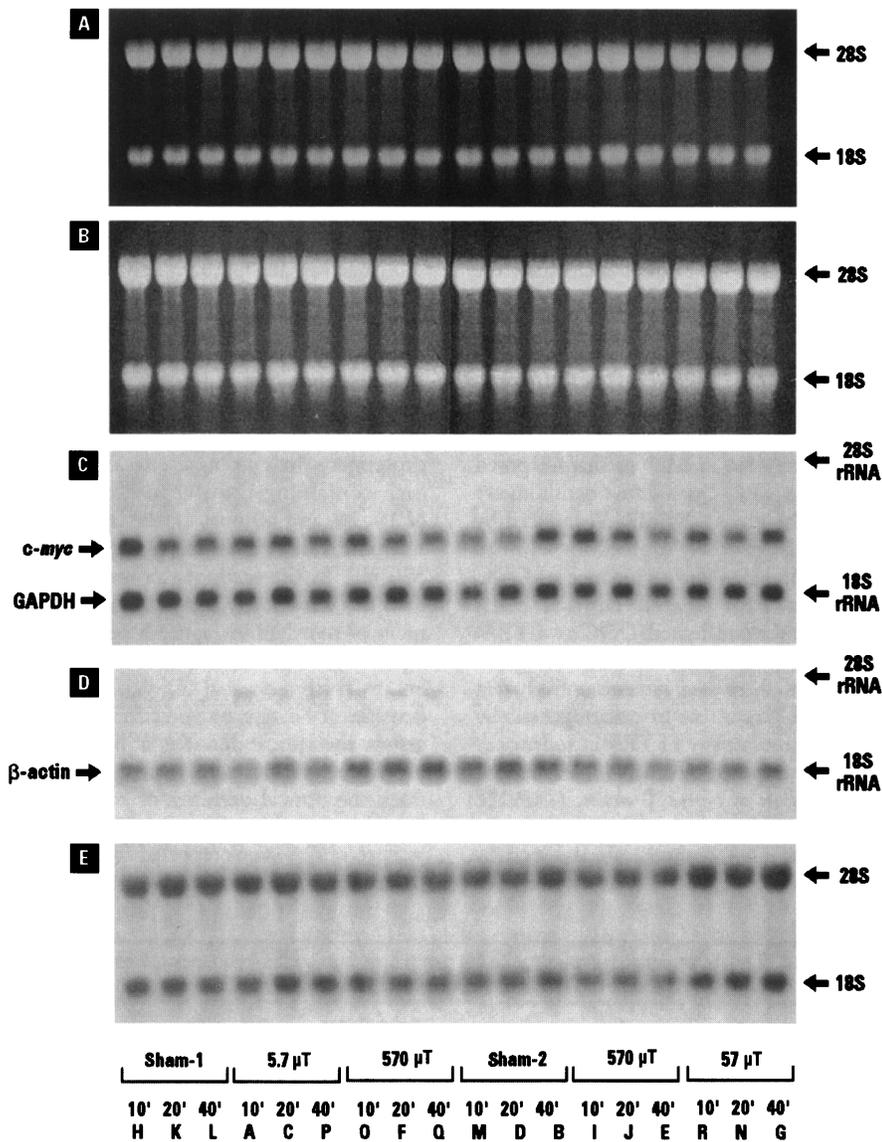


Figure 6. Expression of *c-myc*, GAPDH, and β -actin mRNA and 18S and 28S ribosomal gene transcripts in sham- or magnetic field-exposed HL60 cells. After the coded samples in Figure 5 were analyzed, the group identities were revealed and the RNA samples were grouped according to magnetic field exposure level/duration patterns. (A) Ethidium bromide-stained RNA samples (from Fig. 4) in a denaturing agarose/formaldehyde gel. (B) Ethidium bromide-stained RNA samples following transfer onto the membrane are shown as controls for the amounts of RNA loaded in each lane (10 μ g/lane). (C) Levels of *c-myc* mRNA and GAPDH mRNA in sham or magnetic field-exposed HL60 cells. Stripped membrane was sequentially hybridized with 32 P-labeled human probes for β -actin (D), 18S, and 28S ribosomal gene transcripts (E) as described in Materials and Methods. The letters (bottom of figure) are an alphanumeric code according to a computer-generated sequence of random numbers.

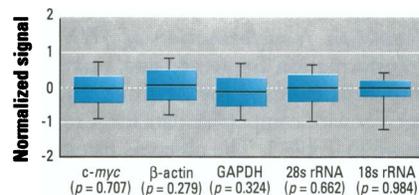


Figure 7. Normalized-signal (Z-score) distributions of autoradiographic densities of the multiple coded samples such as those shown in Figure 5. The box plots show the median, the upper and lower quartiles (the box), and the 10th and the 90th percentiles (the bars). *p* is the one-sample *t*-test two-tailed probability that the group mean is zero (*p*-values of less than 0.05 would customarily be required to reject this hypothesis).

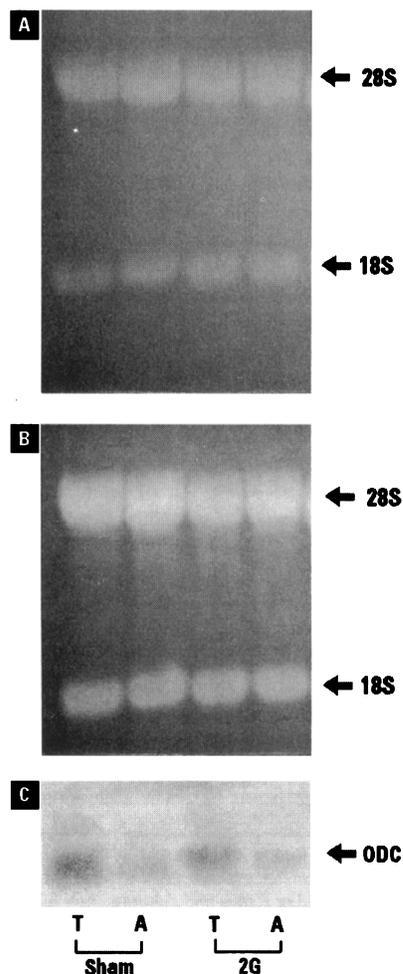


Figure 8. Expression of ODC mRNA in actively growing C3H/10T1/2 cells after sham exposure or 60-Hz MF exposure (200 μ T = 2 G for 24 hr). Within less than 15 min following exposure to the magnetic field, cells were treated with 0.3 μ g of 12-*O*-tetra-decanoylphorbol-13-acetate in acetone per milliliter (T) or acetone (A) for 30 min. (A) Ethidium bromide-stained RNA samples in a denaturing agarose/formaldehyde gel; (B) Ethidium bromide-stained RNA samples following transfer onto the membrane are shown as controls for the amounts of RNA loaded in each lane (20 μ g/lane); (C) ODC transcription in sham or 60-Hz magnetic field (2 G)-exposed HL60 cells.

Results

Neoplastic transformation of SHE cells.

Transformation frequency and survival of SHE cells exposed for 24 hr to 200 μ T, together with comparable data for X rays, are shown in Table 1. The observed plating efficiencies of 20 to 35% (Table 1) were similar to values reported by LeBoeuf et al. (21,22).

Determination of transformation frequency in this study involved morphological evaluation of nearly 20,000 colonies from nine experiments. Eight transformed colonies (Fig. 3) were found in the 1.5-Gy group and no transformed colonies were found in sham or MF groups. Transformation frequency at a moderately cytotoxic X-ray dose of 1.5 Gy was 2.86×10^{-3} per clonogenic cell (Table 1). This estimate is about five times the frequency reported previously for SHE cells grown in conventional media, pH 7.1–7.3, (20); no other X-ray data are available for comparison, but we note that enhanced carcinogen-induced morphological transformation and low spontaneous background were expected for SHE cells grown at pH 6.7 (21,22).

Neoplastic transformation of C3H/10T1/2 cells.

Transformation frequency and survival of MF-exposed C3H/10T1/2 cells with and without the promoter TPA, together with data for X irradiation, are shown in Table 2. More than 500 dishes per group were accumulated to establish the effects of MF with or without TPA with some degree of statistical certainty.

Under the present experimental conditions, there were no significant differences in plating efficiency among MF-exposed and sham-exposed cells, as expected. Positive control results with X rays are in agreement with observations from related experiments using the C3H/10T1/2 assay in this and other laboratories (23–26, 65–69). The effect of TPA on sham-exposed cells was not significant (sham-irradiated control

vs. sham-irradiated control + TPA; $p = 0.35$), whereas it produced the expected enhancement of transformation induced by X rays (X irradiated vs. X irradiated + TPA; $p < 10^{-6}$) (25–28). Consistent with the results obtained using the SHE assay, MF and 0 μ g TPA/ml post-treatment produced no significant effect on transformation (sham-irradiated control vs. MF; $p = 0.70$). Similarly, the treatment MF and 0.1 μ g TPA/ml post-treatment did not increase the transformation frequency above the corresponding control level (sham-irradiated control + TPA vs. MF + TPA; $p = 0.59$). An approximately twofold effect of TPA in MF-exposed cells was not significant (MF vs. MF + TPA; $p = 0.13$).

Expression of ODC in C3H/10T1/2 cells.

As shown in Figure 8, Northern blot analysis of ODC mRNA demonstrated the presence of two distinct ODC mRNA transcripts, approximately 2.2 and 2.7 kb in size. Two ODC mRNA transcripts have been demonstrated in a number of different tissues; the 2.2-kb band is typically weaker than the 2.7-kb band in fibroblasts (76). Compared to basal levels, we observed an increased expression of 2.2- and 2.7-kb ODC mRNA transcripts immediately following TPA application for 30 min; no similar increases in ODC mRNA were seen in MF- or sham-exposed cells without TPA. Quantitative densitometry indicated that the ODC mRNA induced by TPA in cells previously exposed for 24 hr to MF was similar to the abundance of ODC mRNA in TPA-treated control cells. Therefore, we confirmed ODC as a TPA-inducible but not an MF-inducible gene. According to these data, it seems unlikely that MFs could play a role in carcinogenesis by mimicking the activity of TPA in rodent cell systems, as supposed by others (3,4,8,42,63).

Expression of *c-myc*, β -actin, GAPDH, and ribosomal genes in HL60 cells (coded data).

Figures 4–7 demonstrate the sequential steps of processing and analysis of MF

and sham-irradiated samples of RNA. One-microliter volumes of RNA samples, separated on a non-denaturing 1% agarose gel directly following RNA extraction from a flask of cells (each representing one experimental group), but before the RNA sample concentrations were measured and equalized, are shown in Figure 4; this demonstrates that approximately equal numbers of cells were collected in each group.

Representative Northern blot analyses from our present studies of the expression of *c-myc* mRNA, GAPDH mRNA, and β -actin mRNA in coded samples are displayed in Figure 5. In this example, Figure 5A shows equal amounts of ethidium bromide-stained RNA samples separated on a denaturing formaldehyde-agarose gel. Figure 5B represents RNAs shown in Figure 5A after transfer on the nylon membrane. The membrane in Figure 5B was hybridized with two 32 P-labeled DNA probes for human *c-myc* (exon 3) and GAPDH (Fig. 5C) together and sequentially rehybridized with the DNA probe for β -actin (Fig. 5D) and cDNA probe from reverse transcribed total RNA as described in Materials and Methods (Fig. 5E).

The example of the data in Figure 5 was obtained in one day from a single experiment designed as a series of two runs, each containing three exposure durations of 10, 20, and 40 min. In the first run, the magnetic flux densities were 0, 5.7, and 570 μ T. In the second, the magnetic flux densities were 0, 57, and 570 μ T. Disagreement of our data in Figure 5 with the data reported by Goodman et al. (34) is apparent, even from a visual comparison of band densities for *c-myc* and β -actin in the same group: the optical densities of bands resulting from hybridization with *c-myc* do not track the optical densities of bands resulting from hybridization with β -actin, contrary to observations reported for *c-myc* and β -actin by these authors (34).

Table 1. The frequency of neoplastic transformation of Syrian hamster embryo cells exposed to 60-Hz magnetic field or X rays and cultured at pH 6.7

Treatment ^a	PE or SF \pm SE ^b	Total colonies	Total positive colonies	TR \pm SE (10 ⁻³) ^c
O	0.25 \pm 0.07	8424	0	0
MF	0.28 \pm 0.10	8475	0	0
X	0.78 \pm 0.05	2802	8	2.86 \pm 1.0

^aExperimental groups were defined as follows: O, sham-irradiated control; MF, 24-hr exposure to 60-Hz MF at 200 μ T; X, X irradiation at 1.5 Gy.

^bPlating efficiency (PE) or surviving fraction (SF) \pm standard error (SE) from pooling the results from nine independent experiments on survival and transformation.

^cTransformation frequency (TR) per surviving cell \pm SE derived from total number of colonies and total number of positive colonies.

Table 2. Effect of post-irradiation treatment with the tumor promoter on the frequency of neoplastic transformation of C3H/10T1/2 cells exposed to 60-Hz magnetic field or X rays

Treatment ^a	PE or (SF \pm SE) ^b	Total dishes	Total dishes with foci	Mean no. viable cells/dish	TR \pm SE (10 ⁻⁴) ^c
O	0.58 \pm 0.13	569	3	292	0.18 \pm 0.11
O + TPA	0.49 \pm 0.15	574	7	268	0.46 \pm 0.17
MF	0.54 \pm 0.14	574	3	250	0.21 \pm 0.12
MF + TPA	0.58 \pm 0.19	629	10	281	0.57 \pm 0.18
X	(0.74 \pm 0.10) ^b	243	17	275	2.64 \pm 0.64
X + TPA	(0.73 \pm 0.08) ^b	248	62	295	10.1 \pm 1.2

^aExperimental groups were defined as follows: O, Sham-irradiated control; MF, 24-hr exposure to 60-Hz magnetic field at 0.2 mT; X, X irradiation at 1.5 Gy. After treatment, cells were plated in medium containing acetone or 0.1 μ g 12-*O*-tetra-decanoylphorbol-13-acetate/ml in acetone (TPA).

^bPlating efficiency (PE) or surviving fraction (SF) \pm standard error (SE) from pooling the results from nine independent experiments on survival and transformation.

^cTransformation frequency (TR) per surviving cell (48) \pm SE derived from total number of dishes and total number of positive dishes (49).

Z-score distributions of the coded image data for *c-myc*, β -actin, GAPDH, and ribosomal genes pooled from several similar experiments (including independent interlaboratory comparisons of Northern analysis of the RNA samples from the same experiment) are shown in Figure 7. Analysis of the entire data set by ANOVA yielded $p = 0.833$ ($F = 0.364$; $df = 4$) for comparison among gene groups and $p = 0.217$ ($F = 1.302$; $df = 53$) for comparison within groups, confirming that the group means were equal (similarly large p -values were obtained for the data subsets for individual experiments). Box-whisker plots of Z-score distributions of individual groups are shown in Figure 7. One sample t -test performed on the group data provided the following two-tail probabilities that the group mean is zero: *c-myc* group, $p = 0.707$; β -actin group, $p = 0.279$; GAPDH group, $p = 0.324$; 18S rRNA, $p = 0.984$; and 28S rRNA, $p = 0.662$ (53 df in each data subset). Taken together, these analyses show no statistically significant departures from random variability. Therefore, our data contradict previous conclusions for *c-myc* and β -actin (32–34).

Expression of *c-myc*, β -actin, GAPDH, and ribosomal genes in HL60 cells (uncoded data). The data-handling methods used in the original publications (34) as well as in the recent papers reporting the replication results (12–14) can only be used if the group identities are known. Figure 6 shows independent Northern blots of the RNA samples in Figure 4 after the group identities were revealed.

MF-group means of replicate ratios of optical densities of a band from an MF-exposure group to the appropriate control group band, along with the coefficients of variation, are summarized in Table 3. In terms of the coefficients of variation, the normalized autoradiographic data for the 18S ribosomal transcript and a metabolic

transcript, GAPDH, were the least heterogeneous, perhaps justifying their use as reference genes, as reported in the recent MF-related work (12,14). Normalized data sets for the *c-myc*, β -actin, and 28S rRNA gene transcripts were similar to each other but were more heterogeneous than the other two. Less than 20% of variations in the relative levels of 28S ribosomal gene, *c-myc* and β -actin transcripts among nine MF-groups in Table 3 have no recognizable biological consequences (77–79).

In terms of the band density ratios shown in Table 3, the variation of five transcripts, 18S rRNA, 28S rRNA, *c-myc*, β -actin, and GAPDH was not associated with MF exposure parameters and remained close to basal levels. Thus, the results from the present experiments offer no support for the previous findings that MFs affect transcription of *c-myc* and β -actin (32–34).

Expression of *c-fos* in HL60 cells. In control treatments, TPA and hyperthermia significantly increased *c-fos* mRNA abundance above basal levels in control cells (Fig. 9); these results are consistent with the data for these agents in the literature (51–55). The negative result for X irradiation at 1.5 Gy was expected; the threshold X-ray dose for visualization of the *c-fos* transcript in HL60 cells is 5 Gy (55). The phenotypic consequences of expression of *c-fos* were observed in the course of the present study: hyperthermia induced apoptosis as demonstrated by the DNA fragmentation assay, and TPA treatments caused dramatic population-wide differentiation as evidenced by changes in HL60 cell morphology and adhesion of cells to a plastic substrate (data not shown).

The responses of HL60 cells to positive control treatments and two MF flux densities are shown in Figure 10. In Figure 10B,

we compared the *c-fos* hybridization signals in the RNA positive control samples with the *c-fos* hybridization signals in the RNA samples from sham-exposed and MF-exposed HL60 cells. The high levels of responsiveness to TPA and hyperthermia were not observed with MFs. In fact, the *c-fos* transcription was undetectable in the MF samples. These negative results for the *c-fos* mRNA induction by our MF exposures of HL60 cells were contrary to the reports by others (32,33,35,38).

Discussion

In designing these experiments, we were forced to limit our choices of MF exposure parameters, as mentioned in the Introduction. Overall, we used MF parameters shown by others to be critical in influencing biological responses. In the case of transformation experiments, we considered animal studies that suggest a tendency toward a dose response in terms of MF flux density and exposure duration (3–6), so that higher-level, longer exposures would increase the likelihood of seeing a positive effect. Since the cell cycle time of C3H/10T_{1/2} cells is approximately 20 hr (19,66,69), our 24-hr exposures at 200 μ T could be taken as emulating a lifetime MF exposure of mice scaled to a mouse single cell. Surprisingly, previous studies of gene expression in cultured cells failed to identify critical MF exposure parameters. For example, positive results on the transcription of *c-myc* and histone (35), as well as on ODC enzymatic activity (42,43), have indicated that a brief exposure to a magnetic field is enough to evoke the same level of effect as a prolonged exposure. In other studies, no differences in gene responses were observed in several extremely low frequencies (up to 100 Hz) and MF flux densities (32–43). For our present work involving gene expression in HL60 cells, we selected nine 60-Hz MF exposure parameters tested by Goodman et al. (34).

Table 4 shows the results with MFs for the series as a whole. Our findings, presented in this paper, did not provide any significant

Table 3. Gene expression following exposure of HL60 cells to 60-Hz magnetic fields (MF)

Flux density (μ T)	Exposure duration (min)	Relative abundance of mRNA ^a				
		<i>c-myc</i> ^b	β -actin ^b	GAPDH ^b	28S rRNA ^b	18S rRNA ^b
5.7 (12) ^c	10	0.89 (0.23)	0.97 (0.29)	0.99 (0.13)	0.99 (0.14)	1.03 (0.07)
	20	1.07 (0.27)	0.96 (0.21)	1.04 (0.08)	0.94 (0.20)	0.98 (0.05)
	40	1.13 (0.25)	1.00 (0.39)	1.06 (0.10)	1.10 (0.14)	1.04 (0.06)
57 (12)	10	1.02 (0.20)	1.01 (0.20)	1.02 (0.18)	0.99 (0.13)	0.98 (0.09)
	20	1.06 (0.17)	1.00 (0.29)	1.01 (0.22)	1.02 (0.09)	1.00 (0.05)
	40	0.96 (0.18)	0.95 (0.25)	0.93 (0.17)	1.07 (0.14)	0.97 (0.08)
570 (12)	10	0.91 (0.17)	0.94 (0.23)	1.02 (0.13)	1.00 (0.21)	0.99 (0.06)
	20	1.09 (0.20)	1.08 (0.32)	0.97 (0.19)	0.98 (0.28)	1.03 (0.08)
	40	1.13 (0.32)	1.05 (0.31)	1.11 (0.16)	0.99 (0.23)	1.07 (0.05)

^aHybridization signal of the RNA sample extracted from HL60 cells exposed to 60-Hz MF, which was normalized to hybridization signal in a corresponding control group.

^bValues shown as mean and coefficient of variation (in parenthesis).

^cNumber of independent estimates of the effect of 60-Hz MF from three independent experiments at the field levels and exposure durations shown; in each of three experiments, one flux density group was replicated, providing a within-experiment variation in two groups of samples independently exposed to the same flux density (see text and Fig. 6).

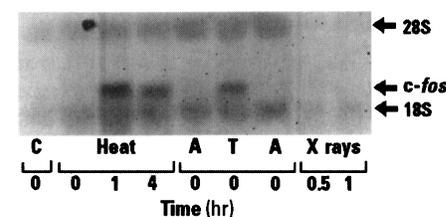


Figure 9. Expression of *c-fos* mRNA in HL60 cells following positive control treatments. Heat, 45°C for 9 min; T, 50 ng 12-*O*-tetra-decanoylphorbol-13-acetate in acetone/ml of medium; A, acetone for 3 hr; X rays, 1.5 Gy. Control (C) or experimental HL60 cells were lysed at postexposure times indicated.

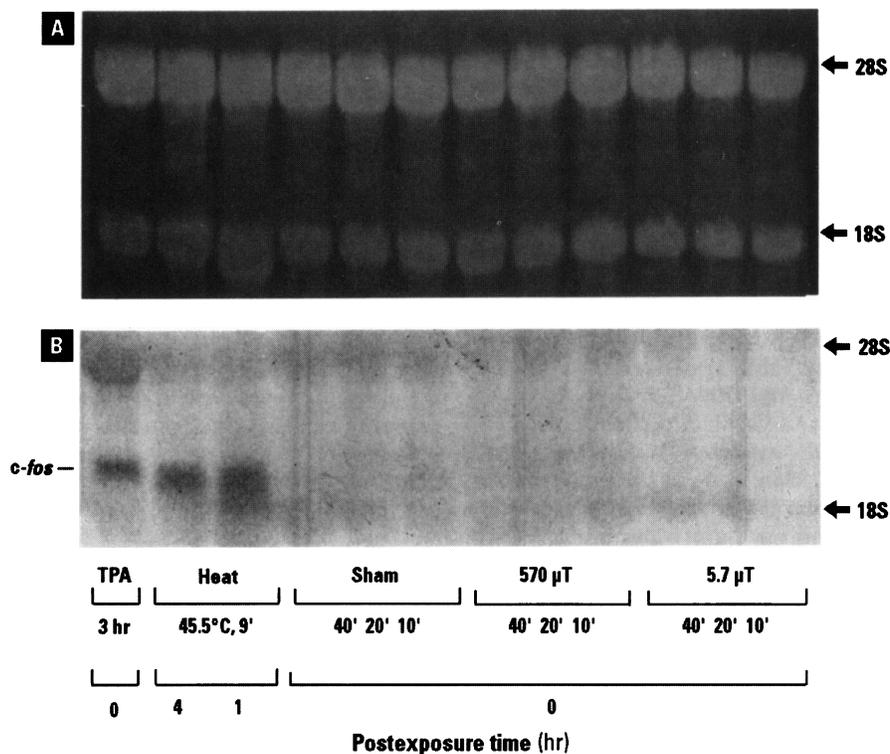


Figure 10. Expression of *c-fos* mRNA sham-exposed or 60-Hz magnetic field (MF)-exposed HL60 cells. Cells were exposed to MF for 10, 20, or 40 min at 0, 5.7, or 570 μ T; 50 ng 12-*O*-tetra-decanoylphorbol-13-acetate (TPA)/ml for 30 min; or 45.5°C for 9 min and lysed at postexposure times indicated. (A) Ethidium bromide-stained RNA samples following transfer onto the membrane are shown as controls for the amounts of RNA loaded in each lane (20 μ g/lane); (B) levels of *c-fos* mRNA in experimental and control HL60 cells.

Table 4. Summary of results on gene expression and neoplastic transformation following low level exposure to magnetic fields

Cell type	Exposure	Endpoint	Outcome
HL60	5.7–570 μ T (10–40 min)	Gene expression: <i>c-myc</i> <i>c-fos</i> β -actin GAPDH RNA Apoptosis, differentiation	No effect No effect No effect No effect No effect
C3H/10T1/2	200 μ T (24 hr)	Gene expression: ODC Neoplastic transformation Cell killing	No effect No effect No effect
SHE	200 μ T (24 hr)	Neoplastic transformation Cell killing	No effect No effect

evidence that MF exposure of rodent cells at a flux density of 200 μ T affects neoplastic transformation or *ODC* gene transcription. Similarly, MF exposures of HL60 cells at flux densities from 5.7 to 570 μ T did not affect *c-myc*, β -actin, *c-fos*, *ODC*, or GAPDH, as well as 28S and 18S ribosomal genes.

We report here for the first time the MF response data obtained using two classic transformation systems. We found no statistically significant evidence of carcinogenic or initiating activities of MF in these *in vitro*

models for the tested cells and experimental conditions. This can be compared with the conclusion from the promotion study of Cain et al. (8) using a variant assay with preinitiated C3H/10T1/2 cells; interestingly, these authors reported a 1.9-fold effect in TPA-treated MF-exposed groups, which was also not statistically significant. Overall, these *in vitro* results corroborate negative results from animal studies (5,7,9). We did not study MF as a promoter *in vitro*; however, considering the lack of MF influence on

ODC mRNA in C3H/10T1/2 cells, promotional activity of MF seems unlikely, at least at the MF–time combination studied. Moreover, even when positive results for *ODC* have been reported with MF, the effects were transient (42,43). In contrast, only the sustained overexpression of *ODC* is associated with neoplastic transformation (46) and cancer promotion *in vivo* (56,57).

We observed no effects of MFs on transcription of several genes performing a variety of functions within the cell. Our data set is more extensive and better documented than those previously published and includes results on numerous genes, many of the exposure conditions of previous studies, and analysis of coded and uncoded data. Our specific results showing no change in *c-myc*, β -actin, and GAPDH transcription are consistent with recent findings (12–14) but do not confirm the original study of Goodman et al. (34) that we attempted to replicate. The general procedures including the cell type, investigated genes (*c-myc*, β -actin), MF exposure levels, and exposure durations were identical between the Goodman study (34) and our studies, but one must note differences between the two studies. One difference was in the transcript detection technology. Goodman et al. (34) drew their conclusions based on dot blot analysis of total RNA. In the form used by these investigators, dot blots are unsuitable for a quantitative analysis of transcript levels because the equal amounts and integrity of RNA samples cannot be verified. In fact, Goodman et al. (34) stated that they could not confirm the dot blot data by Northern blot analysis of the selected samples. The presence or absence of geomagnetic field in the Goodman study or ours had no apparent influence on the results. Both positive and negative results for β -actin and/or *c-myc* were obtained in previous replication studies that used shielded (13) and unshielded (12,14) MF exposure systems.

Our agreement with other recent results (12,13) should not obscure the fact that differences exist between our study and theirs. Both studies (12,13) used TPA for 30 min as a positive control treatment, but neither study reported HL60 cell differentiation into adherent macrophages as a result of TPA treatments, whereas 20 min of TPA is sufficient to commit HL60 cells to differentiation. The *c-myc* reduction after 90 min observed by Saffer and Thurston (13) demonstrates this differentiation of HL60 cells (51,52). Such positive control cells would have to be trypsinized, scraped off the flasks, or lysed *in situ* for RNA extraction, contrary to the stated methodology wherein all groups were blinded and treated as cells in suspension. Since an increase in *c-myc* and *c-fos* transcription is a hallmark

of differentiation of HL60 cells, this effect should also have been observed with MF in the Goodman experiments (32–35,37).

Another difficulty in the interpretation of the *c-fos* results reported by Karabakhshian et al. (37) is that basal levels of *c-fos* mRNA are uniformly very low across different cell lines, including HL60 cells (51–56). Consequently, a many-fold increase of *c-fos* mRNA is required for *c-fos* transcript visualization after an inducing treatment. For instance, the most effective treatment in our experiments, heat shock (45.5°C for 9 min) 1 hr posttreatment (Fig. 9), represents an estimated 1000-fold increase in *c-fos* transcription (53). In view of these results, reported increases of approximately 20–30% in *c-fos* transcription (relative presumably to basal levels) by MF (20 min exposure at 8 to 80 μ T) in HL60 cells, most recently claimed by Henderson (38), must in reality be considered undetectable. Even more puzzling is the finding by these authors that *c-fos* transcription was decreased by certain combined treatments with MFs (37,38) since basal levels are so low to begin with.

In summary, the negative data on neoplastic transformation and gene expression are inconsistent with the proposed hypothesis in the range of MF exposure parameters studied. Studies of neoplastic transformation *in vitro* deserve to be continued at various MF field/time combinations to determine whether MFs represent a real health risk. The significance of our negative results on gene expression is twofold. First, the findings presented in this paper complement the data already available for *c-myc* and β -actin (12–14) and extend these observations to all the MF exposures studied by the Goodman group (34). In addition, we presented new data for several genes, including *ODC* and *c-fos*, that have not been investigated in previous replication experiments. Our control treatments with TPA, hyperthermia, and X irradiation produced the expected cellular and gene responses expected from the literature data. Thus, our experiments were sufficiently sensitive to detect the reported increases in gene expression after MF exposures. Second, positive molecular data, exemplified by Goodman's data on *c-myc* induction by MFs, provide a mechanistic background for several models of MF carcinogenesis. In view of our present results, the genetic effects of MF remain unconfirmed, thus diminishing the biological plausibility of a causal relationship between MF and cancer.

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