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ARTICLES

Magnetic Field Exposure Enhances mRNA Expression of σ^{32} in *E. coli*

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Abstract The mechanism of interaction between weak electromagnetic fields and cells is not understood. As a result, the health effect(s) induced by exposure to these fields remains unclear. In addition to questions relating to the site of initial magnetic field (MF) interactions, the nature of the cell's response to these perturbations is also unclear. We examined the hypothesis that the cells respond to MFs in a manner similar to other environmental stressors such as heat. Using the bacterium *Escherichia coli,* we examined the mRNA levels of σ^{32}, a protein that interacts with RNA polymerase to help it recognize a variety of stress promoters in the cell. Our data show that the intracellular level of σ^{32} mRNA is enhanced following a 15-min exposure to a 60 Hz, 1.1 mT magnetic field. J. Cell. Biochem. 68:1–7, 1998.

Key words: cell stress; heat shock; σ^{32}; magnetic fields; ribonuclease protection assay

The mechanism of interaction between weak electromagnetic fields and cells remains an intractable issue in cell and molecular biology. The fact that most cellular responses to weak electromagnetic fields are small [Goodman et al., 1995] suggests that the cell perceives and responds to these fields as they would to other environmental stressors. Evidence supporting this suggestion was reported by Goodman et al. [1992], who observed an increase in gene transcripts for the stress protein *hsp70* and other proteins [Blank and Goodman, 1989] when *Drosophila melanogaster* salivary glands were exposed to weak magnetic fields.

In these experiments, we asked whether or not weak magnetic field exposure could elicit an elevated stress response in *Escherichia coli.* The parameter examined was transcription of σ^{32}, a transcription factor that is part of RNA polymerase and an essential component of the cell's stress response. σ^{32} facilitates the recognition and enhances transcription of stress promoters such as the GroES operon, dnaK, and dnaJ [Gamer et al., 1992; Georgopoulos and Ang, 1990; Yura et al., 1993]. To examine magnetic field effects on σ^{32} transcription, bacteria in the log phase of growth were exposed to a 1.1 mT, 60 Hz sinusoidal magnetic field for 15 min, and the level of σ^{32} mRNA was assessed using the RNA protection assay. The data show that under these conditions MFs enhance the expression of σ^{32}.

MATERIALS AND METHODS

Growth of Cultures

E. coli wild type strain MG1655, obtained from Professor Richard Burgess (McArdle Laboratory for Cancer Research, UW-Madison), was the source of RNA in the protection assay. A submerged shake culture was started from frozen stocks; cells were grown overnight in nutrient broth (LB medium) at 36.6°C. The following morning 1 µl of culture was inoculated to 25 ml of LB medium and grown to an $A_{600} = 0.3$. The culture was split into two 10-ml aliquots; each portion was placed in a separate incubator maintained at 36.6°C. The culture in one incubator served as a sham-exposed control, and the other was exposed to magnetic fields for 15 min (see Field Application). The investigator was unaware of each incubator's status during the experiment.

Total RNA Isolation

The procedure of Barry et al. [1992] was used to isolate RNA. Following MF-exposure, four aliquots were removed from each flask, placed

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in 1.5-ml Eppendorf centrifuge tubes, and centrifuged at 8,000*g* for 5 min at room temperature. The pellets were resuspended in 40 µl diethylpyrocarbonate (DEPC, Sigma, St. Louis, MO) and 0.3 µl of 0.5% DEPC-treated water. Ice-cold acetone (200 µl) was added, mixed, and recentrifuged at 10,000*g* for 5 min. The supernatants were removed and the pellets resuspended in 40 µl of DEPC-H_2O and 3 µl of a Proteinase K (Promega, Madison, WI) solution (100 mM Tris-HCl [pH 7.2], 50 mM NaCl, 0.2% sodium dodecyl sulfate, 200 µg/ml Proteinase K). Following a 10-min incubation at 4°C, 3.5 µl of 0.5% DEPC, 200 µl 65°C phenol (pH 5.2), 150 µl chloroform, and 120 µl DEPC-H_2O were added. The suspensions were mixed and centrifuged at 12,000*g* for 5 min; 1 ml of 100% ethanol was added to precipitate total RNA overnight at −20°C. The RNA solutions were centrifuged at 4°C for 15 min; ethanol was removed, and the pellet was dried in a SpeedVac™, resuspended in 100 µl DEPC-H_2O and stored at −20°C until use. The purity of the RNA was assessed using the ratio of A_{260}/A_{280} = 1.8–2.0. The integrity of the RNA was determined by examining the ribosomal RNA bands in a 1% agarose formaldehyde denaturing gel.

Generation of the RNA Probe

The bacteria containing the σ^{32} plasmid (pSigma32, obtained from Professor Carol Gross, University of California/San Francisco) were grown in 10 ml of LB media containing 50 µg/ml of ampicillin. The plasmid was isolated using a modified alkaline lysis miniprep protocol [Xiang et al., 1994]; the concentration and purity were determined spectrophotometrically (1 A_{260} = 50 µg DNA). The size of the supercoiled plasmid was analyzed on a 1% agarose gel.

To create a runoff transcript and synthesize the antisense RNA probe, the plasmid was linearized by digestion for 1 h with the restriction enzyme *BamHI* at 37°C using standard protocols (Promega). Following digestion, 20 mg/ml of Proteinase K (Sigma) was added for an additional 30 min. The linearized plasmid was isolated by adding an equal volume of 25:24:1 phenol:chloroform:isoamly alcohol and centrifuging at maximum speed (~12,000*g*) in a microfuge for 30 s at room temperature. The extraction was then repeated, followed by a final extraction in chloroform. The DNA was precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of cold absolute ethanol (−20°C). Following centrifugation (15 min, 4°C) the supernatant was removed, and the pellet was washed in 70% ethanol and air dried. The DNA was resuspended in TE (10 mM Tris-HCL, 1 mM ethylenediamine tetraacetic acid, pH 8.0) to a concentration of 1 µg/ml.

The linearized template pSigma32 DNA was transcribed using the Ambion (Austin, TX) MAXIscript™ T3 in vitro transcription kit, resulting in a 114 bp probe. Briefly, 1 µg of linearized DNA was used in each 20-µl reaction. Following the addition of the σ^{32} template, the other reagents were added to a final concentration of 10 mM dithiothreitol, 2.5 µl σ^{32}P UTP (Amersham, Arlington Heights, IL) (800 Ci/mol, 20 mCi/ml), 5 µl of 0.5 mM nucleotides (GTP, ATP and CTP, [Promega]), 1X transcription buffer (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ 5 mM DTT, 50 µg/ml bovine serum albumin), 28 U ribonuclease inhibitor (Promega), 10–20 u T3 RNA polymerase, and water to a final volume of 20 µl. The reaction mix was incubated at room temperature for 1 h. Two units of RNAse-free DNAse I were added to the reaction mix followed by a 30-min incubation at 37°C.

Following incubation, an equal volume of gel-loading buffer was added and the tube heated for 3 to 5 min at 90°C to destroy secondary RNA structure and to insure that only single-stranded RNA was present. Two microliters were removed from the mixture and counted to determine the percent incorporation of the isotope. The remaining portion of the transcription reaction was purified by loading onto a 75-mm 8M urea, 8% polyacrylamide gel using a Bio-Rad (Richmond, CA) Mini PROTEANII™ electrophoresis unit run at 250 V for 20 min to 1 h. The gel was removed, wrapped in Saran Wrap™, and exposed to Fuji Medical X-ray film for 1–5 min at room temperature. Using the autoradiogram as a template, the bands representing the full-length probe were cut out, submerged in 350 µl of probe elution buffer (500 mM ammonium acetate, 1 mM EDTA, 0.2% SDS), and briefly vortexed. To insure maximum recovery of the probe, the ammonium acetate solution was incubated overnight at 37°C. An autoradiogram of the isolated probe is shown in Figure 1.

Fig. 1. An 8M urea/8% polyacrylamide denaturing gel of Psigma32 linearized with *BamHI.* The linearized DNA was used as a template to generate a 114-bp antisense probe that was used to hybridize to σ^{32} mRNA in the Ribonuclease Protection assay.

Specific Activity of Probe

To affect specific hybridization with the σ^{32} mRNA, the probe must be in 3–10-fold molar excess. To determine the specific activity, 198 ml of TE buffer containing 100 µg tRNA as a carrier was added to 2 µl of the probe mixture prior to its gel purification. The mixture was vortexed and 100 µl was removed and counted. Two milliliters of 10% trichloroacetic acid (TCA) was added to the remaining 100-µl sample. Following a 5-min incubation at 4°C, the mixture was pipetted to a 13-mm nitrocellulose filter (3-µm pore size) supported on a Hoeffer (San Francisco, CA) vacuum manifold. After collecting the filtrate by vacuum, the filter was washed once with 10% TCA and twice with 2 ml 95% ethanol air dried and counted. The specific activity was calculated as described in the Ambion protocols.

RNAse Protection Assay

The Ambion RPAII™ standard protocol was used as described by the manufacturer; in all experiments yeast RNA was hybridized to the antisense probe and functioned as a negative control. In the standard procedure, the probe and samples are precipitated with 5M ammonium acetate (NH_4OAC) prior to resuspension in hybridization buffer. Hybridization was performed in microfuge tubes at 42°C for 20 h. To digest unhybridized single-stranded RNA, 200 µl of an RNAse A and T_1 mixture (Ambion) diluted 1:100 in RNAse digestion buffer (Ambion) was added to each tube; tubes were incubated for 30 min at 37°C. The mix was precipitated with 300 µl of inactivation/precipitation mix (Ambion); 100 µl of ethanol was also added to improve recovery of fragments of at least 100 bases. As a control, one tube with yeast RNA was filled with an equal amount of RNAse digestion buffer but no RNAse.

Analysis of Protected Fragments

The precipitated DNA was centrifuged in a Brinkman (Westbury, NY) microfuge for 15 min at 4°C. After traces of supernatant were removed, the pellet was resuspended in 8 µl of gel loading buffer; 0.5 µl of a cyclin B probe was added as an external gel-loading control (kindly provided by Professor C.-M. Chen, U.W.-Parkside). Samples were loaded onto a 0.75-mm 8M urea/8% polyacrylamide gel (Bio-Rad Mini-PROTEANII™ electrophoresis unit) and run at 250 V for 45 min. The gel was wrapped in plastic wrap and exposed to X-ray film (Fuji Fisher Scientific, Itasca, IL) for 2 h at −70°C. Following exposure, the film was developed and analyzed.

Data Analysis

Prior to the actual analysis of the gels, experiments were run in which increasing amounts of hybridization mixtures (increasing amounts of radioisotope) were electrophoresed and overlain with film to establish the linear response of the autoradiographic film. The RPA bands were examined and quantified using a ChemiImager 2000 (AlphaInnotech, San Leandro, CA). Using the densitometer software, outlines with equivalent areas were drawn around the bands of interest, and the intensity of the total area was determined. Spot intensities were used to normalize the data for loading differences. To allow for daily differences in the probe activity and the amounts of RNA, the data from each experiment were collectively analyzed using a paired *t*-test.

Field Application

Cultures were exposed to 60 Hz, 1.1 mT sinusoidal magnetic fields. AC fields are generated

by dual-filament-wound Helmholtz coils mounted on a wooden frame surrounding a water-jacketed cylinder into which the culture flasks were placed. The water jacket isolates the cultures from any local heating from the coils and, more importantly, provides more precise temperature control than the incubator itself. Exposed and control (sham-exposed) cultures were in separate incubators. The coils produce a field in the vertical direction. Cells were in the region within the coils where fields are homogeneous to better than ±5%. The two filaments surrounding exposed cultures were connected to generate magnetic fields that add; in the sham-exposed (control) coils, the two filaments generate fields in opposite directions, which cancel each other to better than one part in 10^4. Both filaments in the exposed and both in the sham-exposed coil are wired in series and carry the same current; this arrangement ensures that both coils contribute more equivalent heating and vibration effects [Kirschvink, 1992], though the contributions were not identical. During field-off intervals, neither control nor exposure coils were energized. The coils are driven by a Techron Model 7541 (Elkhart, IN) power amplifier, which receives an input signal from a Wavetek Model 125 (San Diego, CA) arbitrary waveform generator.

Magnetic field excitation waveforms and amplitudes were monitored by an oscilloscope. The fields were checked using a Hall-effect probe magnetometer (F.W. Bell, Model 9640, Orlando, FL) and a fluxgate magnetometer that can measure weak ac and dc fields (Bartington, Model Mag-03MC100, Redwood City, CA). The two measuring systems were cross-compared and checked against the calculated field from a 20-cm radius Helmholtz coil.

In all experiments, the identity of the exposed and control incubators was controlled using a concealed switch. A random number table was consulted at the start of each exposure sequence to determine the switch setting for that set of experiments. The investigators did not know which cultures were exposed or control until the end of the experimental series.

RESULTS

Once the experiments were completed, the code was broken and the normalized densities of the samples were analyzed to assess whether or not MFs significantly altered the levels of σ^{32} mRNA. The data show that when cells are exposed for 15 min to 60 Hz, 1.1 mT (RMS) magnetic fields, the transcription of σ^{32} is significantly enhanced ($P < 0.05$). These data are summarized in Figure 2.

Determining whether σ^{32} mRNA was being enhanced by MFs requires that the RNA being isolated is of high quality, intact, and equivalent in non-exposed and MF-exposed samples. The need for extracting equivalent RNA was especially important because we lacked a reliable internal control; we know of none in *E. coli* that has been shown definitely not to respond to MFs. To circumvent this problem, we were extremely careful to insure that the cell densities in the cultures used in the experiment were equivalent by growing bacteria to log phase and then splitting the culture into two equivalent aliquots; new growth and field exposure occurred during the next 15-min period. In essence, cells started at the same point in growth and were only separated for a total of 15 min before RNA isolation.

Although this procedure insures an equivalent start to the experiment, it does not assure equivalent isolation of RNA from the control and exposed flasks. The isolated RNA was quantified by uv spectroscopy; the integrity of the isolated RNA's samples was determined by examining the RNA on a 1% agarose formaldehyde gel. In examining the gels, both equivalence of intensity and the appearance of both ribosomal bands were indicative of a successful isolation (Fig. 3).

Another potential source of error would be placing unequal amounts of sample on the gel used to assess the levels of σ^{32} transcription, resulting in an over- or underestimation of the amount of protected message. To address this problem, a radiolabelled 150-bp cyclin RNA was added to both hybridization samples prior to electrophoresis. The cyclin b marker was the selected probe because it was larger than the plasmid's 114 bp and therefore could be easily resolved on the gel. In addition to the controls for pipetting and isolating samples, negative controls involving hybridization and digestion to yeast total RNA were also run. In these experiments, yeast RNA should not hybridize to the σ^{32} probe and would not be expected to be protected from RNAse digestion. A typical gel (Fig. 4) shows the yeast standards, the external controls, and the RNAse-protected fragments.

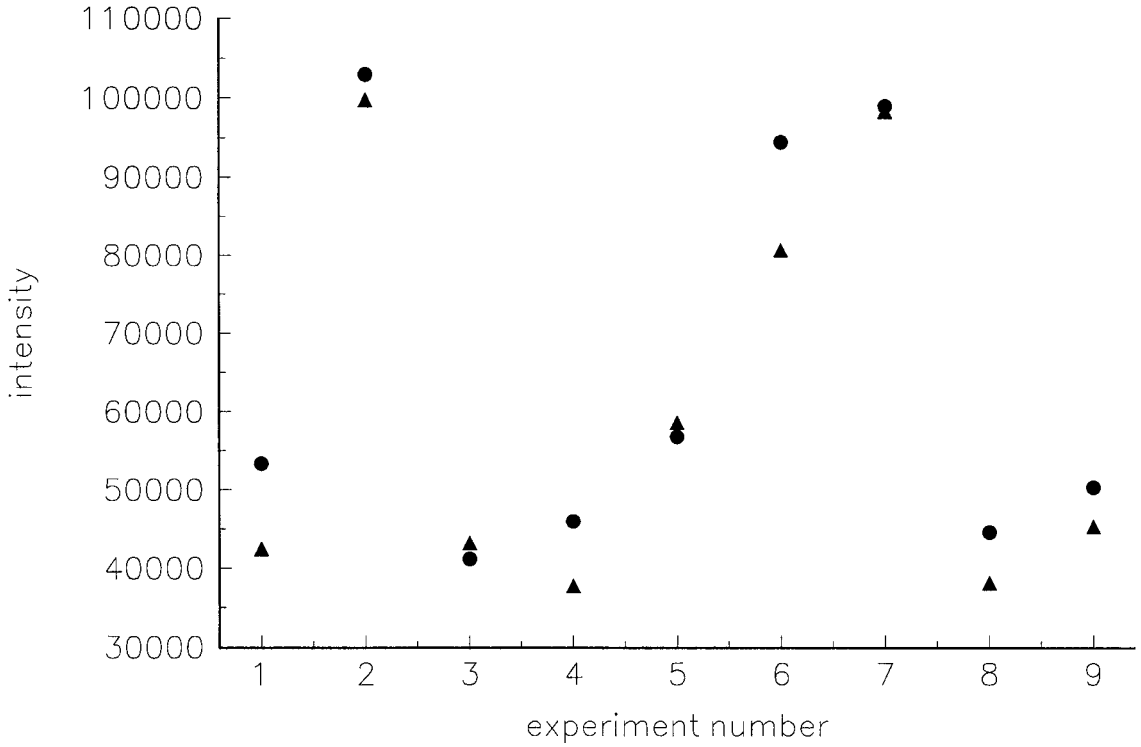

Fig. 2. A summary of 9 individual RPA experiments probing for σ^{32} mRNA; MF-exposed (●) and non-exposed (▲) sham controls. Using a paired *t*-test the MF-exposed cells were found to have significantly more σ^{32} mRNA than the non-exposed sham controls ($P < 0.05$).

DISCUSSION

A review of the literature shows that *E. coli* responds to a variety of environmental stimuli, such as altered pH, osmolarity, heavy metals, and anaerobiosis, by inducing stress proteins (often referred to as heat shock proteins) [for reviews see Lindquist, 1986, 1988; Mager and DeKruuff, 1995]. Although these previously mentioned stressors induce the "heat shock response," it is generally weaker and involves only a subset of the heat-stress proteins [Neidhardt and VanBogelen, 1987]. Functionally, σ^{32} competes with σ^{70} (the "normal transcription factor") to form the holoenzyme RNA polymerase ($\alpha_2\beta,\beta' + \sigma$ factor). As a result of this competition for core polymerase, and the paucity of σ^{32} proteins available, only a few "maintenance" promoters are recognized and expressed at low temperatures. Proteins that are normally expressed in unstressed cells include the chaperone-like *GroES* and *GroEL* (members of the *Hsp60*-like class of proteins). When cells encounter an environmental stress, both the amount and stability of σ^{32} mRNA are enhanced [Straus et al., 1987] and subsequently the amount of σ^{32} protein.

The underlying assumption of our experiments is that weak fields are perceived by cells in a manner similar to any other environmental stress. This hypothesis was originally suggested by Goodman et al. [1992, 1994] and Blank et al. [1993]. To test this theory, experiments were undertaken to examine the well-characterized response of *E. coli* to environmental stressors. Since the normal intracellular levels of σ^{32} is low (10 to 30 molecules) [Craig and Gross, 1991], the first events following application of a stressor one would expect would be an increase in the σ^{32} mRNA. This rise would precede the appearance of the elevated protein.

Reports by Goodman et al. [1992, 1994] and Blank et al. [1993] suggest that exposure to MFs at field strengths of 80 μT can elicit a stress response in eukaryotic *hsp* RNAs and proteins. Although we have applied field intensities approximately 10 times those of Goodman and colleagues, the data are consistent in showing a significant enhancement of the stress-

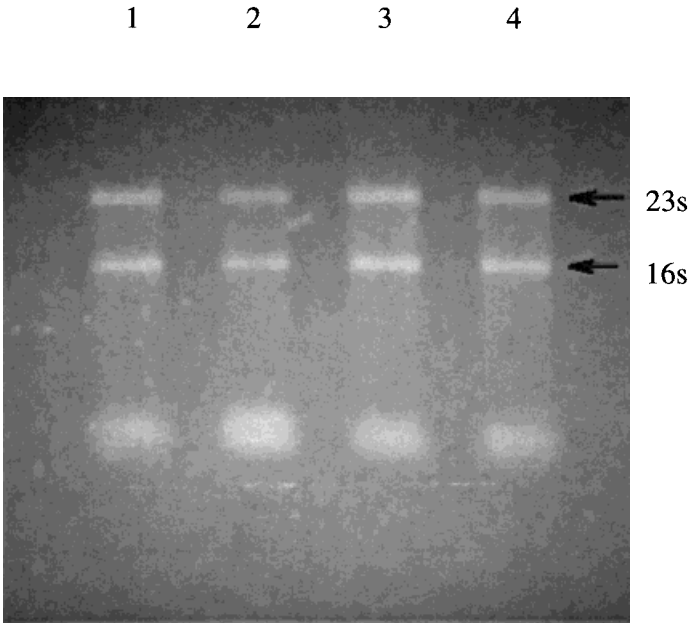

Fig. 3. A 1% ethidium bromide-stained agarose/formaldehyde gel showing the integrity of the isolate RNA. The isolated RNA was quantified by uv spectroscopy. The experiments were continued if discrete ribosomal bands (23S and 16S) were visible.

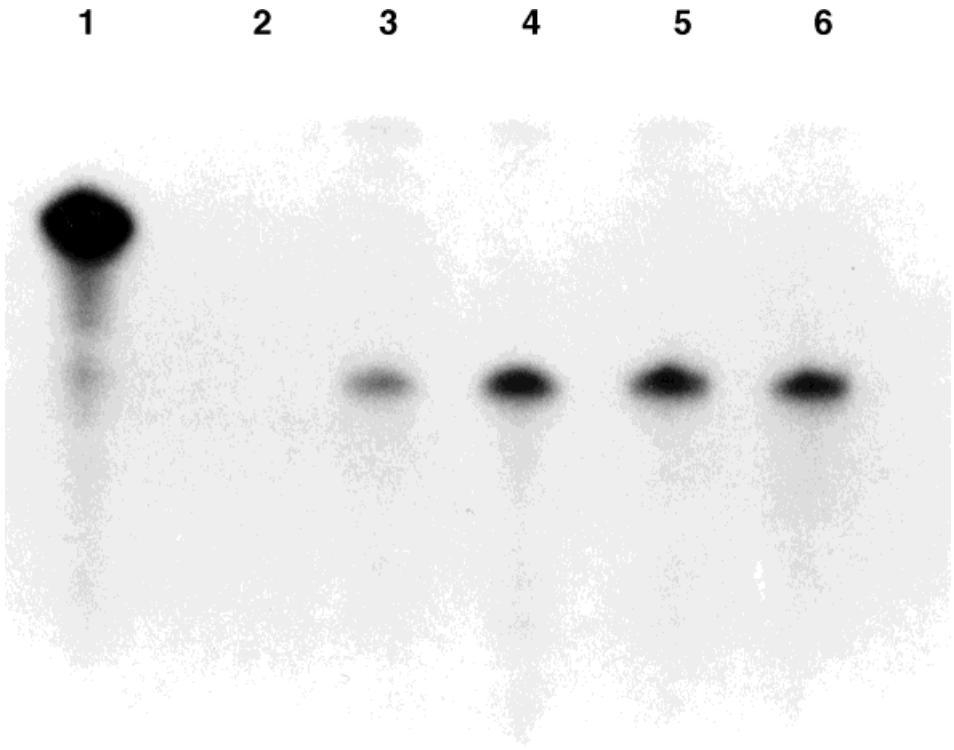

Fig. 4. A typical autoradiogram from a ribonuclease protection assay. **Lane 1** is the free probe, **lane 2** represents the free probe following RNAse digestion. **Lanes 3, 4, 5,** and **6** show the known 250-bp cyclin B probe that was used to normalize the RPA data. In this gel, lanes 3 and 4 represent the non-exposed control and lanes 5 and 6 the MF-exposed cultures.

related σ^{32} mRNA following a 15-min MF-exposure. Given that σ^{32} is required to recognize and transcribe stress proteins in bacteria, these data extend and support their conclusion that cells perceive and respond to applied MFs as they would any environmental stressor.

A re-examination of our previously published 2-D PAGE experimental data in *E. coli* [Goodman et al., 1994] showed that the stress proteins *htpG, grpE,* and *htpR* (σ^{32}) were elevated. At the time, we were not aware of the significance of this finding, although we knew that the cells had not been subjected to any heat shock. That this 2-D gel data is real is supported by subsequent and independent experiments in which the proteins identified by 2-D PAGE data as elevated by MF-exposure, specifically α polymerase and topoisomerase, were subsequently shown to be enhanced in cell-free expression studies [Goodman et al., 1993].

In summary, we initiated this study to determine whether the cell perceived and responded to an applied magnetic field in a manner analogous to any exogenous stressor. In *E. coli,* this response has been well characterized and is known to begin with an increase of σ^{32}, a protein that recognizes stress promoters and facilitates their transcription by RNA polymerase. Using the RNAse protection assay, we obtained data that show enhanced transcription of σ^{32} following a 15-min exposure to MFs. We intend to extend these experiments to quantify the comparison between MFs and other stressors, such as heat shock and/or osmotic shock. For instance, we wish to ascertain how much the growth temperature would have to be increased to induce a similar elevated response in σ^{32}. Such a comparison will help add perspective to the health-effects debate.

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