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BIOLOGICAL EFFECTS OF ELF-EMF ENHANCED STRESS RESPONSE: NEW INSIGHTS AND NEW QUESTIONS

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ABSTRACT

The effect of extremely low frequency electromagnetic fields (ELF-EMF) on gene expression and early development has been investigated in two different transgenic animals, the nematode *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively. ELF-EMF enhanced biological reactions considerably in the presence of a second stressor (mild heat shock). In *C. elegans*, this effect could be demonstrated at the level of heat shock protein (hsp) gene expression by means of a lacZ reporter gene controlled by an hsp 16 or hsp 70 promoter. In *Drosophila*, the same experimental strategy led to ELF-EMF induced developmental defects, as well as to a considerable retardation of development. The findings are discussed with respect to possible molecular mechanisms that might explain the observed synergistic ELF-EMF induced enhancement of the stress response. An experimental approach is suggested which may help to unravel the involved signal transduction pathways.

INTRODUCTION

The technological advances of the last decades were, to a large extent, based on the usage of electricity. Everybody is exposed to anthropogenic electro-

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magnetic fields of various frequencies and intensities, and this raised concerns about possible negative effects on human health. Many studies have been conducted addressing whether extremely low frequency electromagnetic fields (ELF-EMF) of 50 Hz (Europe) and 60 Hz (U.S.A.) produce significant biological effects. Despite strong research efforts, progress has been slow. The reasons for this are many. Since the biological targets of ELF-EMF are not known, it is difficult to devise convincing experiments. Furthermore, the observed effects are often transient and difficult to monitor, and when criteria are used that are relevant for the assessment of environmental hazards, for example, mutagenicity or the induction of developmental lesions, the effects typically have been small, difficult to reproduce, and in many cases statistically insignificant. Another disturbing observation is that the biological effects of ELF-EMF do not follow a simple dose–response relationship that is characteristic of chemical environmental hazards. These and other problems have thwarted a molecular analysis of the causal effects of ELF-EMF on cells. The numerous and quite diverse findings concerning the effects of extremely low frequency electric and magnetic fields on living systems and the problems in interpreting the data have been discussed extensively (reviewed in refs. 1 and 2).

From these observations, the picture emerges that cells are physiologically well buffered against negative effects of ELF-EMF. We speculated that clearer biological effects of ELF-EMF might be obtained if the cells were put under stress and thus depleted of their regulatory capacity. Under these circumstances, an additional weak stressor like ELF-EMF might produce large effects. We decided to make use of the heat shock response and speculated that a moderate and controlled stress (hereafter referred to as ''costress'') might ''sensitize'' the animals to ELF-EMF exposure. We considered this effect possible in view of the finding of Goodman *et al.* (3) that ELF-EMF induces heat shock proteins (hsp) in the absence of any thermal stress. Biological effects of the heat shock response have been studied successfully in a large number of different cell types and different organisms. As a result, experimental systems are readily available to analyze the ELF-EMF mediated heat shock response and its biological consequences.

We decided to use invertebrate model systems and not an in vitro system as a first approach, since permanent cell lines typically show a weaker heat shock response than cells of experimental animals or of primary cultures subjected to the same stressor. The invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* were our test systems of choice, since suitable genetic strains are available and transgenic animals can be produced routinely if this is desirable for future work.

In this communication, some recent experiments will be briefly summarized, in which the effects of ELF-EMF were studied under thermal costress conditions. Based on the encouraging results, possible molecular mechanisms will be discussed with the aim toward devising a successful experimental strategy that overcomes the obstacles that have hindered progress in the field in the past.

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MATERIALS AND METHODS

Exposure to the Stressors

An experimental setup was used that allowed us to expose the animals to magnetic flux densities up to 150 μT (50 Hz) with an accuracy of ±2%. The ambient temperature in exposure chambers made of plastic could be controlled with an accuracy of about ±0.1°C. Technical details have been published by Junkersdorf *et al.* (4).

Animal Stocks

A transgenic *C. elegans* stock (pPCZ1) was used in which a lacZ reporter gene was placed under the control of the hsp 16 promoter (from *C. elegans*) so that the expression of the lacZ gene could be used to quantify the heat shock expression (5). This was achieved by scoring the percentage of lacZ expressing animals in the population of exposed animals. Another transgenic *C. elegans* stock (pD2047; also referred to as CB 4027) in which the lacZ reporter gene was under the control of the hsp70 promoter (from *Drosophila*) was used to quantify the lacZ expression photometrically (6). Further details have been published elsewhere (4).

The transgenic *Drosophila* stock, engrailed-lacZ, was used to monitor effects of ELF-EMF on early development. In this stock the lacZ reporter gene is placed under the control of the *engrailed (en)* promoter. The segmental β-galactosidase expression pattern facilitates scoring of developmental defects. Staged embryos from the same batch of flies were divided up into 3 groups: one group served as control (25°C), another group was thermally stressed (34 to 37°C in different experiments; see Table 1), and the third group was exposed to the stress temperature as well as to ELF-EMF (100 μT, 50 Hz). Following exposure to the stressor(s) the embryos were left to develop for 16 h at 18°C. The embryos were fixed, stained with X-gal as a substrate to visualize β-galactosidase activity, and pattern defects as well as the developmental stage were recorded (see ref. 7 for details).

RESULTS AND DISCUSSION

ELF-EMF Enhances the Heat Shock Response in *C. elegans*

Based on the finding that ELF-EMF induces the heat shock response (3,8), we chose to use the transgenic strain pPCZ1 of the nematode *C. elegans* for our studies since the expression of a lacZ reporter gene under the control of the hsp 16 promoter (see Materials and Methods) allowed us to easily quantitate the heat shock response by simply scoring the fraction of stained animals expressing β-galacosidase. The method was introduced by Stringham and Candido (5).

Our attempts to study the effect of ELF-EMF under thermic costress conditions required that we first carefully define the costress conditions. We reasoned

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that a suitable costress would be a significant heat shock response, which should be below 50% of the maximal inducible level (all animals expressing β-galacosidase). Conditions were defined in which about 40% of all animals expressed the reporter gene (29°C for 1 h). It turned out that it was essential to control the temperature with an accuracy of 1/10°C since only 1°C more or less than 29°C provided unsuitable costress conditions. When the nematodes were additionally exposed to ELF-EMF (100 μT) the percentage of stained nematodes rose from 40% to 87% and hence more than doubled (4)! When 50 μT and 150 μT were tested, the percentage of stained animals was 78% and 89%, respectively. Presumably, much smaller magnetic flux densities than 50 μT would have shown an effect, but this has not yet been systematically tested. Exposing the animals to ELF-EMF at the control temperature (18°C) did not increase the percentage of stained animals.

The ELF-EMF enhanced heat shock response can also be elicited using the hsp70 promoter from *Drosophila* (strain pD2047), thus confirming the results obtained with the hsp16 promoter construct. However, the costress conditions had to be defined for the pD2047 strain again, and 30°C proved to be the optimal costress temperature during the ELF-EMF exposure (4).

Most of the experiments with *C. elegans* and all of the experiments with *Drosophila* described below were carried out at flux densities of 100 μT. No attempt was made to increase the field strength. It was pointed out before that a dose-response relationship cannot be expected in the study of ELF-EMF effects. The work of Bessho *et al.* (9) is very telling in this respect. These authors subjected *C. elegans* to extremely high magnetic flux densities with a peak flux density of 1.7 T (60 Hz). In these experiments the chamber of the nematodes had to be cooled with chilled water of a flow rate of 90 L/min since without the cooling system the temperature in the chamber would have increased to about 600°C. Despite these extremely strong fields the treatment was well tolerated by the animals and only modest and reversible biological effects were recorded.

ELF-EMF Affects *Drosophila* Development under Costress Conditions

While the gene expression studies summarized above are useful to identify and quantify different stressors acting singly or synergistically at the transcriptional level, possible effects of the stressors on the animal's physiological function, vitality, or development must be addressed in different types of experiments.

We chose to study the early development of *Drosophila* using the same combination of stressors as in the experiments with *C. elegans* described above. Transgenic strains were used for the analysis in which a lacZ reporter gene was placed under the control of promoters of genes involved in segment formation during embryogenesis (e.g. *engrailed, fushi tarazu*, or *sloppy paired 2*). The char-

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acteristic segmental β-galactosidase staining patterns greatly facilitated the identification and scoring of developmental defects. Figure 1 illustrates the experimental approach. Any deviation from the normal pattern was scored as abnormal. In *Drosophila*, a small percentage of anomalies is typically observed in untreated controls, and their frequency depends on the strain used as well as the developmental history of the flies used for the egg deposition (crowding conditions of larvae, food availability, temperature, etc.). For this reason it is important to use the same batch of flies for all experiments whose results are to be compared directly.

In a series of experiments, staged embryos were exposed for 30 min to ELF-EMF (100 μT) in the presence or absence of thermal costress (7). The costress temperature was not as critical as in the experiments with the nematode *C. elegans* (see above) and 34 to 37°C produced the expected effects. The analysis was limited to the early phase of embryogenesis, i.e., cellularization (2 h 30 min–3 h), early gastrulation (3 h–3 h 30 min), and late gastrulation (3 h 30 min–4 h).

The observed defects included lacking segments, fused segments, or irregular and patchy expression of β-galactosidase. Since it was not our aim to score for specific defects like phenocopies of pattern mutants (see below), no attempt was made to classify the observed anomalies in separate categories. Exposure to just ELF-EMF (without thermal stress) did not result in any apparent increase in pattern defects above control levels. This finding is in keeping with the results of Nguyen *et al.* (10) who subjected embryonic cells of *Drosophila* to weak electromagnetic fields for 16 to 18 h (10 or 100 μT, 60 Hz) and did not obtain any evidence of developmental toxicity in their test systems. An exposure of 24 h or even for the entire *Drosophila* life cycle did not increase the number of develop-

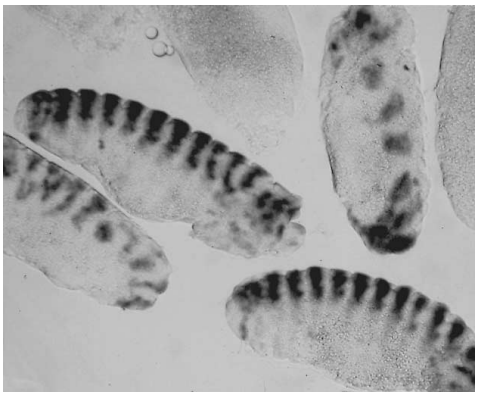

Figure 1. Following exposure to the stressors for 30 min (ELF-EMF and raised temperature), the *Drosophila* embryos of the strain engrailed-lacZ were left to develop for 16 at 18°C, fixed and stained for β-galactosidase activity using X-gal as a substrate. The photograph shows two short germ band embryos with a normal segmentation pattern; in other embryos the pattern is disturbed and characterized by an abnormal pattern of β-galactosidase expression.

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Table 1. Effect of Heat and ELF-EMF on the Formation of Embryonic Pattern Anomalies in *Drosophila*[a]

		25°C (Control) (%)	Stress Temperature (%)	Stress Temperature + 100 µT (%)
2h 30 min–3h:	34/35°C	100	152 ± 85	256 ± 70
	36/37°C	100	513 ± 274	997 ± 294
3h–3h 30 min:	34/35°C	100	94 ± 93	163 ± 59
	36/37°C	100	98 ± 70	526 ± 625
3h 30 min–4h:	34/35°C	100	125 ± 68	140 ± 64
	36/37°C	100	175 ± 140	375 ± 387

[a] The number of anomalies in the controls (25°C) was used as reference and set to 100%. The standard deviation is given. The magnetic flux density was 100 µT.

mental defects by their rather crude scoring system (curly wings and abdominal errors) which was originally designed to assess effects of chemical teratogens. These experiments were carried out at a temperature of 24°C.

In our experiments, we followed a different experimental strategy. The results gave clear evidence that ELF-EMF increased the frequency of abnormally developing embryos under costress conditions (Table 1). The variation between different experiments was considerable, yet under all conditions tested the enhancing effect of ELF-EMF was apparent. The earliest stage tested was most sensitive to developmental disturbances. At this critical stage of development, the activation of the embryonic genome and the genetic expression of major patterning genes are initiated. With respect to the costress, the higher temperatures (36–37°C, data pooled) gave clearer results than the lower temperatures tested (34–35°C).

Another striking effect of ELF-EMF was observed during the course of the experiments: the exposed embryos' development slowed down considerably. The inhibitory effect on the rate of development is illustrated in Figure 2. Similar experiments with other genotypes gave essentially the same results (not shown). The large fraction of embryos in the youngest age group (2 h 30 min–3 h) which could not be classified reflect the high sensitivity to stressors and the resulting lethality (see above). The delayed development is particularly obvious in the two older age groups. While the costress treatment of 30 min alone had only a minor effect on the rate of development as compared to the stress-free controls (36°C compared to 25°C), the exposure to ELF-EMF had the effect that almost all embryos were in the long germ band stage at the end of the incubation period, whereas the group exposed only to the costressor was almost exclusively in the more advanced short germ band stage (Fig. 2). The development from a typical long germ band stage (e.g., stage 11) to a characteristic short germ band stage (e.g., stage 13) takes about 3 h at 22°C (11), and at the culture temperature of 18°C the period between these stages of development is even longer. Hence, the inhibitory effect

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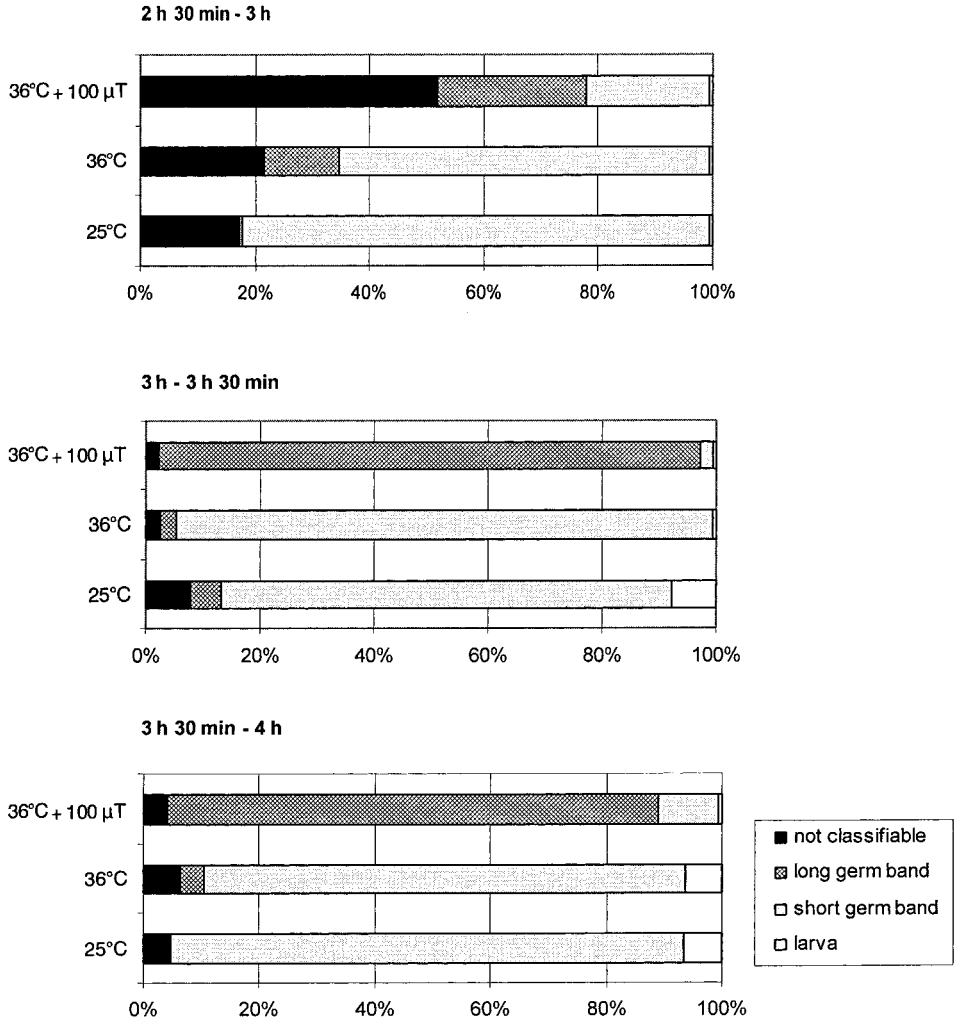

Figure 2. After exposure to the indicated stressor(s) followed by stress-free development for 16 h at 18°C, the developmental stage of the developing embryos was determined and classified into the four categories as indicated. Note that ELF-EMF strongly inhibits development in the presence of the costressor (36°C) since most embryos have reached the extended germ band stage, while in the control embryos (36°C) most embryos are in the more advanced short germ band stage. The data were taken from Michel and Gutzeit (7) and redrawn to illustrate the effect of ELF-EMF on the rate of development.

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lasted considerably longer than the exposure period (30 min). Much smaller effects of ELF-EMF on the rate of embryogenesis have been noticed before in other species (e.g., ref. 12).

How Does ELF-EMF Interact with Thermal Stress?

The experimental strategy to study the biological effects of ELF-EMF in the presence of a costressor proved to be successful, and clear biological effects were obtained. In a recent study, Tsurita *et al.* (13) used a very similar approach. They studied the expression of hsp70 in human tissue culture cells stimulated by strong repetitive pulsed magnetic fields (34 mT, 50 Hz). A clear stimulatory effect of the fields was only obtained when at the same time the temperature was raised from 37°C to 42°C, thus providing an efficient costress in our terminology.

The experimental results described above suggest that the stressors act synergistically so that at some point the signal transduction pathways are likely to converge. Evidence was presented by Lin *et al.* (8) that ELF-EMF (60 Hz, 8 μT) may act in a similar way as hyperthermia by activation of heat shock factor 1 and binding to the heat shock element present in the promoters of stress-inducible genes. However, the energy density of magnetic and thermal stimuli that evoke a stress response differ by 14 orders of magnitude (14). Hence it seems improbable that weak ELF-EMF could account for (partial) unfolding of proteins. Incorrectly folded proteins are known to elicit the heat shock response (15). If ELF-EMF cannot trigger the heat shock response in this way, there should be other targets and signal transduction pathways to mediate the response. This is also suggested by the puzzling observation that ELF-EMF effects are not characterized by a dose-response relationship that is usually expected in toxicity testing (see above). The suggested presence of ''energy windows'' gave rise to molecular models that invoked a resonance effect (see, for example, ref. 16 and discussion in refs. 2 and 17). Be that as it may, between the target and the effects on the transcriptional level, an amplification cascade is likely to be present.

Interestingly, evidence was presented suggesting ''that the binding of *c-myc* protein on the hsp70 promoter is integral in the regulation of magnetic field–induced hsp70 gene expression'' (14). This finding and the assumptions and facts mentioned above are incorporated into a general interaction model (Fig. 3) that despite many uncertainties may be useful in defining the experimental strategy in future work.

How could the observed developmental effects be explained? A strong heat shock at critical phases of development is sufficient to cause malformations. Sublethal treatment of *Drosophila* may produce anomalies that mimic the effects of known mutations and are hence referred to as phenocopies. Early stages of embryogenesis are particularly susceptible to phenocopy induction. The developmental lesion closely corresponds to well-known patterning genes in segment formation and segment specification (18 and references cited in that paper). The molecular reactions that give rise to the defined defects is not known, but since

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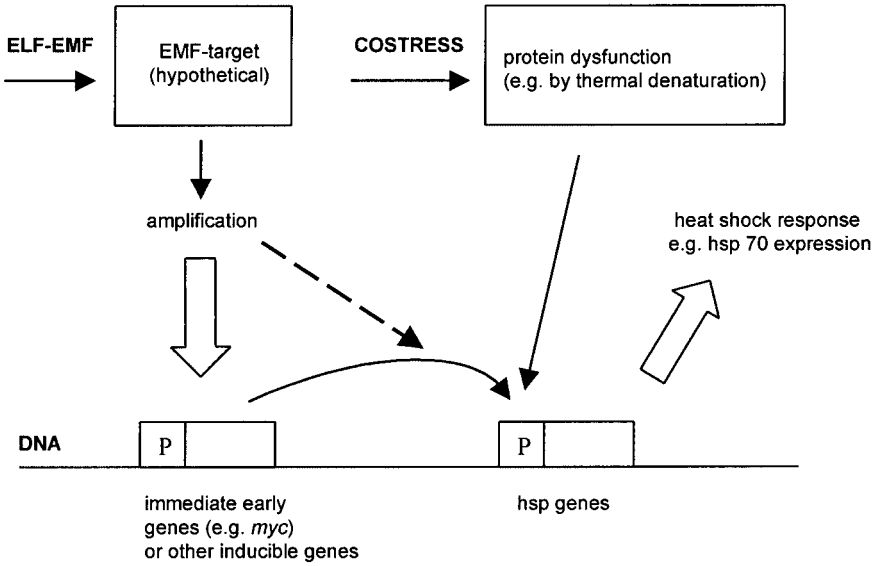

Figure 3. Possible molecular interactions that might result in the observed synergistic interaction of stressor (ELF-EMF) and costressor (heat). The dashed line indicates a possible pathway for which, however, the experimental evidence is weak. P: promoter of the respective genes.

the heat shock response involves complex reprogramming of cellular activities including transcription and translation, it is plausible that pattern gene products may reach critical levels, thus leading to the observed anomalies.

The finding that ELF-EMF delays early development of *Drosophila* can also be explained by the effects of a strong heat shock. The cytoskeleton is known to be severely affected by heat shock, and cell cycle arrest is commonly observed (reviewed in ref. 19). At the optimal temperature for phenocopy induction (which is close to the lethal temperature), the recovery of gene expression is delayed by 3–5 h. These data correspond well with the observed developmental delay in our experiments. Furthermore, *Drosophila* tissue culture cells which overexpress hsp70 at normal temperatures show a reduced rate of cell growth (20).

The combined effects of both stressors, i.e., mild heat shock and ELF-EMF, resemble a single strong thermic stress in their biological reactions. We obtained no evidence of an ELF-EMF–induced specific phenotype. In view of the limited number of morphological criteria used and the lack of molecular data it would certainly be premature to suggest that ELF-EMF specific effects cannot be produced. However, the available evidence suggests that using our experimental strategy ELF-EMF merely enhances the heat shock reaction (costress).

Where To Go from Here?

The relevance of interacting stressors in our environment affecting all organisms is a point that needs to be addressed in future studies. Since the heat shock

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response can be elicited not only by thermal stress but also by a large number of environmental chemicals, costress conditions and the interaction of different chemical and physical stressors appears to be a realistic scenario. The apparent synergistic interaction of stressors poses a formidable challenge for a realistic risk assessment of the stressors.

Wiegant *et al.* (21) showed that cells react with a strong heat shock response if they are first subjected to a moderate heat shock, followed by treatment with low doses of a chemical stressor. Presumably, the authors observed the same phenomenon of costress that we have analyzed in this study. Interestingly, in their experiments, the second stressor with the enhancing effect determined the type of stress reaction, as indicated by the characteristic expression levels of the different heat shock proteins, demonstrating the molecular character of the stress reaction. This observation is particularly interesting, since it could help to unravel the molecular interaction of stressors, including the biological effects of ELF-EMF. Conceivably, ELF-EMF activated genes (see Fig. 3) could enhance transcription of an already activated set of heat shock genes (costress) in a differential way, thus providing an adequate and optimal defense reaction to the second stressor.

The observed clear biological effects of ELF-EMF under costress conditions facilitate a molecular approach. First of all, we need to know more about the signal transduction cascade and the inferred signal amplification downstream of the ELF-EMF target. By means of the recently introduced cDNA array technique, changes in the expression of hundreds of relevant genes can be identified at the same time. Studies on this level of complexity will, no doubt, be useful in future work on the activation of stress-related gene transcription and the involved signal transduction pathways that are activated by the stressors.

The identification of the molecular target(s) of ELF-EMF appears to be a major challenge which, if completed successfully, may pay a rich scientific dividend. The collaboration of cell biologists with biochemists and physicists is likely to be the most promising approach.

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