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# Specific Region of the c-*myc* Promoter Is Responsive to Electric and Magnetic Fields

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**Abstract** The level of c-*myc* transcripts is increased in cells exposed to extremely low frequency (elf) electromagnetic (EM) fields at 60 Hz. The aim of the present experiments was to determine if regulatory regions upstream of the c-*myc* gene modulate the response to EM fields. DNA upstream of P1 of both mouse and human c-*myc* genes was transfected into cells as CAT constructs. The presence of DNA 5′ to the human or mouse myc genes results in increased expression of CAT following 20 min exposures of cells to 60 Hz elf EM fields. Specific portions of the human upstream DNA were deleted and introduced into cells. The region responsive to EM fields is located between −353 and −1,257 relative to the $P_1$ promoter. 

**Key words:** c-*myc* promoter, CAT expression, low frequency electric and EM fields, transcription

The possibility of health risks resulting from exposure to electric and magnetic (EM) fields provides a strong motivation to determine how such fields interact with cells. Initially, an important clue to understanding how cells respond to EM fields was the finding that transient increases in specific transcripts occur in cells exposed to extremely low frequency [(elf) (>300 Hz)] EM fields. This response has been observed in a variety of cell types including dipteran salivary gland cells [Goodman et al., 1983, 1992a,b, in press], yeast cells [Weisbrot et al., 1993a], and human cells [Goodman and Henderson, 1991; Goodman et al., 1992c,d; Czerska et al., 1992; Phillips et al., 1992; Liburdy et al., 1992]. The effect of EM fields is probably directly at the transcriptional level [Goodman et al., 1983; Phillips et al., 1992]. The initial evidence came from analysis of transcription autoradiograms of dipteran salivary gland cells. The presence of increased grain density over specific chromosome regions indicated a direct influence of low frequency EM fields on transcription per se rather than, for example, an increase in RNA stability, or the release of RNA storage forms [Goodman et al., 1983, 1992a,b, in press]. Other experimental evidence for effects at the transcription level is derived from nuclear run-off analyses. Increased transcription of c-*myc*, c-*fos*, c-*jun*, and protein kinase C was observed in a derivative of human T-lymphoblastoid cells [Phillips et al., 1992].

Transcript levels for c-*myc* are increased in a variety of cell types exposed to a 60 Hz sinusoidal EM field, and under different experimental conditions. Increased c-*myc* transcript levels have been measured following short exposures of thymocytes stimulated with Con A to EM fields [Liburdy et al., 1992]. The increase in transcript levels is coordinate with an increase in intracellular calcium, which implies an interaction of EM fields with the cell membrane. The increase in transcripts following a 60 Hz EM field exposure is rapid, within 4–8 min [Goodman et al., 1992c]. Taken together, these results strongly suggest that regulatory pathways are implicated in the response of the cell to low energy *EM* fields.

DNA upstream of c-*myc* was transfected into both mouse (stable transfectants) and human cells (transient transfectants) as a CAT construct. The presence of upstream DNA in CAT constructs resulted in increased expression of

Abbreviations: CAT, chloramphenicol transferase; elf, extremely low frequency; EM, electric and magnetic; Hz, Hertz.

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CAT after exposure of cells to 60 Hz EM fields. Further experiments showed that the upstream regulatory region responsive to EM fields in human cells is approximately 900 base pair (bp) and located between the restriction sites ClaI and PvuII.

## MATERIALS AND METHODS

### Cell Culture and Transfections

HeLa cells are maintained in D-MEM (Gibco), 10% fetal calf serum (FCS). Mouse myeloma (PX3) cells are grown in IMDM (Gibco). All media included 10% FCS and 1% Penn-Strept.

Transfections of cells used lipofectin (HeLa cells) and electroporation (mouse cells). Mouse cells were stably transfected [Muller et al., 1990]. For electroporation, the cells were seeded at $5 \times 10^5$ per 25 cm plate and incubated overnight in medium. After washing, the cells were suspended at $5 \times 10^5$ cells per ml in cold PBS. Twenty micrograms of linearized pSV2-neo DNA (linearized with PstI) was added to 1 ml of cells, and the cells placed on ice for 15 min. Electroporation used 220 volts, 960 capacitance. The cells were returned to ice for 30 min after electroporation. They were washed in 10% FCS IMDM and subcloned by placing the cells in a 96 well plate for 24 hr in selective media. After 24 hr, the media was replaced. The cells were maintained for about 4 weeks in 0.5 mg/ml G418. Eight resistant clones were pooled and maintained as cell lines.

For transient transfections, HeLa cells were placed in 10% FCS EMDM for 3 hr before transfection [Malone et al., 1989; Zhang et al., 1993]. Ten micrograms of DNA was mixed well in 100 μl of OPTI-MEMI (serum free). Other procedures are as described with the Gibco lipofectin transfection kit. From the final mixture 2.75 ml was added to a culture dish containing $8 \times 10^5$ cells/ml in a T-25 dish. The mixture was incubated overnight or 15 hr. After 15 hr, the media was replaced with normal media. The cells were harvested for CAT assay 48–63 hr after transfection.

### Plasmid Construction

Murine c-*myc* DNA (1.8 kilobase) (gift of Dr. K. Calame, Columbia University) was subcloned into PUC-18 containing CAT by blunt end ligation. CAT was originally excised from pSV2-CAT using a HindIII-EcoRI digest. Portions of the human myc upstream regulatory promoter regions were obtained from Dr. R. Dalla-Favera. These included the following regions: (1) a 2.8 kb HindIII to PvuII digest (HPV); (2) a 1.7 kB ClaI to PvuII fragment (CPV); and (3) a PvuII to PvuII fragment of 0.86 kb. (PPV) Blunt end ligation into $pSV_2$ CAT used a HindIII linker.

### CAT Assay

CAT was measured by enzymatic determinations. Acetylated $^{14}$C-chloramphenicol was measured by thin layer chromatography [Gorman et al., 1982]. Assays contained 30–60 μg of extract protein as measured by the BioRad Protein assay kit. After chromatography, spots were quantitated using a beta scanner, or by scintillation counting. CAT activity was calculated as the percentage of chloramphenicol converted to the acetylated form.

### RNA Extraction and Analysis

RNA isolation and purification has been described [Goodman et al., 1992c].

### Quantitation of Transcript Levels

For dot blot analysis [Muller et al., 1982], 4 μg of total RNA was used from each sample for dot blot and diluted by half for each point. Each sample was probed for c-*myc* and CAT. CAT DNA was labeled in vitro with $^{32}$P-dCTP using the random primer method [Weinberg and Vogelstein, 1983] (Amersham Random Primer Kit). Hybridization was performed at 45°C for 12 hr and the membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1 SSC, and 1% SDS. The membrane was exposed overnight at −70°C. Dot blot quantitation was obtained by measuring the radioactivity of each dot using a Packard Tri-Carb 4530 scintillation counter. The sensitivity of the dot blot process was measured by dotting equal amounts of control RNA onto a filter and quantitating the radioactive counts. The standard error is about 5%. Two randomly chosen areas of each filter were measured for background. Proportionality was observed between experimental and control dots at the various concentrations. All samples were examined for DNA contamination, and monitored for RNA breakdown, using agarose gel electrophoresis before hybridization studies.

### Northern Hybridization

For Northern hybridization, 15 μg of total RNA was used for each sample; 50 ng of CAT DNA was labeled as a probe using the random

primer method. Hybridization was at 45°C for 12 hr and membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1 SSC, and 1% SDS. Membrane was exposed overnight at −70°C. Probe DNA was isolated by agarose gel electroelution and subsequently purified (Geneclean, La Jolla, CA). DNA probes were labeled in vitro with [$^{32}$P]dCTP via the random primer method.

### Composition of EM Signals

All exposures used a continuous sinusoidal 60 Hz field generated by a pair of Helmholtz coils (Electro-Biology Inc., Parsippany, NJ) [Goodman et al., 1992c]. EM fields used were 8 or 80 μTelsa. The calculated corresponding induced electric fields were 50 and 500 μV/meter [Bassen et al., 1992]. Exposures were for up to 20 min in duration.

### Conditions of EM Field Exposure

HeLa or mouse myeloma (PX3) cells were changed to new media 3 hr before exposure in 100 × 20 mm culture dishes. PX3 cells from a single flask were divided into two flasks to be used as experimental and control cells ($1 \times 10^6$ cells/ml). Cells from three flasks of transfected HeLa cells were combined into two flasks for exposures. One flask was placed in the exposure apparatus while the remaining flask served as a simultaneous control. Details on the placement of cells, physical separation of experimental and control flasks, and composition and construction of the mu metal container shielding the exposure signal are described in Goodman et al. [1992c]. Control cells were placed in a mu metal box in the same incubator as experimental cells to minimize potential thermal differences that could arise by using separate incubators. The signal generator was placed outside the incubator. All experiments were carried out at 37°C. Temperature was monitored using a Physitemp thermocouple temperature probe (Physitemp Instruments Inc., Clifton, NJ) which is sensitive to 0.1°C.

## RESULTS

### Construction of Transfectants in Mouse Myeloma Cells; DNA Upstream of the *myc* Promoter Increases the Expression of CAT in Myeloma Cells Exposed to a 60 Hz EM Field

Stable transfections of mouse myeloma cells (PX3) used a CAT construct containing 1.8 kb of the murine promoter including exon 1 and flanking sequences to −1,141 relative to $P_1$ (Fig. 1). The relative quantity of the c-*myc* transcript was the same in both transfected and nontransfected cells. Parental and transfected mouse myeloma cells were exposed to a 60 Hz sinusoidal field to test the response relative to time of exposure for CAT expression (Fig. 2A–D).

The maximum effect on either c-*myc* or CAT transcript increase in murine myeloma cells was at an EM field of 80 μT and 20 min of exposure. CAT enzymatic activity was also increased in cells exposed to 60 Hz EM fields under the same conditions (Fig. 3A,B). The results clearly indicate that DNA upstream of the c-*myc* promoter is responsive to the EM field in stably transfected cells.

### Transient Assays Using HeLa Cells; DNA Upstream to the *myc* Gene Increases the Expression of CAT in Human Cells Exposed to a 60 Hz Sinusoidal EM Field

Transiently transfected HeLa cells were exposed to a 60 Hz sinusoidal field at both 8 and 80 μT. The maximum exposure conditions were at 8 μT for 20 min (Fig. 4). Three chimeric con-

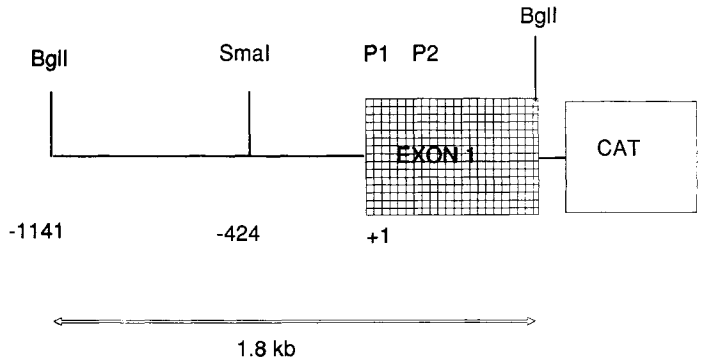


**Fig. 1.** Diagram of murine c-*myc* upstream regulatory region CAT construct.

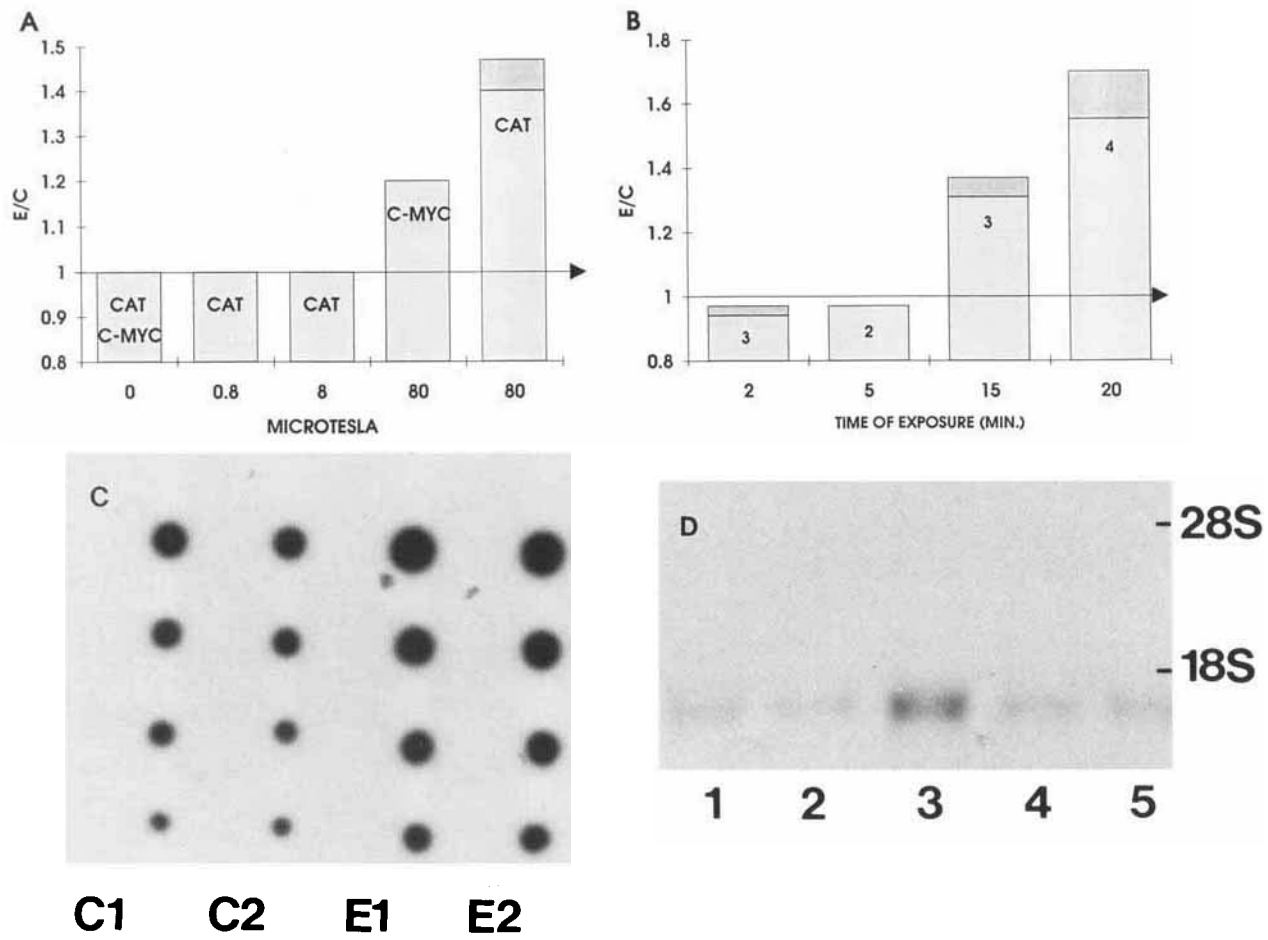


**Fig. 2.** Determining exposure conditions for maximum transcript levels in stably transfected mouse cells. The results in **A** and **B** are expressed as the ratio of experimental to control (E/C). The dark portion of each bar represents the standard error of the mean where three or more experiments were performed. A: The effect of increasing field strength on transcript levels for CAT and c-*myc*. The maximum effect was at 80 μT, 100 μV/m, and 20 min of exposure. Values were determined by dot blot hybridizations using total RNA against c-*myc* or CAT DNA. Control values for myc and CAT are the mean of three experiments; the 0.8 and 8 μT points are the mean of two experiments. The 80 μT points for myc are the mean of two experiments; for CAT, the mean of 10 experiments. B: The effect of increasing time of exposure on transcript levels for CAT and c-*myc* transcripts. The number of experiments represented is given by the number in the bar. **C:** Example of dot blot hybridization of total murine RNA against CAT DNA. CAT expression was measured between unexposed control cells and exposed cells (80 μT for 20 min). CAT DNA was labeled in vitro with $^{32}$P-dCTP using random primer. Hybridization conditions were as described in A. Control cells, C; exposed cells, E. **D:** Northern blot hybridization against CAT DNA. Northern blot hybridization for CAT gene expression was measured in cells exposed to each of three field strengths: 0.8 (lane 1), 8 (lane 2), and 80 μT (lane 3). Lanes 4 and 5 are RNA from unexposed cells. Hybridization was performed at 45°C for 12 hr and the membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1× SSC, and 1% SDS. The membrane was exposed overnight at −70°C.

structs containing portions of the c-*myc* promoter upstream of CAT were used in transient assays (Fig. 5). CAT expression for each construct is given in Table I. The HPV and CPV constructs caused increased expression in the presence of the EM field, but the levels of expression using the PPV construct were the same under control and exposure conditions (Fig. 6). Maximal activity was achieved with a chimera constructed from −2,329 (from $P_1$) to the second PvuII site in exon 1 (HPV) of the c-*myc* gene. Transfection of the constructs containing 1,257 bp of upstream DNA (CPV) showed about 70% of the expected activity; the construct containing the −353 bp (PPV) fragment gave about 50% of the maximum activity. Based on previous reports [Hay et al., 1987], it was expected that the value for the first 353 bp of upstream sequence would be about 10% of the maximum. We were unable to resolve this discrepancy, even

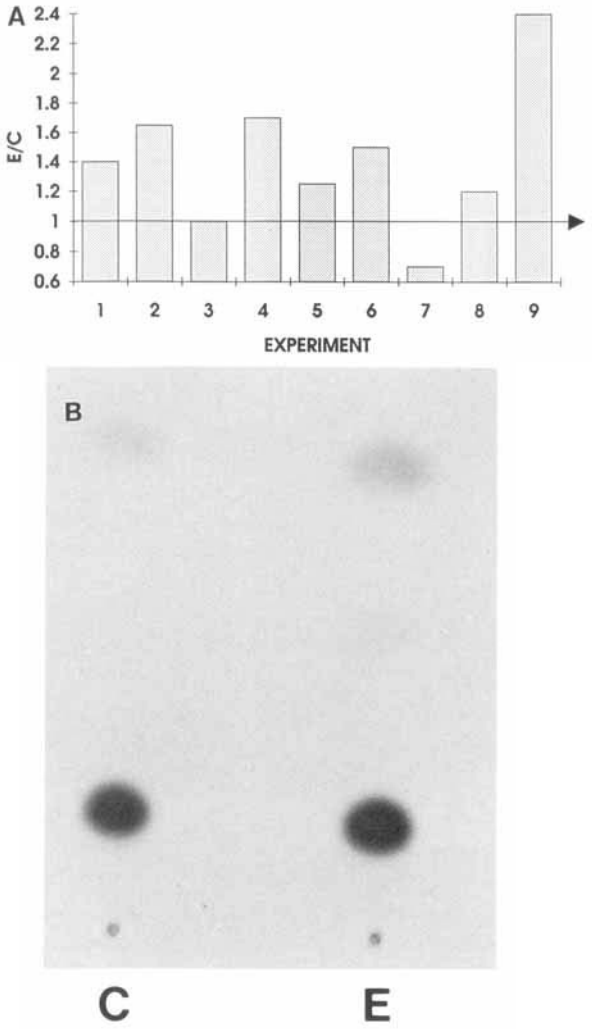


**Fig. 3.** Determination of CAT activity on murine transfected cells in presence and absence of an EM field. Mouse c-*myc* upstream DNA (1.8 kb) in a CAT construct was transfected into mouse myeloma (px3) cells. Acetylated $^{14}$C-chloramphenicol was separated by thin layer chromatography. **A:** The results of a series of experiments where a stable clone of transfected mouse cells was exposed to an 80 μT, 100 μV/m elf EM field for 20 min. The mean ratio of the exposed to control cells for the series was 1.44 ± 0.16 (SE mean). Experiments 1–6 used direct counting of gel; 7–9 used beta scanner for determinations of radioactivity. **B:** Example of results of thin layer chromatography.

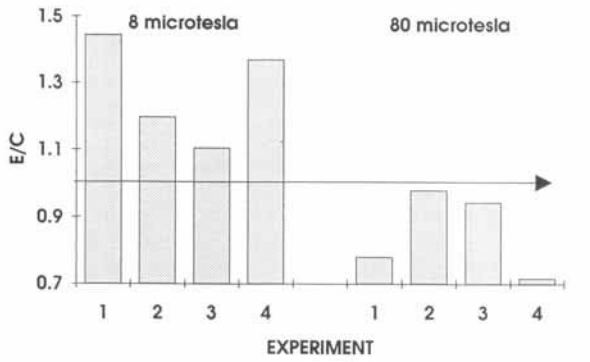


**Fig. 4.** Determination of conditions for EM field exposure in HeLa cells; the effect of increasing field strength on transcript level for CAT. Transfection of the entire 2.3 kb of DNA upstream to the c-*myc* gene was used in these experiments. Exposure of transfected HeLa cells to EM fields was at 8 and 80μT for 20 min. Values were determined by dot blot hybridizations. The mean of the four different experiments (using four separate transfections) for exposure at 8 μT was 1.2 ± 0.07 (SE mean); at 80 μT, it was 0.9 ± 0.06 (SE mean). A significant increase was seen with exposure at 8 μT and 100 μV/m at 20 min of exposure.

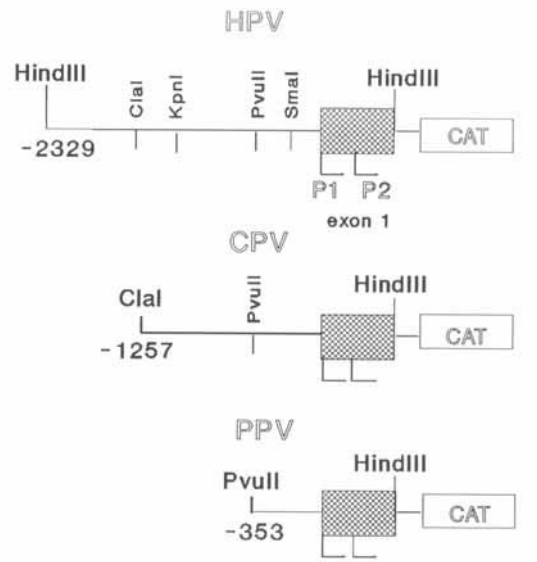


**Fig. 5.** Schematic diagram of the three constructs of human DNA upstream of the c-*myc* gene with CAT.

using a series of dilutions for the CAT assay, and assume that the differences in cell types used in the transfections may be the cause.

## DISCUSSION

A targeted interaction between EM fields and the cell membrane has been proposed as one route by which a cell could respond to EM fields [Adey et al., 1982; Blackman et al., 1989; Wallaczek and Liburdy, 1990]. It is assumed that a change in surface charge influences receptor binding activity either directly or indirectly through changes in the calcium flux patterns of the cell. Identification of both frequency and intensity windows support this idea [Goodman and Henderson, 1991; Blackman et al., 1989; Wei et al., 1990].

The nature of the subset(s) of genes that respond to EM fields has yet to be identified, but exposure of cells to low frequency EM fields does

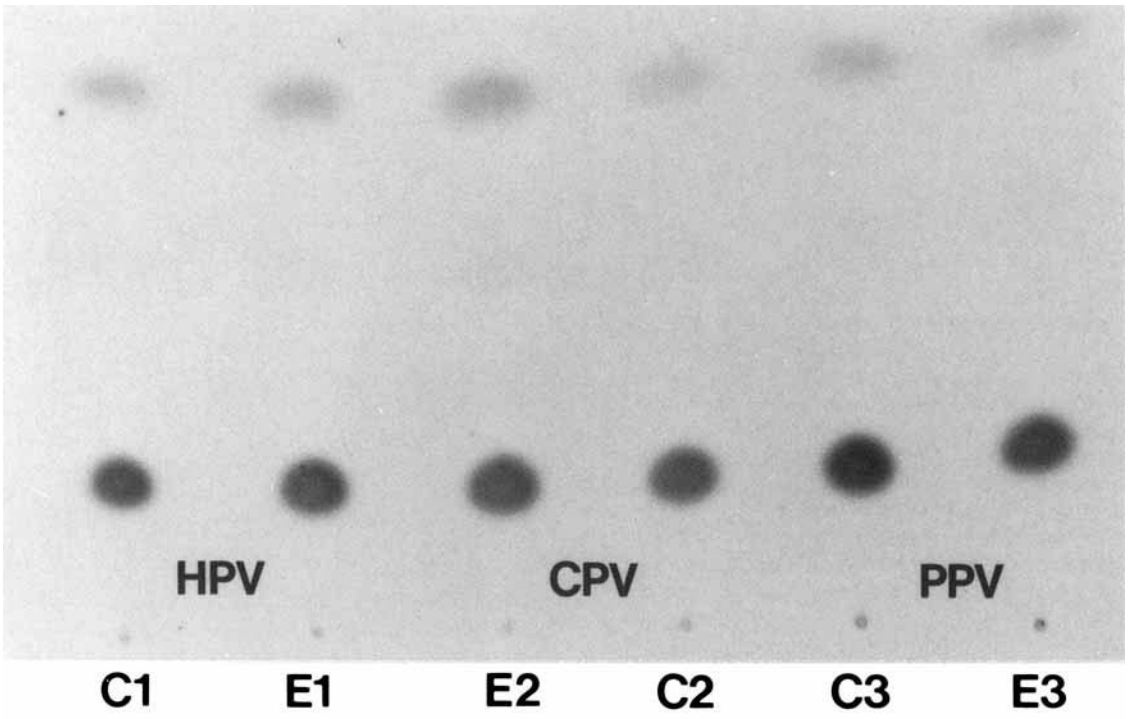


**Fig. 6.** Determination of CAT activity in transfected cells in the presence and absence of a 60 Hz field (8 μT). Three constructs were transfected into HeLa cells. The HPV and CPV constructs caused increased CAT gene expression in the presence of the EM field. Transfected cells using each construct were divided into two dishes for control and exposed conditions. The levels of expression using the PPV construct were the same under control and exposure conditions (see Fig. 5 and Table I). C, control; E, exposed.

**TABLE I. Regulation by Upstream DNA Sequences in c-*myc* Promoter in the Presence and Absence of an EM Field**

| | HPV | | CPV | | PPV | |
|---|---|---|---|---|---|---|
| | C | E | C | E | C | E |
| # Determinations[a] | 13 | 14 | 5 | 5 | 5 | 5 |
| Mean CAT activity | 0.31 | 0.59 | 0.22 | 0.38 | 0.16 | 0.16 |
| SE mean | 0.03 | 0.06 | 0.04 | 0.085 | 0.02 | 0.02 |
| Activity (relative to HPV) | 1 | 1.9 | 0.72 | 1.2 | 0.5 | 0.5 |
| Ratio of activity of exposed and control cells | | 1.9 | | 1.7 | | 1 |

[a]Each determination used a separate transfection (see *Materials and Methods*).
Upstream DNA regions are defined in Figure 6. Exposed cells were placed in a B field of 8 μT for 20 min. Relative CAT activity was determined by chromatographic assay. Radioactivity was determined by beta scanning.

not affect all expressed genes [Phillips et al., 1992; Blank et al., 1992]. An increase in the myc transcript has been measured by several investigators in cells exposed to various electric and/or EM fields. The types of cells affected include derivatives of a human T-lymphoblastoid line [Phillips et al., 1992], Con-A–stimulated rat thymocytes [Liburdy et al., 1992], Daudi cells [Czerska et al., 1991], HL-60 cells [Goodman and Henderson, 1991; Goodman et al., 1992c,d; Weisbrot, 1993b; Blank et al., 1992], SV-40 transformed human fibroblasts [Gold et al., in press], and, in the present study, both HeLa and mouse myeloma cells. These findings are important to determining a mechanistic pattern for the effects of EM fields on cells since regulation of the c-*myc* gene plays an important role in initiation and continuance of normal cell proliferation, as well as in the inception of cancer.

c-*myc* expression is regulated by many factors that include transcriptional initiation and elongation, stability of the mRNA [Levine et al.,

1986; Bentley and Groudine, 1986; Hay et al., 1987, 1989], and downregulation of c-*myc* by retention of pol II at the transcription start site [Strobl and Eick, 1992]. The human gene contains three exons that encode the major product of c-*myc* [Hann and Eisenman, 1984]. Transcription can begin at either one of three sites that are regulated by different promoters, designated $P_0$, $P_1$, and $P_2$ [Battey et al., 1983]. $P_0$ is a minor promoter, accounting for less than 10% of the total transcription from the c-*myc* gene [Bentley and Groudine, 1986]. The majority transcript is controlled by the $P_2$ promoter. The $P_1$ and $P_2$ promoters respond to many of the same positive and negative regulators located upstream of the gene [Hay et al., 1987]. Hay et al. [1987, 1989] identified a region between −293 and +513 (relative to $P_1$) that is sufficient for activity of $P_1$ and $P_2$. There are two additional regions that exert positive effects on $P_1$ and $P_2$ from −353 to −1,257 and −1,257 to −2,329. A negative regulator for both promoters is located at −293 to −353. It contains two or more regulatory regions with binding properties consistent with an AP-1 site and an overlapping octomer site [Hay et al., 1987, 1989]. The positive regulators upstream of the negative regulator region can, at least in part, negate the effect of the negative regulatory element (NRE).

The present results show that at least one effect of EM on cells involves regulation of transcription. The presence of DNA upstream to myc resulted in increased expression of CAT following exposure of cells to a 60 Hz sinusoidal field. An increase in expression was observed when the HPV (−2,359 to +513) and CPV (−1,257 to +513) constructs were present in HeLa cells. There was no difference, however, in the values obtained from exposed and control cells when only the PPV (−353 to +513) construct was present. One conclusion is that a critical sequence responsive to the 60 Hz field lies within the approximately 900 bp region difference between the CPV and PPV constructs. An alternate conclusion, however, is that the negative regulatory element within the PPV construct, in the absence of upstream positive regulators, can override the effect of EM fields.

The function of the myc protein as a transcription factor must involve a myriad of genes, considering the proposed roles for c-*myc* in the cell. An affect of EM fields on this type of regulatory gene is important in light of the diversity of effects in cells and organisms that have been ascribed to EM field exposure. Possible effects on regulatory genes could provide a plausible means of defining altered activity in cells resulting from EM field exposure, as well as a putative role for the involvement of signal transduction pathways. From a biological point of view, this mechanistic approach is reasonable since it is consistent with the observed transcriptional activation, and other biological effects attributed to EM field exposure. Proof of mechanism, however, will require demonstration of a feasible means of interaction at the cell membrane and determination of the pathway(s) from cell surface to the DNA in the nucleus.

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