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Regulating Genes with Electromagnetic Response Elements

Hana Lin,[1] Martin Blank,[2] Karin Rossol-Haseroth,[3] and Reba Goodman[1]*

[1]Department of Pathology, Columbia University Health Sciences, New York, NY 10032
[2]Department of Physiology, Columbia University Health Sciences, New York, NY 10032
[3]Department of Medicine, Columbia University Health Sciences, New York, NY 10032

Abstract A 900 base pair segment of the c-*myc* promoter, containing eight nCTCTn sequences, is required for the induction of c-*myc* expression by electromagnetic (EM) fields. Similarly, a 70 bp region of the HSP70 promoter, containing three nCTCTn sequences, is required for the induction of HSP70 expression by EM fields. Removal of the 900 base pair segment of the c-*myc* promoter eliminates the ability of EM fields to induce c-*myc* expression. Similarly, removal of the 70 bp region of the HSP70 promoter, with its three nCTCTn sequences, eliminates the response to EM fields. The nCTCTn sequences apparently act as electromagnetic field response elements (EMRE). To test if introducing EMREs imparts the ability to respond to applied EM fields, the 900 bp segment of the c-*myc* promoter (containing eight EMREs) was placed upstream of CAT or luciferase reporter constructs that were otherwise unresponsive to EM fields. EMREs-reporter constructs were transfected into HeLa cells and exposed to 8 μT 60 Hz fields. Protein extracts from EM field-exposed transfectants had significant increases in activity of both CAT and luciferase, compared with identical transfectants that were sham-exposed. Transfectants with CAT or luciferase constructs *lacking* EMREs remained unresponsive to EM fields, i.e., there was no increase in either CAT or luciferase activity. These data support the idea that EMREs can be used as switches to regulate exogenously introduced genes in gene therapy. J. Cell. Biochem. 81:143–148, 2001.

Key words: electromagnetic field response elements; gene therapy

Low frequency electromagnetic (EM) fields induce increased expression of the stress response gene HSP70 [Lin et al., 1997; Goodman and Blank, 1998]. There are several parallels in the biochemical pathways induced by EM fields and heat shock, but there are striking differences as well. Both pathways involve the binding of heat shock factor 1 (HSF1) to a heat shock element (HSE), but regulation of HSP70 gene expression by EM fields requires three nCTCTn binding sites in the HSP70 promoter that lie between −230 and −160, upstream from the transcription initiation site. These three nCTCTn sequences appear to act as electromagnetic field response elements (EMREs), since the ability of an EM field to induce stress proteins gradually disappears as the EMREs are mutated one by one [Lin et al., 1998a, 1999]. Removal of EMREs by mutation does not affect the response to heat shock, since the heat shock domain is downstream from the EM field domain in the HSP70 promoter (between −106 and −67) [Lin et al., 1997, 1998b, 1999].

We previously showed that a 900 bp region in the c-*myc* promoter (−1257 to −353) was responsive to EM fields [Lin et al., 1994]. Recent reanalysis of this 900 bp region revealed eight nCTCTn sequences within this DNA fragment. These eight EMREs in the c-*myc* promoter could account for the EM field sensitivity of the c-*myc* gene, and the resultant increased c-*myc* transcript levels in cells exposed to EM fields [Jin et al., 1997].

To determine whether EMREs can serve as switches to regulate exogenously introduced genes, the 900 bp fragment of the c-*myc* promoter was placed upstream of CAT or luciferase reporter constructs that were otherwise unresponsive to EM fields. EMRE-reporter constructs were transfected into HeLa cells and

Abbreviations used: CAT, chloramphenicol transferase; EM, electromagnetic; Hz, hertz (cycles/s); EMRE, electromagnetic field response element.

*Correspondence to: R. Goodman, Columbia University Health Sciences, Department of Pathology, 630 W. 168th Street, New York, NY 10032. E-mail: rmg5@columbia.edu

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transfectants exposed to EM fields. Protein extracted from EM field-exposed transfectants showed increased CAT and luciferase activities, whereas no increase in CAT or luciferase was measureable in the controls. Three kinds of controls were used: transfectants that were sham-exposed; transfectants lacking EMREs; and non-specific protein. These data support the theory that EMREs can be inserted into the promoters of exogenously introduced genes to serve as switches that respond to EM fields. This would provide a new and powerful non-invasive technique for regulating gene expression *during* gene therapy.

MATERIALS AND METHODS

Cell culture and transfections. As previously described, HeLa cells were used for transient transfections and the lipofectin method (Gibco/BRL, Cat. No. 18292-011) was used for transfection as described [Lin et al., 1997, 1998a,b].

900 bp segment from the c-myc promoter. The 900 bp region of the c-*myc* promoter containing eight copies of nCTCTn extends from −353 (*Pvu*II site) to −1257 (*Cla*I site).

***p*ΔH-11-CAT HSP70 deletion construct.** (This plasmid was kindly provided by Dr. R. Kingston, Department of Genetics, Harvard University). A diagrammatic representation of this construct is presented by Lin et al. [1999]. This construct contains the first 111 base pairs upstream from the transcription initiation site and includes the heat shock domain (−106 to −67). There are no nCTCTn binding sites in this construct and it is unresponsive to EM fields [Lin et al., 1999].

Construction of EMRE-CAT expression vector. Plasmid pΔ11-CAT was digested with *Hind*III and *Pvu*II, harvested from gel (Fig. 1A). Two oligonucleotides were used for PCR which allowed us to create two enzyme sites and amplify the 900 bp region from c-*myc* promoter.

1. CCTGAGCTCTTCTTTGATCAGAATCGATA
2. TCTAAGCTTCTTTGATCAGAATCGATG

One microliter of plasmid (digested with *Hind*III and *Pvu*II) was mixed with 3 μl PCR product, placed at 12°C overnight for ligation and transformed using DH52 bacteria. Clone hybridization verified insert.

Construction of EMRE-luciferase expression vector. A luciferase expression vector PGL3 (Promega) was digested with Sac1 and Sma1 and harvested from a gel (Fig. 1B). Two oligonucleotides (see above) were used for PCR which allowed us to create two enzyme sites and amplify the 900 bp region from c-*myc* promoter. One microliter of digested plasmid was mixed with 3 μl PCR product, placed at 12°C overnight for ligation and transformed using DH52 bacteria. Clone hybridization verified insert.

Protein. Protein was extracted and concentrations determined as previously described [Lin et al., 1997, 1998a,b, 1999].

CAT assay. CAT assays were performed as previously described [Lin et al., 1997, 1998a]. Results were quantified using a PhosphorImager and ImageQuant software.

Luciferase assay. Luciferase activity was determined (Luciferase Assay Kit) (Promega #E1501) and results quantified as suggested by Promega.

Magnetic field exposures of transfectants. Transfectants were exposed and sham-exposed as previously described [Lin et al., 1998a,b, 1999].

Heat shock. Samples from cells that had been heat shocked (43°C) served as positive controls for CAT assay. Petri dishes containing transfectants were wrapped in Parafilm, placed in a Mu metal box (to shield them from exposure to the magnetic fields generated by the water bath heating motor) and immersed in the water bath at 43°C for 30 min. Petri dishes were removed from the water bath and, following an additional 30 min at 37°C, protein was extracted [Lin et al., 1997].

Sinusoidal electromagnetic field exposure system. Two fully functional exposure units provided simultaneous sham and experimental exposures. Exposures used Helmholtz coils (Electric Research and Management, Pittsburgh, PA) that consisted of 19-gauge wire bundles wound 164 times around a rectangular form 13 cm long and 14 cm wide with 8 cm spacing. The coils were energized by a function generator (11 MHz Wavetek Stabilized Function Generator; model 21). A digital multimeter was used to measure the field intensity and verify the systems operation (Fluke 87 digital multimeter). Field parameters were monitored with a Hitachi V-1065 100 MHz oscilloscope and calibrated inductive search coil (25X; Electro-Biology Inc., Parsippany, NJ). Detailed description of the exposure system, including

background magnetic fields in the incubater, harmonic distortion, DC magnetic fields, and mean static magnetic fields in the incubator, both vertical and horizontal components, can be found in Jin et al. [1997]. Cells were placed on a Plexiglas stand in a horizontal orientation, i.e., the entire area of the 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 ~1.1 cm; the height of the liquid was 0.6 cm. The calculated electric field was ~11 μV/m for an 8 μT 60 Hz exposure.

Mu metal shielding. Helmholtz coils were enclosed within Mu metal containers to minimize stray fields during EM field exposures. Both active (experimental) and sham-exposed (controls) coils were enclosed in a 30 cm high, 15 cm diameter cylindrical Mu metal container (0.040 inch thickness) (Amuneal Corp. Philadelphia, PA). The 60 Hz shielding factor is (min) 90.1 (39.08 dB). Sham-exposed controls and experimental exposures were performed simultaneously in identical Mu metal containers.

Statistical analyses. A minimum of five experiments were performed to assure statistical significance. Statistical significance is determined by multifactor analysis of variance program (INSTAT).

RESULTS

EMREs Increase Luciferase Activity in Transfectants Exposed to EM Fields

To determine whether the nCTCTn sequences (EMREs) that are EM field responsive would confer EM field responsiveness to a reporter construct lacking these sequences, a 900 bp region from the c-*myc* promoter containing eight copies of nCTCTn, was ligated to a PGL3 plasmid containing a portion of the SV40 promoter and carrying the luciferase gene (Fig. 1B). This plasmid construct was transfected into HeLa cells and the transfectants exposed to 8 μT 60 Hz fields for 30 min, followed by an additional 30 min out of the field prior to protein extraction for the luciferase assay. Luciferase activity increased at an average of 61%. Three sets of controls were used (Fig. 2A): (1) sham-exposed transfectants that served as controls for EM field exposure, and showed no significant luciferase activity; (2) transfectants containing the luciferase reporter construct *without* the 900 bp insert served as controls for background and showed no measurable luciferase activity; and (3) non-specific protein served as negative controls with no measurable activity. These transfectants were not responsive to

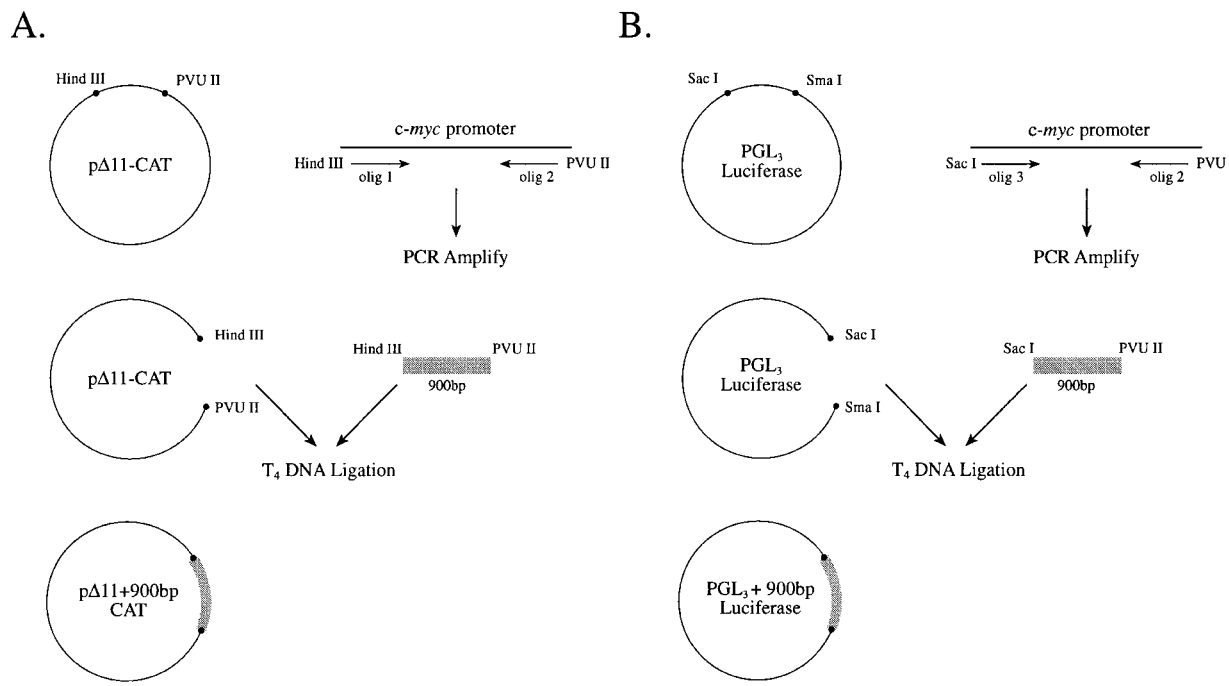

Fig. 1. Construction of EMRE-expression vectors. **A**: pΔ11 + 900 bp + CAT; **B**: PGL_3 + 900 bp + luciferase.

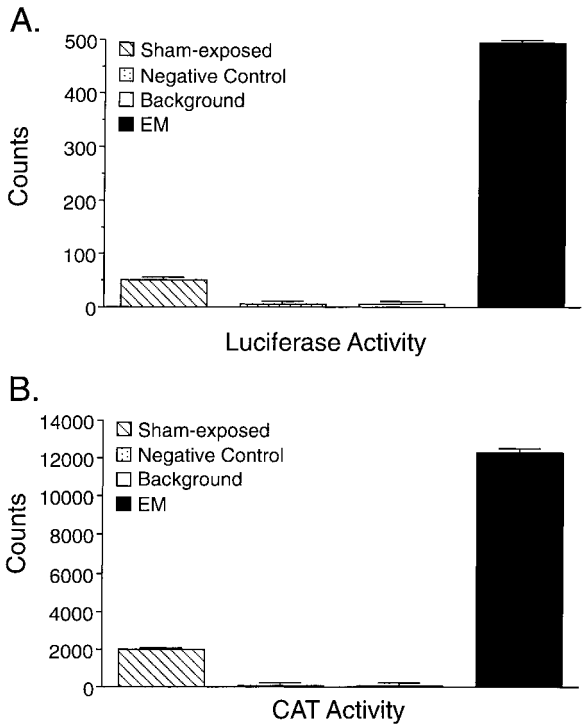

Fig. 2. CAT and luciferase activities. Samples in lane 1 were sham-exposed (30 min); samples in lanes 2, 3, and 4 were exposed to 8 μT 60 Hz EM fields (30 min). **A:** (1) luciferase activity in protein extracted from transfectants containing luciferase construct *plus* the 900 bp insert (sham-exposed); (2) luciferase activity using non-specific protein (negative control) (EM field-exposed); (3) luciferase activity in protein extracted from transfectants containing luciferase construct *minus* the 900 bp insert (EM field-exposed); (4) luciferase activity in protein extracted from transfectants containing luciferase construct *plus* the 900 bp insert (EM field-exposed). **B:** (1) CAT activity in protein from transfectants containing CAT construct *plus* the 900 bp insert (sham-exposed); (2) CAT activity using non-specific protein (negative control) (EM field-exposed); (3) CAT activity in protein from transfectants containing CAT construct *minus* the 900 bp insert (EM field-exposed); (4) CAT activity in protein from transfectants containing CAT construct *plus* the 900 bp insert (EM field-exposed).

heat shock, as expected from the absence of heat shock consensus sequences (nGAAn) in this plasmid construct.

EMREs Increase CAT Activity in Constructs Exposed to EM Fields

In similar experiments with a CAT reporter construct, the 900 bp region from the c-*myc* promoter containing eight nCTCTn was ligated to pΔ11- CAT (Fig. 1A), transfected into HeLa cells, and the transfectants exposed to an 8 μT 60 Hz field for 30 min, followed by an additional 30 min out of the field prior to protein extraction for the CAT assay. There was an average 60% increase in CAT activity. The same three sets of controls described above were employed in these experiments (Fig. 2B): (1) sham-exposed transfectants, served as controls for EM field exposure, and showed no significant CAT activity; (2) transfectants containing the CAT reporter construct *without* the 900 bp insert (pΔ11-CAT) served as controls for background; protein extracts from these transfectants showed no measurable CAT activity; and (3) non-specific protein served as negative controls. Transfectants with and without the 900 bp insert were heat shocked for 30 min at 43°C followed by protein extraction after an additional 30 min out of the heat. There was an average 45% increase in CAT activity in heat shocked transfectants. The pΔ11 plasmid contains the heat shock domain, −106 to −67, therefore response to heat shock served as an additional control.

DISCUSSION

Since EM fields penetrate tissues without attenuation, they must penetrate to the cell nucleus with its DNA and interact with moving charges there [Blank and Goodman, 1999]. That there are conducting electrons in DNA has been shown by Porath et al. [2000], who have made direct measurements of electrical transport through DNA, and by Wan et al. [1999], who have measured the dynamics of DNA-mediated electron transfer at the femtosecond level. Conduction in DNA appears to depend on specific structure, since different DNA sequences have different conductivities [Meggers et al., 1998]. Therefore, EM fields could theoretically interact preferentially with specific DNA sequences, and the nCTCTn sequences (EMREs) in the HSP70 and c-*myc* promoters used in these studies may be such sequences.

Of course, at this stage, it is possible for some unidentified indirect mechanism to be at play, but we have shown that these sequences are critical for EM field responsiveness in our experiments, and other data appear to support this. Verdugo-Diaz et al. [2000], in totally unrelated investigations, showed that low frequency EM field stimulation in nigro-striatal lesioned rats with chromaffin transplants in-

duced changes in the subventricular zones and led to significant motor improvements in a rat Parkinson model. A second report from the same laboratory [Olivares-Banuelos et al., 2000] has used differential display to analyze possible alterations in DNA of EM field-exposed chromaffin cells. Differential bands observed in the EM field-exposed group show changes in gene expression induced by EM fields. One specific differential band in the EM field-exposed samples, containing 349 bp, was sequenced. In an independent analysis of this DNA fragment we have identified three copies of the EM field response element (nCTCTn) that we describe in this report. A computer search is currently underway to determine whether this 349 bp DNA fragment is contained in the promoters of any known genes, possibly a specific gene related to the differentiation process of chromaffin cells.

Advantages of Using EMREs for Gene Therapy

Gene therapy was proposed about 20 years ago as a way to ameliorate genetic defects by providing a source for missing essential genetic components. The injection of *copies of the gene* responsible for the production of a specific protein directly into the targeted area by means of a viral vector was considered a mode of insuring that the protein required would be synthesized at the site where it was needed. This approach offered a distinct advantage over prior conventional treatment of metabolic diseases, which required continuous injection of *gene product* from exogenous sources.

The principle behind gene therapy is simple, but practical application has been difficult. Failure of early gene therapy was mainly due to three problems:

1) difficulties in efficiently transducing primary quiescent human cells in vivo;
2) strong immune responses to the gene therapy vectors, as well as to the foreign therapeutic transgenes that rapidly eliminated transgene expressing cells in humans;
3) the ability of many cell types to shut off the viral promoters that controlled transgene expression in humans.

One positive outcome of these early efforts at gene therapy was the demonstration that introducing cloned genes into humans could be safe, with little or no morbidity. More recently, new vectors have been engineered, including adenoviruses and even naked DNA, enhancing the efficiency of in vivo gene delivery and reducing the immunogenicity of vectors and transgenes.

We have demonstrated that EM fields induce gene expression [Goodman and Blank, 1998; Lin et al., 1999] and that activation of the gene by EM fields requires specific EMREs, which control genes when placed upstream of reporter constructs. Their ability to confer EM field responsiveness suggests the use of EMREs in the control and regulation of gene therapy. The characterization of a cellular promoter system that can be regulated, such as described here, provides a novel, noninvasive technique for the regulation of transgene expression in humans without interfering with normal physiologic function. The applied EM field can be directed to the region where the gene product is needed and, since the EM field intensities needed to affect EMREs are well below the human perception threshold, their introduction and presence would not be felt by the patient. An example of such application would be the introduction of an exogenous insulin gene containing one or more EMREs placed upstream of the gene. Regulation would be provided by the simple and safe application of EM fields. The whole operation would be made automatic by having the EM field generating circuit activated by an implanted glucose sensor responsive to pre-set blood glucose levels.

How Many EMREs are Required for EM Field Responsiveness?

Our results show that the eight nCTCTn sequences (EMREs) in the 900 bp DNA fragment from the c-*myc* promoter are effective in regulating CAT or luciferase activity. However, not all eight EMREs may be needed for a response [Lin et al., 1999]. We previously demonstrated that the EM-induced expression of HSP70 is mediated through *three* EMREs in the human HSP70 promotor. EM field exposure of HSP70 promoter constructs, linked to a CAT reporter gene and containing all three sites, showed more than a threefold increase in CAT activity. Yet, the presence of even one site was sufficient for a 1.5-fold increased CAT response. These data show that even a single EMRE can promote interaction with EM fields. The data also suggest that the level of interaction appears to be roughly proportional to the number of EMREs.

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