1082–1084 *Nucleic Acids Research, 1997, Vol. 25, No. 5*

Transcriptional modulation of viral reporter gene constructs following induction of the cellular stress response

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Received October 18, 1996; Revised and Accepted January 14, 1997

ABSTRACT

In this study, we report that commonly used methods of transient transfection induce the cellular stress response and a recovery period is required following transfection when analyzing cellular stress responsive genes. Four transfection methods were examined for their ability to induce the stress response by measuring the expression of heat shock protein (hsp) 72. We demonstrate that electroporation increases expression of hsp 72 in HUT 78 cells. Additionally, DEAE–dextran and liposome-mediated transfection resulted in increased hsp 72 expression in an adherent cell line (HeLa). Liposome-mediated transfection differentially induced cell stress, dependent on the transfection time in serum-free culture conditions. The stress responsiveness of two viral promoters, the HTLV-1 long terminal repeat and CMV immediate early transcriptional unit were examined. We found the maximal stress-mediated enhancement of transcription with both promoters did not occur until the cells recovered for 24 h following transfection.

A variety of physiologic events modulate gene transcription in eukaryotic cells including cellular differentiation or response to cell activation signals (1). These events ultimately increase or decrease transcription by activating factors which subsequently bind response elements of the gene. Transient transfection assays are an important tool for investigating transcription, however changes in the physiologic state of the cell may influence or mask transcription of a particular gene. Several methods of transient transfection assays are available. Knowledge of the physiologic state of cells following the application of these techniques facilitates accurate interpretation of data from such studies.

In this study, we addressed the modulation of viral promoter expression following induction of the cellular stress response, a physiologic response to adverse changes in the cellular environment. Cells can initiate a stress response when exposed to a variety of metabolic and chemical insults, including hyperthermia, oxidizing agents, transition series metals, drugs affecting membrane structures (i.e. ethanol), serum stimulation, mitogens and lymphokines (2). However, it was unclear if commonly used methods of transfection can induce the cellular stress response, thus affecting transcriptional assays. To address this problem, we evaluated four commonly used methods (liposome-mediated, calcium phosphate, electroporation and DEAE–dextran) of transient transfections for their ability to induce the expression of hsp 72, which is a highly inducible member of the hsp 70 family and a sensitive indicator of stress response induction (3).

We determined hsp 72 expression following electroporation in HUT 78 cells (4) because we had previously optimized electroporation with this cell line (5) and the relative ease of performing electroporation with a suspension culture compared to an adherent cell line. Twenty-four hours following electroporation, the expression of hsp 72 was determined by flow cytometric analysis as previously described (5) and the data presented as the percent expression as compared to non-transfected controls. Transfection of HUT 78 cells by electroporation resulted in increased expression (41%) of hsp 72 (as compared to non-transfected, non-stressed controls) (Fig. 1A).

HeLa cells were transfected by liposome-mediated transfer using a commercially available product (Transfectam®, Promega, Madison, WI). Hela cells were transfected for 3, 6 or 24 h following the recommended assay protocol for media without serum by the manufacturer. Following transfections, media was removed and cells returned to regular culture conditions (Eagles's minimal essential medium supplemented with 10% FCS, 1.2 mM glutamine, 60 U/ml penicillin and 60 μg/ml streptomycin) and hsp 72 expression determined 24 h later. In HeLa cells the serum-free conditions of liposome-mediated transfection resulted in an increased expression of hsp 72 which correlated with the amount of time the cells were cultured in serum-free media (Fig. 1B). Serum-free conditions for 3 h did not result in hsp 72 expression as determined by flow cytometry. Cells left in contact with serum-free medium for 6 and 24 h had a mean expression (16 and 37%) of hsp 72 respectively (Fig. 1B).

Hela cells were transfected by calcium phosphate mediated transfer (5 Prime→3 Prime, Inc., Boulder, CO) or DEAE–dextran-mediated transfer (Promega, Madison, WI) using the

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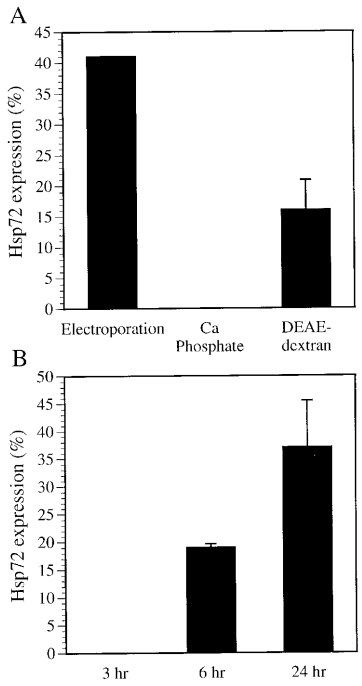

Figure 1. Transient transfection methods differentially induce hsp 72. Immunofluorescent staining for hsp 72 was determined by flow cytometric analysis and data presented as percent expression over non-transfected controls. (**A**) The expression of hsp 72 was determined 24 h following calcium phosphate and DEAE–dextran methods of transfection in HeLa cells. Percent expression of hsp 72 was determined in HUT 78 cells following electroporation. (**B**) Hsp 72 expression was determined following liposome-mediated transfection of varied times in serum-free conditions. × ± s.e.m., $n = 3$ (liposome-mediated, calcium phosphate, DEAE–dextran).

recommended assay protocol by the manufacturer. Calcium phosphate-mediated transfections did not elicit increased expression of hsp 72 in HeLa cells (Fig. 1A). DEAE–dextran-mediated transfection resulted in a mean expression of 16% of hsp 72 in transfected cells as compared to non-transfected controls (Fig. 1A).

While many chemical or physical agents are known to induce the stress response, the mechanisms in which they mediate the response with subsequent enhancement of hsp gene expression is complex and not completely understood (2). Electroporation mediates entry of plasmid DNA into cells by generating an electric field which creates cellular pores through which the DNA diffuses. However, the high-voltage electrical pulse can result in membrane disruption and alteration of protein structure (i.e. denaturation) (6) which are known to induce the stress response (7–9).

Serum-free conditions greatly enhance transfection efficiency when using liposome-mediated DNA delivery system (10). Liposome-mediated transfections in serum-free conditions are often performed overnight, however we found that the degree of expression of hsp 72 correlated with the amount of time the cells remain in serum-free conditions before returning to normal culture condition (i.e. serum-stimulation). Serum-stimulation following serum-free conditions is a potent inducer of the stress response (11). Therefore, when analyzing stress responsive genes, long transfection times with this method may inhibit or mask a stress-mediated response.

The liposome-mediated DNA delivery system resulted in the greatest transfection efficiency in HeLa cells as compared to calcium phosphate and DEAE–dextran (data not shown). This was determined by comparing reporter gene activity following transfection with an SV40 luciferase reporter plasmid (pGL2-Control, Promega, Madison, WI). Because of the pronounced induction of hsp 72 in HUT 78 cells following electroporation, we excluded this method from promoter analysis studies. A 3 h transfection time with liposome-mediated transfer (i.e. serum-free conditions) did not result in increased expression of hsp 72 by flow cytometric analysis (Fig. 1B), therefore we chose this transfection protocol for subsequent kinetic experiments.

Even though no increase in expression of hsp 72, as compared to controls, was found with a 3 h transfection time, we wanted to establish if the cells still required a recovery period before stress induction. To test the influence of an optimized transfection method on promoters known to be responsive to cellular stress, we examined both the human T-cell lymphotropic virus type 1 long terminal repeat (HTLV-1-LTR) (12) and human cytomegalovirus immediate early transcriptional unit (CMV-IE) (13). The CMV-IE luciferase plasmid contains the immediate early gene cloned into the *Xho*I–*Sal*I site of a luciferase reporter plasmid (pGL2-Basic). The stress response was induced in HeLa cells with 640 μM of Na arsenite as previously described (12) at 6 or 24 h following transfection. Luciferase activity was measured by liquid scintillation counting following the manufacturer's recommendations (Promega) in cell extracts collected at 3, 6, 12 and 24 h post-stress and compared to non-stressed extracts. Both promoter constructs were non-responsive to cell stress when only allowed a 6 h recovery prior to induction of the cellular stress response by Na arsenite (Fig. 2A and B). However,when the cells remained in normal culture conditions for 24 h before induction of stress, an increase in promoter activity was observed at 3, 6, 12 and 24 h following transfection (Fig. 2C and D).Our data indicates that maximal stress responsive gene activity with the HTLV-1-LTR and CMV-IE promoters is dependent on the cells being maintained in normal culture conditions for 24 h before induction of the cellular stress response. The lack of promoter responsiveness with only a 6 h recovery period may be related to multiple variables,which includes phase of cell cycle (e.g. G_1/S block) or development of thermotolerance initiated by the serum-free to serum-stimulation conditions of the liposome-mediated transfections (14). Thus, the cells must be allowed to recover >6 h before maximal induction of the stress response can be elicited.

In conclusion, we found that certain transfection techniques do result in induction of the cellular stress response. Therefore, when analyzing stress-mediated changes in gene expression by transient transfection methods, the technique itself may mask any observed changes. Additionally, we demonstrate that the maximal stress-mediated enhancement of transcription with both promoters did not occur until the cells recovered for 24 h following transfection. In this study we used two viral promoters as a model system to study stress-mediated enhancement, however our data have important implications for investigations of any cellular stress responsive genes employing these commonly used transfection methods.

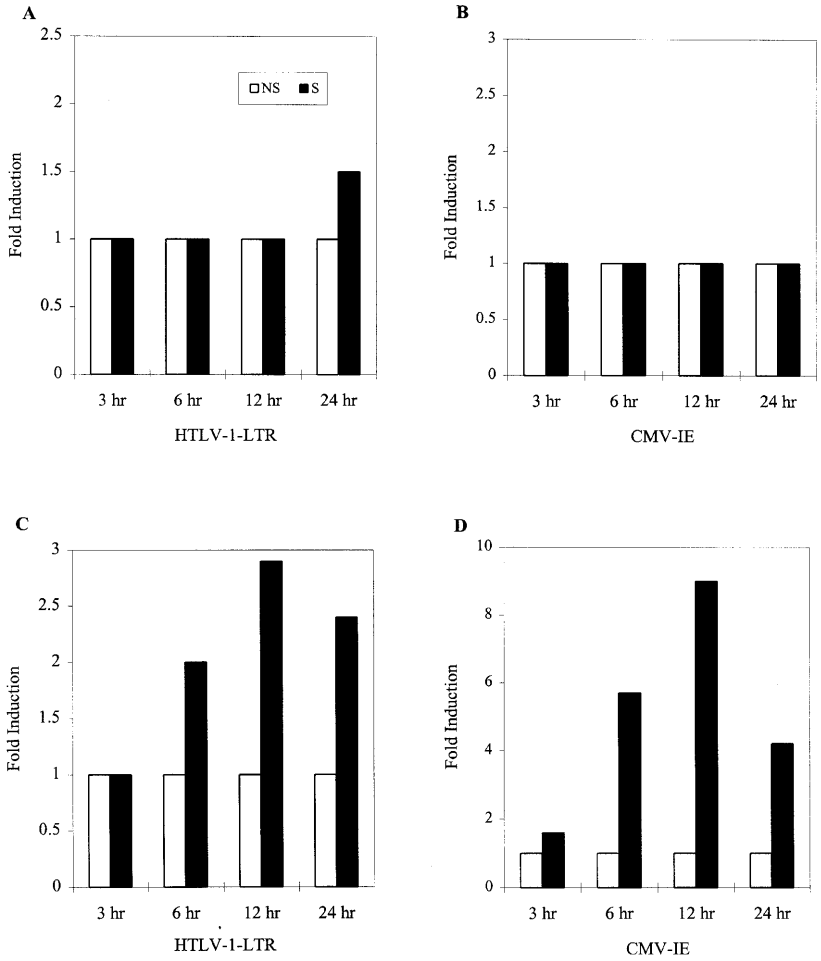

Figure 2. Maximal stress induced increase in promoter activity was detected after HeLa cells rested for 24 h following liposome-mediated transfection. The stress response was induced at 6 h (**A** and **B**) or 24 h (**C** and **D**) following transfection with an HTLV-1-LTR reporter construct and a CMV-IE reporter construct. Reporter gene activity was determined at 3, 6, 12 and 24 h following stress induction. Luciferase activity of extracts from non-stressed transfected cells was assigned a value of 1.0. The data is presented as the fold increase in luciferase activity.

ACKNOWLEDGEMENT

This work was supported by the National Institutes of Health (National Institute of Allergy and Infectious Diseases grant K11 AI01190).

REFERENCES

1 Tjian, R. and Maniatis,T. (1994) *Cell* **77**, 5–8.
2 Nover, L. (1991) Inducers of hsp synthesis: heat shock and chemical stressors, in Nover, L. (ed.), *Heat Shock Response.* CRC Press, Inc. Baca Raton, Florida, pp. 6–40.
3 Welch, W. J., Kang, H. S., Beckmann, R. P. and Mizzen, L. A. (1991) Response of mammalian cells to metabolic stress; changes in cell physiology and structure/function of stress proteins, in Kaufmann,S.H.E. (ed.) *Current Topics in Microbiology and Immunology,* vol. 167. Springer-Verlag Berlin, Heidelberg, pp. 31–55.
4 Gazdar, A. F., Carney, D. N., Bunn, P. A., Russell, E. K., Jaffe, E. S., Schechter, G. P. and Guccion, J. G. (1980) *Blood* **55**, 409–417.
5 Andrews, J. M., Oglesbee, M. J., Trevino, A. V., Guyot, D. J., Newbound, G. C. and Lairmore, M. D. (1995) *Virology* **208**, 816–820.
6 Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P. H. (1982) *EMBO J.* **1**, 841–845.
7 Edington, B. V., Whelan, S. A. and Hightower, L. E. (1989) *J. Cell. Physiol.* **139**, 219–228.
8 Thomas, B. P. and Mathews, M. B. (1984) *Mol. Cell. Biol.* **4**, 1063–1072.
9 Lee, K.-J. and Hahn, B. M. (1988) *J. Cell. Physiol.* **136**, 411–420.
10 Barthel, F., Remy, J. S., Loeffler, J. D. and Behr, J. P. (1993) *DNA Cell Biol.* **12**, 553–560.
11 Wu, B. J. and Morimoto, R. I. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6070–6074.
12 Andrews, J. M., Newbound, G. C., Oglesbee, M. J., Brady, J. N. and Lairmore, M. D. (1997) *J. Virol.* **71**, 741–745.
13 Geelen, J. L. M. C., Boom, R., Klaver, G. P. M., Minnaar, R. P., Feltkamp, M. C. W., Van Milligen, F. J., Sol, C. J. A. and Van der Noordaa, J. (1987) *J. Gen. Virol.* **68**, 2925–2931.
14 Missen, La. A. and Welch, W. J. (1988) *J. Cell Biol.* **106**, 1105–1116.