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Myc-Mediated Transactivation of HSP70 Expression Following Exposure to Magnetic Fields

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Abstract We investigated c-*myc* protein-binding sites on the HSP70 promoter as modulators of the induction of HSP70 gene expression in response to magnetic field stimulation (8μT at 60Hz) and whether the presence of c-*myc* protein potentiates transactivation of HSP70 expression. A 320 base pair region in the HSP70 promoter (+1 to −320) was analyzed. This region contains two c-*myc*-protein binding sites with consensus sequences located at −230 and −160 nucleotide positions (relative to the transcription initiation site) and overlapping with the region reported for the regulation of HSP70 gene expression by c-*myc* protein. This promoter region is upstream of other regulatory sequences, including the heat shock element (HSE), AP-2, and serum response element (SRE). Transfectants containing both c-*myc* protein-binding sites, HSP-MYC A and HSP-MYC B, and exposed to magnetic fields showed a 3.0-fold increase in expression of CAT activity as compared with sham-exposed control transfectants. Transfectants containing one c-*myc* binding site, HSP-MYC A, and exposed to magnetic fields showed a 2.3-fold increase in CAT expression. Transfectants in which both HSP-MYC A and HSP-MYC B binding sites were deleted showed no magnetic field sensitivity; values were virtually identical with sham-exposed controls. If the c-*myc* expression vector was *not* co-transfected with the constructs containing *myc*-binding sites, there was no difference in the expression of CAT activity between magnetically stimulated and sham-exposed controls, although both responded to heat shock. These data suggest that endogenous elevated levels of *myc* protein contribute to the induction of HSP70 in response to magnetic field stimulation. J. Cell. Biochem. 69:181–188, 1998.

Key words: magnetic fields; HSP70 gene expression; human HSP70 promoter; c-*myc* protein binding sites; cellular stress

Environmental hazards such as changes in temperature, hypoxia, heavy metals, and other foreign chemicals, exert complex and damaging metabolic effects. The expression of stress genes is frequently used as an assay to monitor the impact of environmental toxicity on animal and plant life [Dilorio et al., 1996]. Whether magnetic field exposure is a hazard remains a contentious public health issue. However, it is possible to determine if magnetic fields are interpreted by cells as a physiological stress. If magnetic field stimulation is a cellular stress, then activation of the stress response could be expected; i.e., increased levels of stress proteins would be observed in cells and tissues exposed to magnetic fields. We found that cells respond to magnetic field stimulation by activation of heat shock factor (HSF) and increased heat shock element (HSE)-binding [Lin et al., 1997], by the induction of elevated transcript levels for the gene HSP70 [Goodman et al., 1994] and by increased hsp70 protein synthesis [Goodman and Henderson, 1988; Blank et al., 1994]. Moreover, stimulation with magnetic fields confers a temporary stress resistance on cells that is strongly reminiscent of the phenomenon of acquired thermotolerance in the case of thermal stress [Gerner and Schneider, 1975; Li and Werb, 1982; Landry and Chretian, 1983].

A substantial body of evidence shows significant biological changes in cells stimulated by low-energy, low-frequency magnetic fields [reviewed in E.M. Goodman et al., 1995; Hong, 1995]. In

Abbreviations: CAT, chloramphenicol acetyltransferase; HSE, heat shock element; HSF, heat shock factor; HSP70, heat shock 70 gene; hsp70kDa, heat shock 70 protein.

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addition to the induction of HSP70 gene expression, magnetic field exposure increased the steady-state transcript levels of several immediate early response genes, including increased levels of c-*myc* mRNA in HL60 cells [Lin et al., 1996; Jin et al., 1997]; a magnetic field responsive region was identified upstream of the c-*myc* gene [Lin et al., 1994].

Our rationale for further investigation of a link between magnetic field exposure and c-*myc* protein was based in part on reports of a trans-regulatory relationship between c-*myc* and hsp70: nuclear colocalization of *myc* proteins with HSP70 in *myc*-overexpressing cells [Koskinen et al., 1991]; c-*myc* protein activation of the human HSP 70 promoter [Kingston et al., 1984], and the binding of c-*myc* protein complex to two sites in the HSP70 promoter region [Taira et al., 1992]. Increased c-*myc* transcript levels in HL60 cells exposed to magnetic fields have been reported [Lin et al., 1996; Jin et al., 1997]. In this study, we focused on the *myc* protein-binding sites on the HSP70 promoter as requisites for magnetic field responsiveness.

Our initial experiments reconfirmed that transcript levels of HSP70 were elevated in response to magnetic field stimulation and that levels of the 70kDa heat shock protein were increased as previously reported [Goodman et al., 1994; Blank et al., 1994]. We next analyzed a 320bp region upstream of the human HSP70 gene that contains two c-*myc* protein binding sites homologous to the c-*myc* protein-complex-binding sequence in the c-*myc* gene, C**CTCT**CA and C**CTCT**GA, located at nucleotide positions −230 and −160, respectively [Taira et al., 1992]. These two regions overlap with the region reported for the regulation of inducible HSP70 gene expression by c-*myc* protein in the human HSP70 promoter [Kingston et al., 1984] and are upstream of other regulatory sequences, including two heat shock elements (HSEs), as well as AP-2 and a serum response element (SRE).

Constructs containing portions of DNA upstream of human HSP70 gene fused to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) were co-transfected with *myc* protein expression vector into HeLa cells as transient transfectants. Transfectants exposed to magnetic fields had an average 3.0-fold increase in expression of CAT activity in constructs containing both c-*myc* protein-binding sites (HSP-MYC A and HSP-MYC B), as compared with sham-exposed control transfectants. Deletion of 132 base pairs containing MYC A (as well as binding sites for the transcription factors SP-1, AP-2, and HSE) reduced CAT activity in magnetic field-exposed transfectants to 2.3-fold. Deletion of 77 additional bps, which included both HSP-MYC A and HSP-MYC B binding sites (and HSE, SP-1, and AP-2 binding sites), resulted in no magnetic field induction of CAT activity. In the absence of c-*myc* co-transfection, none of the HSP70-CAT constructs responded to magnetic fields. Transfectants with each of the three constructs expressed increased CAT activity when they were heat shocked (with or without c-*myc* protein). Magnetic field induction of HSP70 gene expression appears to be mediated through the binding of c-*myc* protein to *myc* protein-binding sites on the HSP70 promoter.

MATERIALS AND METHODS

HL60 Cells

HL60 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (Gibco/BRL, Gaithersburg, MD) and 100 U/ml penicillin and streptomycin (Gibco/BRL) at 37°C (NuAire incubator). Cells were prepared for each experiment 16 hours prior to an experiment by aliquoting cells from a single T75 flask into individual T25 flasks, ensuring that control and experimental samples derived from the same original batch of cells and that cell concentrations were identical. The following morning one flask was used for determining cell concentration and cell viability while other flasks were designated experimental, sham-exposed, or sham/sham. Cells for experimental and sham-exposed samples, in T25 flasks, were exposed to magnetic fields at cell densities of 1 x 10^6 cells/ml in 15ml/flask. All cells were maintained in mu metal containers for 2 days before experiments.

Electromagnetic Field Exposure System

Magnetic fields were generated by a pair of double-wound Helmholtz coils (164 turns of 19-gauge copper wire around a 13 x 14cm Plexiglas form (Electric Research Management, Pittsburgh, PA). The Helmholtz coils were shielded in mu metal containers (Ammuneal Manufact. Corp., Philadelphia, PA) within the incubator. The sinusoidal field was generated by a Wavetek function generator (Wavetek model 21, 11MHz) connected to a power regulator. The function

generator and power regulator were situated outside the incubator. Signal parameters were monitored by a calibrated inductive search coil (Electro-Biology, Inc., Parsippany, NJ) with a Hitachi (Danbury, CT; V-1065, 100MHz) oscillscope. There are two identical sets of coils, and each can be set for either active (exposure) or zero field (sham-exposed mode). Controls used the sham-exposed mode where the direction of current flow creates a zero field.

To ensure that no heating resulted from the coils, temperatures were monitored with a thermocouple probe (PhysiTemp, Inc., Hackensack, NJ) attached to the coils throughout all exposures (sensitivity $\pm$ 0.1°C).

Mu Metal Shielding

Both active (experimental) and sham-exposed coils used as controls are enclosed in a 30cm-high, 15cm-diameter cylindrical mu metal container (.040″ thickness). The 60Hz shielding factor is (min) 90.1 (39.08dB). Sham-exposed controls and experimental exposures are performed simultaneously in identical mu metal containers.

Exposure Conditions for RNA and Protein Determinations

Previous studies established the optimal magnetic field exposure conditions for inducing HSP70 transcripts and hsp70 protein [Goodman et al., 1994; Lin et al., 1996; Jin et al., 1997]; i.e., 8μT field strength at 60Hz. To determine magnetic field stimulation on HSP70 transcript levels, HL60 cells were exposed for 20 minutes and immediately prepared for RNA isolation as previously described [Lin et al., 1996; Jin et al., 1997]. To determine protein levels, cells were exposed to magnetic fields for 20 minutes followed by an additional 20-minute recovery period before preparation of protein lysates as previously described [Lin et al., 1997]. Control cells were sham-exposed at the same time in the same incubator and shielded in an identical mu metal container. Samples were not blinded.

Heat Shock Conditions

Cells in T25 flasks, wrapped in Parafilm and placed in a mu metal container, were immersed in a water bath at 43°C. For RNA analysis, cells were heat shocked for 20 minutes; for protein analysis, cells were heat shocked for 20 minutes followed by an additional 20-minute period at 37°C. Temperature was monitored with the PhysiTemp thermocouple probe.

RNA Isolation

SDS/phenol extraction procedure was performed as described [Lin et al., 1996; Jin et al., 1997].

Northern Blot Hybridization

Total RNA was analyzed with Northern blot hybridization on Hybond N (Amersham, Arlington Heights, IL). The HSP70 DNA probe was generously provided by Dr. Jing Wu, Department of Medicine, New York University. β-2microglobulin was used as a reference control since it has been shown to be unresponsive to magnetic field stimulation [Jin et al., 1997]. DNA probes were labeled in vitro (Random Primer, BMB) with α-^{32}P-dCTP (DuPont/New England Nuclear, Boston, MA; 6,000 Ci/mM) to a specific activity of a minimum of 5–8 x 10^8 dpm/μg. Ten micrograms of RNA from total sample was denatured by formamide and formaldehyde at 65°C (15 minutes). RNA was blotted on to Hybond N membrane, baked 15 minutes at 80°C and UV (312nm) cross-linked 5mins. Prehybridization in Hybrisol I (catalogue no. S4041, Oncor, Gaithersburg, MD) was for 2 hours. Approximately 40ng of the ^{32}P-labeled probe was added (this was dependent on the specific activity of the probe); hybridization was overnight at 45°C with Hybrison TM 1 (Oncor). The membrane was washed at 65°C for 30mins followed by two 30min washes with 2X SSC (1% SDS); final washes with 0.1X SSC and 0.1% SDS (30 minutes at 65°C). The membrane was exposed overnight at −70°C to DuPont Reflection NEF496 autoradiographic film.

The intensity of the radiolabeled transcripts was quantified on a PhosphorImager 400A (Molecular Dynamics, Sunnyvale, CA).

Western Blot

Lysates from whole cells were prepared as described previously using a modification of Mosser et al., 1988 [Lin et al., 1997] and analyzed using the ECL detection system (Amersham). Anti-hsp70 antibody was from StresGen Inc. (Vancouver, Canada; Catalogue no. SPA 820; Lot 702409).

HeLa Cells and Transfections

HeLa cells were maintained in DMEM (Gibco/BRL) with 10% fetal calf serum (Gibco/BRL) and 100 U/ml penicillin and streptomycin (Gibco/BRL). Transient transfections of HeLa cells used the lipofectin method as described (Gibco/BRL, Catalogue no. 18292-011). HeLa cells were used for all transfections because HL60 cells have been shown by us and others (e.g., Gibco) to be impossible to transfect under any conditions.

DELETION CONSTRUCTS

Deletion constructs were generously provided by Dr. R. Kingston, Department of Genetics, Harvard University. A diagrammatic representation of these constructs is presented in Figure 1 and they are:

1. pΔH-17. This construct extended from +1 to −320 in the HSP70 promoter and contained both HSP-MYC A and HSP-MYC B binding sites at nucleotide positions −230 and −160.
2. pΔH-18. This construct, containing 188bp, retained HSP-MYC B (at −160). The deletion of 132bp included the deletion of HSP-MYC A from pΔH-17.
3. pΔH-11. This construct contained 111bp. The deletion of 209bp included the deletion of HSP-MYC B from pΔH-18. There were no *myc* protein-binding sites in this construct.

C-*myc* Protein Expression Vector

The expression vector used here [described in Wei et al., 1993] was generously provided by Dr. R. Dalla-Favera, Columbia University.

EXPOSURE OF TRANSFECTANTS

Transfectants from three flasks were combined into two flasks. One flask was exposed to an 8μT 60Hz magnetic field (at 37°C) for 20 minutes followed by a 20-minute recovery period at which point the samples were processed for total protein [Lin et al., 1997]. The cells in the second flask served as a simultaneous sham-exposed control. Transfectants were also heat shocked at 43°C for 20 minutes followed by a 20-minute recovery (at 37°C) and lysates prepared for CAT assay.

CAT Assay

CAT assays [Zhang et al., 1993] were performed by chromatography. After chromatography, the spots were quantified on a PhosphorImager 400A. CAT activity was calculated as the percentage of chloramphenicol converted to the acetylated form.

RESULTS

HSP70 RNA Accumulation in HL60 Cells Stimulated by Magnetic Fields

Total RNA was prepared from HL60 cells exposed to an 8μT 60Hz sinusoidal magnetic field for 20 minutes and from sham-exposed control cells. These results reconfirmed previous data showing increased HSP70 transcript levels [Goodman et al., 1994]. The average increase (from four experiments) was 1.68 ± .17 (experimental/control)(Fig. 2). No increase in HSP70 transcripts was measurable in the sham-exposed control samples.

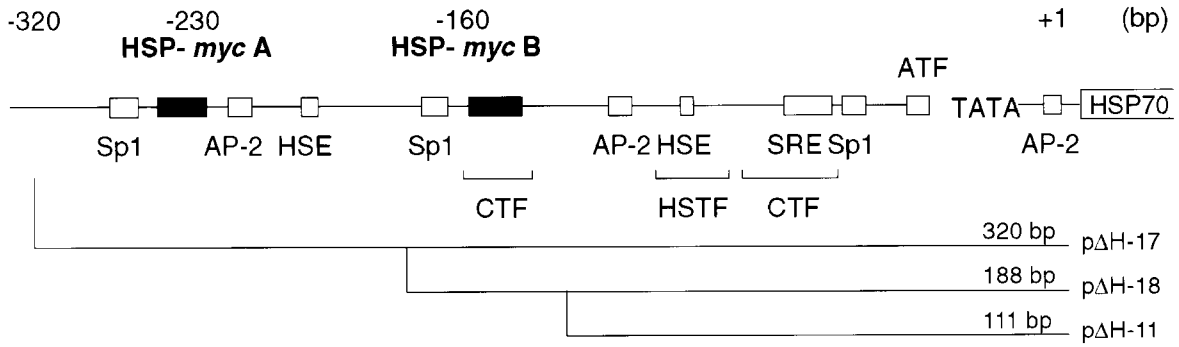

Fig. 1. Schematic diagram of three constructs for human HSP70 promoter region +1 to −320, showing number of base pairs in each construct. HSP-MYC A and HSP-MYC B *myc* protein-binding regions are shown as solid boxes. Open boxes are other transcription factor binding sites.

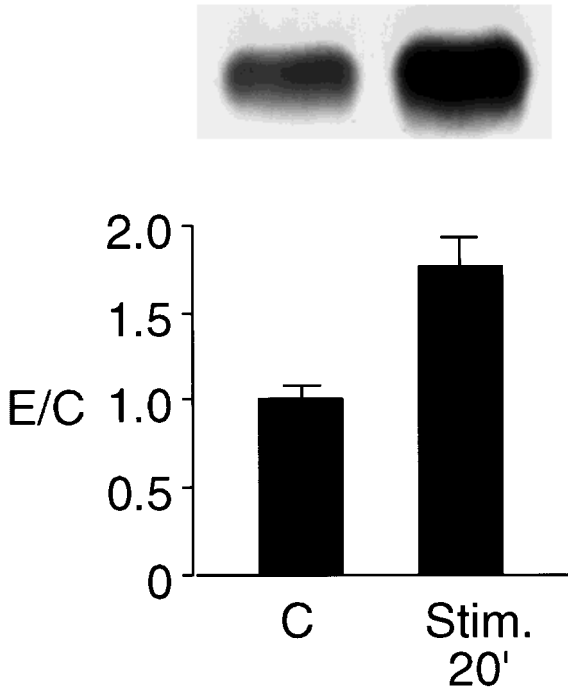

Fig. 2. Transcriptional activation of HSP70 in HL60 cells stimulated by magnetic fields. Total RNA was prepared from HL60 cells exposed to an 8μT 60Hz sinusoidal magnetic field for 20 minutes and from sham-exposed control cells. Transcript levels for HSP70 were analyzed by Northern blot hybridization. β-2microglobulin was used as an internal control. An average increase of 1.68 ± .17 (from four separate experiments) was measured in the steady-state transcript levels for HSP70. No increase in HSP70 transcripts was measurable in the sham-exposed control samples.

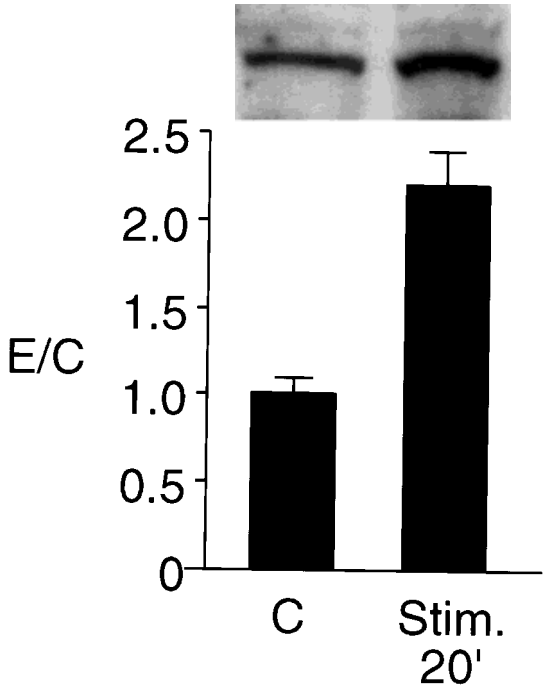

Fig. 3. Translational activation of hsp70 protein in HL60 cells stimulated by magnetic fields. Total protein was isolated from HL60 cells exposed to an 8μT 60Hz sinusoidal mnagnetic field for 20 minutes. After each exposure cells were incubated for 20 additional minutes before protein extraction. Levels for hsp70 following 20 minutes of exposure (plus 20 minutes recovery) averaged 2.19 ± .20. No increase in hsp70 was measurable in the sham-exposed control samples.

Accumulation of hsp70 Protein in HL60 Cells Stimulated by Magnetic Fields

Total protein was isolated from HL60 cells exposed to an 8μT 60Hz sinusoidal magnetic field for 20 minutes. After each exposure cells were incubated for 20 additional min before protein extraction. The average increase was 2.19 ± .20 (experimental/control) from four experiments (Fig. 3).

Human HSP70 Promoter Contains Two *myc* Protein-Binding Sites That Regulate HSP70 Expression in Response to Magnetic Field Stimulation

Constructs of DNA upstream of human HSP70 gene fused to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) were co-transfected with *myc* protein expression vector into HeLa cells as transient transfectants (diagram of constructs in Fig. 1). HeLa cells were used for transfections since HL60 cells proved impossible to transfect.

pΔH-17. This construct extends 320 base pairs upstream from the transcription initiation site of HSP70 gene. When this construct was co-transfected with c-*myc* protein into HeLa cells and exposed to magnetic fields (8μT 60Hz for 20 minutes), a 3.0-fold average increase in the expression of CAT activity was measured as compared with sham control transfectants (Fig. 4). In addition to the two *myc* protein binding sites at −230 and −160, there are other binding sites, e.g., HSE and AP-2. However, their importance is secondary since transfectants lacking c-*myc* protein expression vector were unresponsive to magnetic fields.

pΔH-18. This construct, extending 188 base pairs upstream from the transcription initiation site of HSP70 gene, is 132bp less than pΔH-17; c-*myc* protein-binding site HSP-MYC A (as well as SP-1, AP-2, and HSE) has been deleted. When this construct was co-transfected with c-*myc* protein into HeLa cells and exposed to magnetic fields (8μT 60Hz for 20

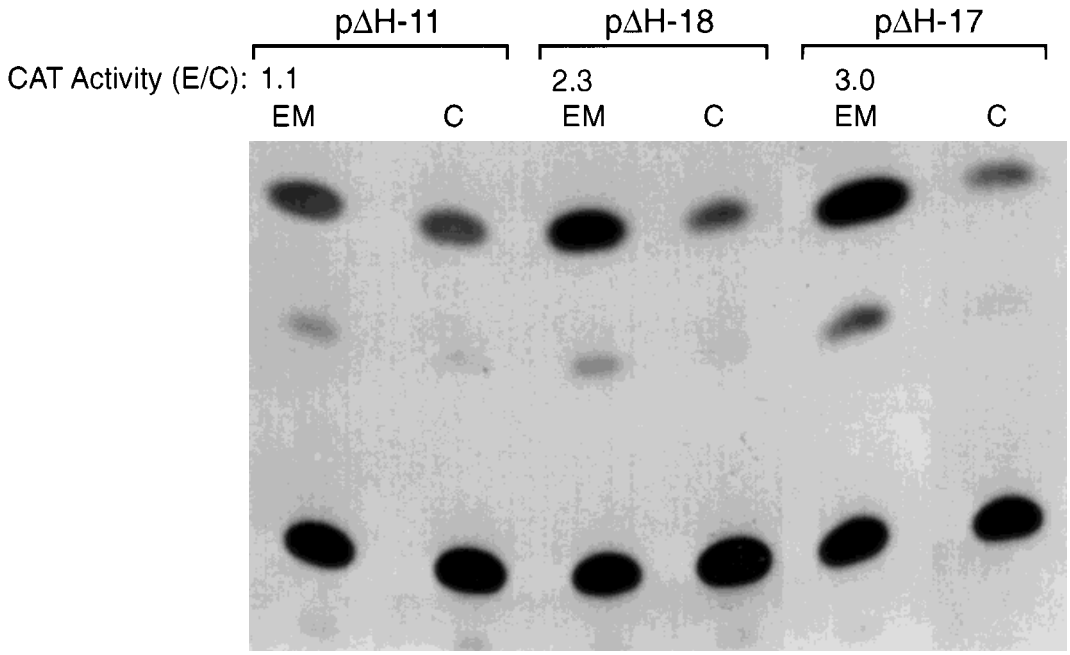

Fig. 4. CAT activity with three deletion constructs. CAT activity expressed as the ratio of experimental to control (E/C). Constructs of DNA upstream of human HSP70 gene fused to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) were co-transfected with *myc* protein expression vector into HeLa cells as transient transfectants. Transfectants were exposed to an 8μT 60Hz magnetic field for 20 minutes, then allowed to recover for 20 minutes before preparation of total protein for analysis of CAT activity. **pΔH-17:** 320bp upstream from the transcription initiation site of HSP70 gene; contains *both* HSP-MYC A and HSP-MYC B binding sites. Co-transfection of c-*myc* protein induced a 3.0-fold increase in the expression of CAT activity when transfectants were exposed to an 8μT 60Hz magnetic field as compared with sham control transfectants. **pΔH-18:** 188bp upstream from the transcription initiation site of HSP70 gene; contains HSP-MYC B binding site. Binding site HSP-MYC A has been deleted. Co-transfection with c-*myc* protein expression vector induced a 2.3-fold increase in the expression of CAT activity when transfectants were stimulated with magnetic fields as compared with sham control samples. **pΔH-11:** 111bp upstream from transcription initiation site of HSP70 promoter. Both HSP-MYC A and HSP-MYC B were deleted. When this construct was co-transfected with c-*myc* protein, CAT activity was 1.1 under both magnetic field-exposed and sham-exposed control conditions.

minutes), a 2.3-fold average increase in the expression of CAT activity was measured as compared with sham control transfectants (Fig. 4).

pΔH-11. This construct (111bp of HSP70 promoter), 77bp less than pΔH-18,was co-transfected with c-*myc* protein expression vector. In this construct both HSP-MYC A and HSP-MYC B were deleted. CAT activity was negligible under both exposed and sham control conditions (Fig. 4). Transfectants lacking c-*myc* protein expression vector were also unresponsive to magnetic fields.

When each of these three constructs was heat shocked (43°C for 20 minutes plus 20 minutes of recovery at 37°C) there was a 4.25-fold average increase in CAT expression (with or without c-*myc* protein expression vector; 4 experiments).

DISCUSSION

Cells respond to magnetic field stimulation in a variety of ways, including induction of stress proteins, e.g., hsp70, and several immediate early genes, including c-*myc* [Lin et al., 1996; Jin et al., 1997]. However, the sensing and signal transduction pathways responsible are only now beginning to be understood. The induction of c-*myc* and the presence of c-*myc* binding sites in the HSP70 promoter prompted us to investigate whether magnetic field-stimulated induction of HSP70 is mediated by c-*myc*.

In this study we show that *myc* protein and two *myc* protein-binding sites on the HSP70 promoter are magnetic field-responsive, and our results suggest that the binding of c-*myc* protein on the HSP70 promoter is integral in the regulation of magnetic field-induced HSP70 gene expression. Although it has not been deter-

mined whether the HSP-MYC sequences in the HSP70 promoter region function as c-*myc* protein-dependent transcriptional regulatory elements, their responsiveness to magnetic field exposure is suggestive.

The conditions for magnetic field induction of HSP70 gene expression differ from those described for the induction of heat shock [Morimoto, 1993], which requires a consensus sequence composed of pentanucleotide modules, nGAAn, arranged as contiguous inverted repeats, that bind heat shock factor (HSF). Perhaps, as suggested for light activation in Chlamydomonas [Kropat et al., 1995], regulation of HSP70 expression by magnetic fields is mediated by two types of DNA-binding factors: one confers activation (possibly recognizing the consensus sequence CTCT for positive regulation); the other confers de-activation. Taira et al. [1992] showed that CTCT, protected in methylation interference experiments, is required for sequence-specific protein binding to both HSP-MYC A and HSP-MYC B and that c-*myc* protein binds to these HSP-MYC A and HSP-MYC B sequences in a sequence-specific manner.

That magnetic field induction of HSP70 occurs by a different pathway than that used by heat shock is not surprising, since the magnetic stimulus is a small perturbation and evokes responses at an energy density 14 orders of magnitude lower than heat shock and may affect different cellular components. Table I lists the lowest input for magnetic and thermal stimuli that evoke a stress response in controlled experiments with Diptera [Blank et al., 1994].

It is now accepted that many other environmental stressors induce the HSP70 gene in addition to heat shock, cadmium, and serum; e.g., pesticides, anoxia, hypoxia. The cis regulatory region in the HSP70 upstream region is extremely compact [reviewed in Lis and Wu, 1993]. Wu et al. [1986] have suggested that each stressor may activate distinct transcription factors that recognize separate promoter elements. This could be the case with magnetic field-induction of the stress response where at least one pathway used in magnetic field stimulation appears to require *myc* protein binding to CTCT consensus sequences on the HSP70 promoter. Alternatively, the various stressors may sort into classes and activate the HSP70 promoter through a limited set of pathways. These data lead to the conclusion that the induction of HSP70 heat shock gene is mediated by c-*myc* protein in conjunction with magnetic field stimulation. It remains to be determined whether magnetic field induction of HSP70 gene expression occurs through the heat stress-sensing pathway or acts through an independent signal transduction chain.

TABLE 1. Lowest Input for Magnetic and Thermal Stimuli That Evoke a Stress Response

Energy	Input	Energy density (joules/m^3)
Magnetic	0.8 μT	2.6×10^{-7}
Thermal	+5.5°C	$2.3 \times 10^{+7}$

While our data do not provide a direct link between magnetic field exposure and clinical pathology, they clearly show important and fundamental physiological changes at the cellular level. Further, the presence of stress proteins has been used as a diagnostic and/or prognostic clinical indicator in breast cancer [Thor et al., 1991; Tandon et al., 1990; Ciocca et al., 1993] and has been shown to contribute to cellular resistance to cytotoxic drugs [Ciocca et al., 1992; Osterreich et al., 1993].

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