Journal of Cellular Biochemistry 75:170–176 (1999)

A Magnetic Field-Responsive Domain in the Human HSP70 Promoter

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Abstract HSP70 gene expression is induced by a wide range of environmental stimuli, including 60-Hz electromagnetic fields. In an earlier report we showed that the induction of HSP70 gene expression by magnetic fields is effected at the level of transcription and is mediated through c-*myc* protein binding at two nCTCTn sequences at −230 and −160. in the human HSP70 promoter. We report on the identification of a third c-*myc* binding site (between −158 and −162) that is an important regulator of magnetic field-induced HSP70 expression. We also show that the heat shock element (HSE), lying between −180 and −203, is required for induction of HSP70 gene expression by magnetic fields. The HSE centered at −100 alone is insufficient. J. Cell. Biochem. 77:170–176, 1999. © 1999 Wiley-Liss, Inc.

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

Genes encoding heat shock proteins are rapidly induced in response to perturbation of the physiological state of the cell. This *de novo* induction of stress genes leads to elevated synthesis of stress response proteins that protect cells from the initial stress, subsequent stress, and even lethal stress. Some of these proteins also act as molecular chaperones; they enable cellular proteins to achieve correct functional conformation and to reach their final destination in the cell.

Low-frequency low-energy 60-Hz magnetic fields induce the expression of both HSP70 and c-*myc* genes [Goodman et al., 1994: Jin et al., 1997]. Further, a 900-base pair (bp) region of the c-*myc* promoter, containing eight nCTCTn sequences, was shown to be magnetic field-sensitive [Lin et al., 1994]. The transregulatory relationship between c-*myc* and hsp70, which has been described in great detail [Koskinen et al., 1991; Kingston et al., 1984; Kaddurah-Daouk et al., 1987; Taira et al., 1992], prompted us to examine the interaction of magnetic fields with the c-*myc* protein complex-binding sequences in the human HSP70 promoter (HSP-MYC-A and HSP-MYC-B) containing nCTCTn nucleotide sequences [Taira et al., 1992]. We asked whether c-*myc* protein and its binding sequences at HSP-MYC-A and HSP-MYC-B [Taira et al., 1992] are required for magnetic field-induced HSP70 gene expression. Transfections with deletion constructs of the HSP70 promoter together with site-directed mutagenesis showed that transactivation of HSP70 expression requires co-transfection of c-*myc*-protein with constructs containing nCTCTn c-*myc* binding sequences for magnetic field responsiveness [Lin et al., 1998a].

Examples of the similarities between the induction of the human stress gene HSP70 by heat shock and magnetic fields include:

1. Heat shock factor 1 (HSF1) trimerization and HSF1 binding to a heat shock element (HSE) [Lin et al., 1997].
2. Induction of elevated HSP70 transcript levels [Goodman et al., 1994].
3. Induction of increased hsp70 protein synthesis [Goodman and Henderson, 1988; Blank et al., 1994].
4. Induction of a sustained state of stress resistance in exposed cells [Goodman and Blank, 1998; Han et al., 1998), similar to acquired

Abbreviations used: CAT, chloramphenicol acetyltransferase; HSF, heat shock factor; HSE, heat shock element; HSP70, heat shock 70 gene; hsp70, heat shock 70 protein.

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Received 16 December 1999; Accepted 12 April 1999

thermotolerance [Gerner and Schneider, 1975).

There are, however, distinct differences between magnetic field-induced and heat shock-induced HSP70 gene expression:

1. The stress response is induced by magnetic fields at 14 orders of magnitude lower energy density than heat shock [Goodman and Blank, 1998].
2. Magnetic field stimulation does *not* inhibit normal basal cellular protein synthesis [Goodman and Henderson, 1988; Blank et al., 1994].
3. Magnetic fields induce increased AP-1 binding activity [Lin et al., 1998b]; heat shock does not induce AP-1 activation.
4. c-*myc* protein is required for mediation of HSP70 transactivation by magnetic fields [Lin et al., 1998a]; the response to heat shock does not have this requirement.

Using site-directed mutagenesis and a transient transfection assay, we have identified a third c-*myc* binding sequence required for magnetic field responsiveness, HSP-MYC-A and HSP-MYC-B. This nCTCTn c-*myc* protein-binding sequence, HSP-MYC-C, lying between −158 and −162, serves as a regulator of the magnetic field-induced stress response. Site-directed mutagenesis also showed that the heat shock element (HSE) lying between −180 and −203 is required for induction of HSP70 gene expression by magnetic fields; the HSE at −100 alone is insufficient.

MATERIALS AND METHODS

HSP70 Promoter Deletion Construct pΔH-17

The HSP70 promoter deletion construct pΔH-17 [Kingston et al., 1984](kindly provided by Dr. R. Kingston, Department of Genetics, Harvard University) contains a portion of the HSP70 promoter between +1 and −320 (shown diagrammatically in Fig. 1).

DNA Sequence of Plasmid pΔH-17

The deletion construct pΔH-17 was sequenced by the DNA Facility of the Cancer Center, Columbia University.

Site-Directed Mutagenesis

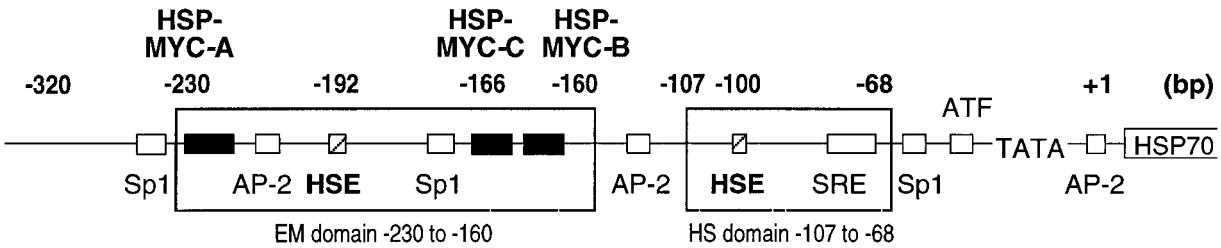
Site-directed mutagenesis in HSP70 promoter deletion construct pΔH-17 was accomplished with Quikchange (Stratagene; cat. no. 200518–5) as follows:

MYC-C. −158 to −162: ACTCTC was mutated to AGTAGTG.

HSE. −180 to −182. GAA was mutated to GAG; −195 to −197. TTC was mutated to CGG; −200 to −203. TTC was mutated to TCT.

Transfections

HeLa cells were used for transient transfections. These cells are routinely maintained in



HSE: -180 to -182. GAA mutated to GAG
-195 to -197. TTC mutated to CGG
-200 to -203. TTC mutated to TCT

MycC: -158 to -162. ACTCTC mutated to AGTAGTG

MycB: -150 to -154. CCTCTAT mutated to CCACTAT

Fig. 1. Schematic diagram of the pΔH-17 construct of human HSP70 promoter lying between +1 and −320. The magnetic field-responsive domain lies between −160 to −230. The heat shock-responsive domain lies between −68 and −107. **HSE;** −180 to −182: GAA mutated to GAG; −195 to −197: TTC mutated to CGG; −200 to −203: TTC mutated to TCT. **MYC-C;** −158 to −162: ACTCTC mutated to AGTAGTG.

Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) with 10% fetal calf serum (FCS) (Gibco-BRL) and 1% penicillin and streptomycin (Gibco-/BRL). The lipofectin method (Gibco-BRL, cat no. 18292–011) was used for transfection as described [Lin et al., 1997, 1998a]. Plasmids containing wild-type and mutated constructs of DNA upstream of human HSP70 gene, fused to the bacterial gene encoding chloramphenical acetyltransferase (CAT), were co-transfected with a *myc* protein expression vector [Wei et al., 1993](kindly provided by Dr. R. Dalla-Favera, Columbia University) as previously described [Lin et al., 1997, 1998a].

Magnetic Field Exposures of Transfectants

For each construct used (mutant or wild type), transfectants were combined in one container and then split into three flasks 48 h later. Transfectants in the first flask (EM) were exposed to an 8-μT 60-Hz sinusoidal electromagnetic field (37°C) for 30 min, followed by a 30-min recovery period; transfectants in the second flask were sham-exposed (S) controls for the same time period as the experimentals; and transfectants in the third flask were heat shocked at 43°C (HS) for 30 min, followed by a 30-min recovery at 37°C. HS samples served as positive controls. Protein for CAT assay was extracted as previously described [Lin et al., 1997, 1998a,b] and protein concentrations determined with a BioRad protein assay kit (BioRad Laboratories).

CAT Assay

CAT assays [Zhang et al., 1993; Lin et al., 1997, 1998a] were performed by chromatography and the spots quantified on a PhosphorImager 400A and ImageQuant software (Molecular Dynamics). CAT activity was calculated as the percentage of chloramphenicol converted to the acetylated form. Results were quantified using a PhosphorImager and ImageQuant software.

Electrophoretic Mobility Shift and Competition Assays

Bandshift assays were carried out as previously described [Lin et al., 1997, 1998a,b] to ascertain sequence specificity. DNA binding-activity was identified using γ^{32}P-ATP-labeled oligonucleotides specific for the sites (wild-type or mutant nCTCTn) to be examined. Oligonucleotides were chemically synthesized (Gibco-BRL) and end-labeled (γ-^{32}P-ATP) for use as probes (~20,000 cpm). The end-labeled (^{32}P-ATP) probe was incubated with 10 μg of total protein from HL-60 cells in the presence or absence of unlabeled competitor oligonucleotides at molar ratios of 100:1 or 10:1.

Electromagnetic Field Exposure System

Helmholtz coils designed by Electric Research and Management (Pittsburgh, PA) were used in the experiments described in this article. The coils are composed of 19-gauge wire bundles wound 164 times around a square form 13 cm long and 14 cm wide with 8-cm spacing. The coils are double-wound to permit energizing the coils in the sham exposure mode without significant field production and as a control for effects of coil heating. The exposure system permits simultaneous sham and experimental exposures under double-blind conditions. The coils are energized by a function generator (11-MHz Wavetek Stabilized Function Generator, model 21). A digital multimeter is used to measure the field intensity and verify the system operation (Fluke 87 digital multimeter). Field parameters are monitored with a Hitachi V-1065 100-MHz oscilloscope and a calibrated inductive search coil (25X; Electro-Biology, Parsippany, NJ). The magnetic field is delivered as a continuous sinusoidal wave at an amplitude of 8 μT and a frequency of 60 Hz. Transfectants in 100-mm Petri dishes were placed on a Plexiglas stand with the coils in a vertical orientation and the dishes in a position horizontal to the coils; i.e., the entire area of the Petri dish was exposed to the field. The bottom of the dish was 2 cm below the axis level. The height from dish bottom to top surface of liquid was about 1.1 cm; the height of the liquid was 0.6 cm. For experiments using 8 μT peak, the calculated E field was ~11 μV/m.

Mu Metal Shielding

Transfectants were shielded during exposures (experimental and sham) in mu metal containers (see Jin et al. [1997], for details on shielding measurements). Temperature was monitored with a Physitemp (BAT-12) (Hackensack, NJ) thermocouple probe (sensitive to ±0.1°C).

Heat Shock

Transfectants in Petri dishes, wrapped in Parafilm, were placed in a mu metal box (to shield them from exposure to magnetic fields generated by the heating device in the water bath) and immersed in the water bath at 43°C for 30 min, followed by an additional 30 min at 37°C before protein extraction.

Statistical Analyses

A sufficient number of experiments were performed to ensure statistical significance. Samples from each experiment were tested three times and experiments repeated a minimum of six times. The data were entered into Excel for analysis and the results examined with a two-tailed *t*-test. Statistical significance is determined by multifactor analysis of variance program (INSTAT). The data are expressed as the ratio of the experimental to the control (E/C) ±SEM.

RESULTS

Induction of HSP70 Expression by Magnetic Fields Requires HSP-MYC-C

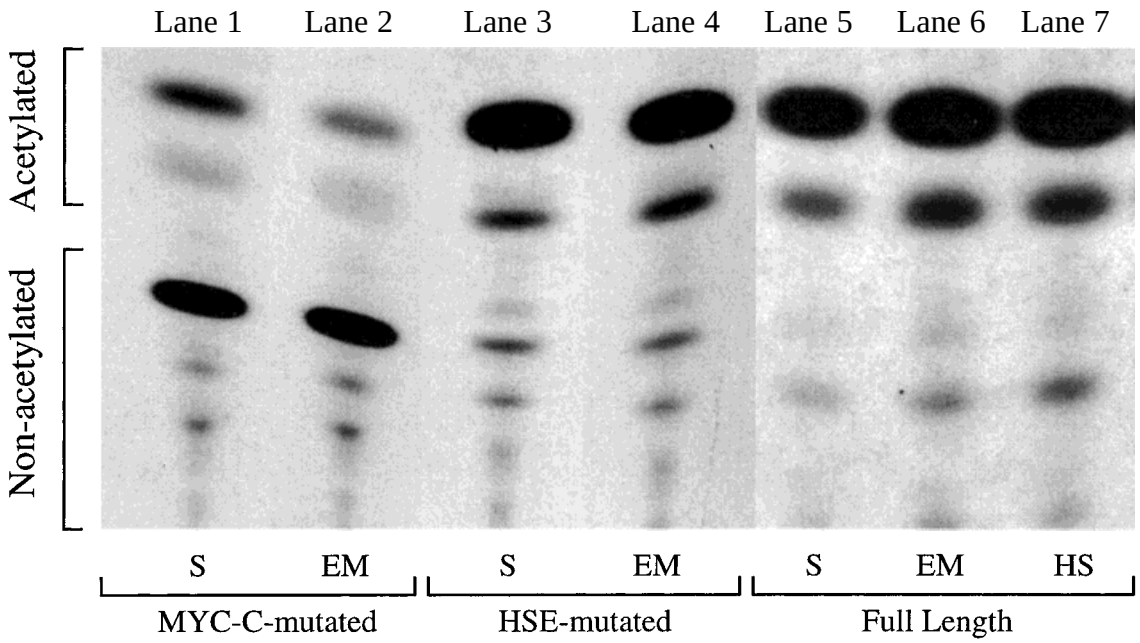
CAT expression was consistently below sham-control levels in transfectants containing construct with mutated nCTCTn sequence between −165 and −169 (i.e., HSP-MYC-C), co-transfected with c-*myc* protein, and exposed to magnetic fields (E/C = 0.72 ± 0.02)(Fig. 2). This c-*myc* binding site apparently serves as an important regulator and is required for magnetic field responsiveness, together with the two other nCTCTn c-*myc* binding sequences: HSP-MYC-A and HSP-MYC-B [Lin et al., 1998a] (Fig. 2).

Induction of HSP70 by Magnetic Fields Is Dependent on the HSE Centered at −192

Mutation of the distal heat shock element (HSE) between −180 and −203 resulted in loss of magnetic field inducibility; CAT activity was determined as E/C = 1.05 ± 0.02 (Fig. 2). This construct was inducible by heat shock at 43°C.

Magnetic Field-Induced Transactivation of HSP70 Requires c-*myc* Protein

Initial experiments replicated and confirmed our previous experimental results [Lin et al., 1998a]. A twofold increase in CAT activity was measured in transfectants containing the pΔH-17 construct (+1 to −320; Fig. 1) co-transfected with c-*myc* protein and exposed to 8-μT 60-Hz magnetic fields (Fig. 2). The level of



S, sham-exposed; EM, magnetic field-exposed; HS, heat shock at 43°C.

Fig. 2. CAT assays, representative of assays in seven different experiments. **Lane 1**, pΔH-17 sham exposed-transfectant (control); **lane 2**, pΔH17 with mutated MYC-C exposed to 8 μT 60 Hz; **lane 3**, pΔH-17 sham-exposed (control); **lane 4**, pΔH17 with mutated HSE-exposed to 8 μT 60 Hz; **lane 5**, pΔH17 sham-exposed (control); **lane 6**, pΔH17 exposed to 8 μT 60 Hz; **lane 7**, pΔH17 exposed to 43°C.

CAT activity remained at sham-control levels in transfectants exposed to magnetic fields *without* co-transfection of c-*myc* protein [Lin et al., 1998a]. CAT activity was increased twofold in transfectants containing the pΔH-17 deletion construct exposed to heat shock (with or without co-transfection of c-*myc* protein).

Competition Experiments Confirmed Identity of nCTCTn Sequence

Bandshift assays were carried out as previously described [Lin et al., 1997, 1998a,b] to ascertain nCTCTn sequence specificity. DNA binding activity was identified using a γ^{32}P-ATP-labeled wild-type oligonucleotide or a labeled oligonucleotide that carried a mutation over the nCTCTn sequence between −165 and −169 (HSP-MYC-C) (Fig. 3); lane 1 shows shifted band using wild-type oligonucleotide for nCTCTn and c-*myc* protein. The mutated MYC-C oligonucleotide (lane 2) showed no shifted band, indicating the absence of binding with c-*myc* protein. Shifted bands were inhibited by competition with excess amounts of unlabeled wild-type oligonucleotides (lanes 4 and 5). An oligonucleotide for NfkB was used as a nonspecific competitor (lane 3) and its presence did not block binding of c-*myc* protein. The positive control was the oligonucleotide specific for HSE with protein from a heat-shocked sample (lane 6).

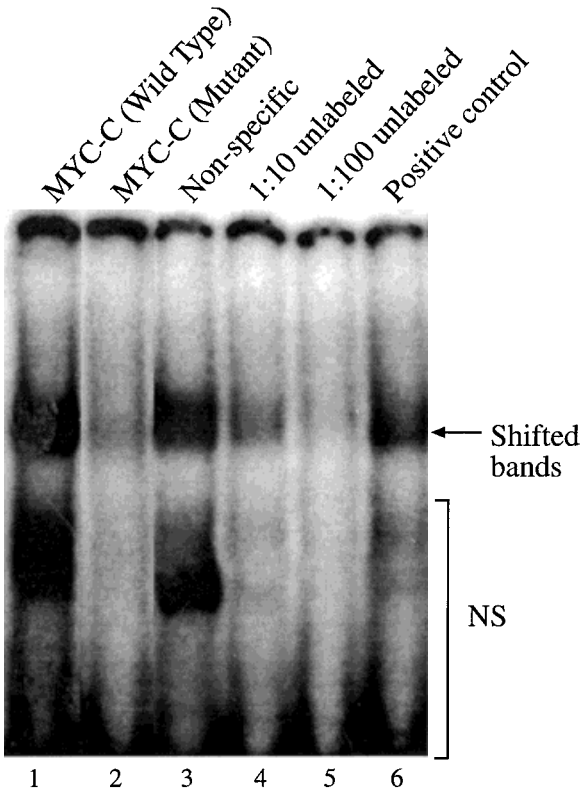


Fig. 3. Competition experiments to confirm that the sequence nCTCTn is required for c-*myc* protein-binding using wild-type, mutant, and nonspecific oligonucleotides. Representative of bandshift assays in six different experiments. **Lane 1**, wild-type oligonucleotide for MYC-C; shifted band shows binding with c-*myc* protein; **lane 2**, mutant MYC-C oligonucleotide; no binding with c-*myc* protein; **lane 3**, oligonucleotide for NfkB used as nonspecific competitor probe (1:100); shifted band shows that c-*myc* protein-binding is not blocked by this nonspecific competitor; **lane 4**, excess amounts of nonlabeled wild-type oligonucleotides (1:10 times); reduction in c-*myc* protein binding; **lane 5**, excess amounts of nonlabeled wild-type oligonucleotides (1:100 times); greater reduction in c-*myc* protein binding; **lane 6**, positive control; heat-shocked sample using heat shock element (HSE) oligonucleotides.

DISCUSSION

The results of experiments that use promoter deletion constructs containing mutations are generally presented in terms of a linear model of the DNA molecule. However, the particular spatial arrangement of the various elements of the promoter, and the association between elements in different parts of the DNA chain, may be critical for the specificity of the binding reactions [Lefstin and Yamamoto, 1998; Williams and Morimoto, 1990].

The *cis*-regulatory region upstream of the HSP70 promoter is extremely compact [Lis and Wu, 1993]. This is confirmed by reanalysis of our previous data using the promoter construct pΔH-18 (+1 to −188). Initially we believed that this construct contained only the HSP-MYC-B binding site, and no HSP-MYC-A. Yet, CAT expression in magnetic field-exposed transfectants showed only a slight reduction in inducible activity [Lin et al., 1998a]. Re-examination of DNA sequencing data, together with the results of the experiments reported in this paper, reveal that the HSP70 promoter construct pΔH-18 contains three nTTCn sequences of the distal HSE (lying between −180 and −204), as well as an nCTCTn sequence (HSP-MYC-C) centered at −165 that serves as an important regulator. These two previously unidentified promoter elements could account for the response we previously reported when transfectants containing this construct were exposed to magnetic fields [Lin et al., 1998a]. These data suggest that partial association, only the nTTCn sequences of the HSE, may be sufficient for magnetic field inducibility of HSP70. Further, there may be cooperative activity between

c-*myc* protein and HSF binding sites lying in close proximity, thus constituting a magnetic field-specific response region.

In this report, we show that when the nGAAn and nTTCn sequences lying between −180 and −204 are mutated, there is no response to magnetic fields (Fig. 2). Although we believe that the conclusions drawn from these experiments provide reasonable insights into the mechanism of magnetic field-activated HSP70 gene expression, conformational changes in one region of the DNA molecule can affect the conformation of other regions and thus contribute to the observed effects.

Further confirmation of the specificity of the HSE binding site (centered at −192) is provided by using transfectants containing the HSP70 promoter construct pΔH-11 (+1 to −111). This construct does not contain the HSE sequences lying between −180 and −204 or any of the HSP-MYC binding sites. However, this construct does contain the heat shock responsive regulatory domain with an HSE centered at −100. Transfectants containing this construct did not respond to magnetic field stimulation (with or without co-transfection of c-*myc* protein), but they did respond to heat shock [Lin et al., 1998a]. Based on evidence from site-directed mutagenesis, the presence of the distal HSE at −192 is apparently critical for magnetic field response. When this HSE is mutated there is no response to magnetic fields even with all three HSP-MYC binding sites present.

In addition to heat shock, many other environmental stressors induce HSP70 gene expression, e.g., cadmium, serum, pesticides, hypoxia. Wu et al. [1986, 1987] postulated that each stressor activates distinct transcription factors that recognize separate promoter elements. This could be the case with the magnetic field stressor. While the magnetic field-induced stress response depends on trimerization of HSF1 and binding to an HSE, the magnetic field regulatory domain (from −160 to −240) in the HSP70 promoter is upstream from the heat shock regulatory domain and contains three c-*myc* binding sites (nCTCTn) in addition to an HSE. These HSP-MYC binding sites in the HSP70 promoter have previously not been regarded as transcriptional regulatory elements. However, their response to magnetic fields suggests that they confer inducible regulation in response to this specific stimulus; nCTCTn sequences together with c-*myc* protein are essential for the response to magnetic fields. It will be of considerable interest to know exactly how cells sense magnetic field disturbance and how these sensing mechanisms coordinate the activities of the HSF and c-*myc* signal transduction cascades.

ACKNOWLEDGMENTS

We thank Dr. Imre Gyuk and the US Department of Energy for support. We also gratefully acknowledge Abraham J. Kremer for his editorial assistance.

REFERENCES

Blank M, Khorkova O, Goodman R. 1994. Changes in polypeptide distribution stimulated by different levels of electromagnetic and thermal stress. Bioelectrochem Bioenerg 33:109–114.

Gerner EW, Schneider MJ. 1975. Induced thermal resistance in HeLa cells. Nature 256:500–502.

Goodman R, Blank. 1998. Magnetic field stress induces expression of *hsp70*. Cell Stress Chaperones 3:79–88.

Goodman R, Henderson A. 1988. Exposure of salivary gland cells to low-frequency electromagnetic fields alters polypeptide synthesis. Proc Natl Acad Sci USA 85:3928–3932.

Goodman R, Blank M, Lin H, Khorkova O, Soo L, Weisbrot D, Henderson A. 1994. Increased levels of hsp transcripts are induced when cells are exposed to low frequency electromagnetic fields. Bioelectrochem Bioenerg 33:115–120.

Han L, Lin H, Head M, Jin M, Blank M, Goodman R. 1998. Application of magnetic field- induced heat shock protein 70 for pre-surgical cytoprotection. J Cell Biochem 71:577–583.

Jin M, Lin H, Han L, Opler M, Maurer S, Blank M, Goodman R. 1997. Biological and technical variables in c-*myc* expression in HL60 cells exposed to 60Hz electromagnetic fields. Bioelectrochem Bioenerg 44:111–120.

Kaddurah-Daouk R, Greene JM, Baldwin AS, Kingston RE. 1987. Activation and repression of mammalian gene expression by the c-*myc* protein. Genes Dev 1:347–357.

Kingston RE, Baldwin AS, Sharp PA. 1984. Regulation of heat shock protein 70 gene expression by c-*myc*. Nature 312:280–282.

Koskinen PJ, Sistonen L, Evan G, Morimoto R, Alitalo K. 1991. Nuclear colocalization of cellular and viral *myc* proteins with HSP70 in *myc*-overexpressing cells. J Virol 65:842–851.

Lefstin JA, Yamamoto KR. 1998. Allosteric effects of DNA on transcriptional regulators. Nature 392:885–888.

Lin H, Goodman R and Henderson AS. 1994. Specific region of the c-*myc* promoter is responsive to electric and magnetic fields. J Cell Biochem 54:281–288.

Lin H, Opler M, Head M, Blank M, Goodman R. 1997. Electromagnetic field exposure induces rapid, transitory heat shock factor activation in human cells. J Cell Biochem 66:482–488.

Lin H, Head M, Blank M, Han L, Jin M and Goodman R. 1998a. *Myc*-mediated transactivation of HSP70 expression following exposure to magnetic fields. J Cell Biochem 69:181–188.

Lin H, Head M, Blank M, Han L, Goodman R. 1998b. Magnetic field activation of protein-DNA binding. J Cell Biochem 70:297–303.

Lis J, Wu C. 1993. Protein traffic on the heat shock promoter: parking, stalling and trucking along. Cell 74:1–4.

Taira T, Negishe Y, Kihara F, Igushi-Ariga SMM, Ariga H. 1992. C-*myc* protein complex binds to two sites in human hsp70 promoter region. Biochim Biophys Acta 1130:166–174.

Williams GT and Morimoto RI. 1990. Maximal stress-induced transcription from the human HSP70 promoter requires interaction with the basal promoter elements independent of rotational alignment. Mol Cell Biol 10: 3125–3136.

Wei G, Cechova K, Tassi V, Dalla-Favera R. 1993. Opposite regulation of gene transcription and cell proliferation by c-*myc* and Max. Proc Natl Acad Sci USA 90:2935–2939.

Zhang Y, Shields T, Crenshaw T, Hao Y, Moulton T, Tycko B. 1993. Imprinting of human H19: allele-specific CpG methylation, loss of active allele in Wilms tumor, and potential for somatic allele switching. Am J Hum Genet 53:113–124.