Vol. 212, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 6, 1995 Pages 165-171

TRANSCRIPTIONAL REGULATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE BY 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

Susan E. McNulty*† and William A. Toscano, Jr.†‡

†Center for Bioenvironmental Research and Department of Environmental Health Sciences, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA 70112-2699

Received May 5, 1995

Summary: The action of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on transcription and steady-state mRNA levels, protein expression, and enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured human keratinocytes was examined. GAPDH transcription increased as a function of time of exposure to TCDD, reaching a maximum of 23-fold after 8 days. Northern blot analysis showed a 4-fold increase in mRNA after 8 days exposure. Protein levels were increased 4-fold, and dehydrogenase activity by 5-fold, under the same exposure conditions. GAPDH is commonly thought of as a constitutive housekeeping gene and is often used as a loading control for northern blot analysis. Our data suggest that caution should be used when using GAPDH as an RNA control, especially in studies examining cell proliferation and carcinogenesis.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [EC 1.2.1.12], catalyzes the formation of glycerate-3-phosphate, the first ATP-forming step in the Embden-Meyerhof pathway (1). Action of this enzyme is important in regulating the intracellular redox status of cells. The enzymatic activity of the GAPDH gene product is dependent on its subunit structure. The enzyme exhibits redox activity (GAPDH) when present as a homo-tetramer. When present as a monomer the enzyme exhibits DNA excision-repair activity called uracil DNA glycosylase (UDG) (2). UDG repairs DNA by catalyzing the hydrolysis of the N-glycosidic bond between uracil and deoxyribose when incorporation of deoxyuridine triphosphate or deamination of cytosine residues occur in DNA (2). GAPDH is considered by many to be a constitutive housekeeping enzyme whose activity is unaffected by agents that alter cell proliferation. However, during our studies on the action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in which we used GAPDH as a loading control, we observed changes in the intensity of GAPDH in samples of RNA from TCDD-exposed cultures.

2,3,7,8-TCDD is the most potent congener of a related group of polychlorinated compounds known collectively as dioxins. Studies in animals have shown dioxins elicit pleiotropic toxic actions. These include thymic involution, loss of spleen and lymph tissue, hyperplasia and hypertrophy of some tissues including the gastric and intestinal mucosa, urinary tract, lung and skin, as well as tumor promotion (3-5). Associations with lung cancer, soft tissue sarcomas and hepatobiliary carcinoma have

*Present Address: Dept. Biology & Molecular Medicine, UCLA, Los Angeles, CA 90024.
‡To whom correspondence should be addressed. Fax:504-584-1726.

0006-291X/95 $12.00

been found in some epidemiology studies on humans exposed to dioxins (6-8). A definitive role for TCDD in the etiology of human tumorigenesis, however, has not been unambiguously established (9).

Dioxins are neither mutagenic nor do they bind to deoxyribonucleic acid (DNA) directly (3) rather, dioxins exert their toxic actions through the Aryl hydrocarbon (*Ah*) receptor. Dioxins bind to the *Ah* receptor concomitant with dissociation of at least two hsp90 proteins. The ligand-bound complex is transported to the nucleus, where, with the *Ah* receptor nuclear translocation factor, it binds as a heterodimer to a consensus DNA sequence, the dioxin responsive enhancer (DRE) (10-12), inducing transcription of several cytochrome P450 genes (13). Recent studies have demonstrated that expression of several oncogenes and growth factors is also modulated by *Ah* receptor, indicating the toxic mechanism of dioxins lies in alteration of tightly regulated intracellular signaling pathways (14-17).

During the course of studies in our laboratory examining dioxin-modulated transcriptional activation of a number of oncogenes and growth factors in the cultured human keratinocyte cell line, SCC-12F (15), we observed alterations in the level of GAPDH. In this study, we report on the action of dioxin as a modulator of the expression of GAPDH.

Materials and Methods

Cell Culture. SCC-12F clonal human keratinocyte cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5 percent iron-supplemented calf serum (Hyclone) on a feeder layer of lethally irradiated murine 3T3 fibroblasts (18). The cultures were maintained at 37 °C in a humidified atmosphere of 95 percent air and 5 percent CO_2. Confluent cultures were exposed in DMEM to either TCDD (100 nM in 0.1 percent DMSO) or 0.1 percent DMSO.

Nuclear Run-on Analysis. Transcriptional analysis was performed using fractionated nuclei, and transcription was completed in the presence of CTP, GTP, ATP and a limiting concentration of [α-^{32}P]-UTP (19). Quantitation of [α-^{32}P]-UTP incorporated into RNA was made using a liquid scintillation counter. Nitrocellulose filters were bound with Pst I linearized plasmids containing the 1.25 Kb GAPDH insert. For each time point the filters were hybridized at 65 °C for 37 h with equal counts of [α-^{32}P]-UTP-labeled RNA from TCDD- and DMSO-exposed cells. Specific binding of [α-^{32}P]-UTP-labeled RNA was visualized using autoradiography. Relative densities were determined using a BioRad GS-670 imaging densitometer and Molecular Analyst software.

RNase Protection Assay. Following four days TCDD exposure in serum-free medium, the mRNA was measured by RNase Protection Assay. A transcribeable pIBI30 vector that contained the 1.25 Kb GAPDH insert was linearized using Sma I. T7 polymerase was used to synthesize an [α-^{32}P]-UTP labeled anti-sense RNA GAPDH probe. This was purified on a 5 percent polyacrylamide gel containing 8M urea, eluted and hybridized at 45 °C for 16 hours with 9 µg cellular mRNA. Following hybridization, single stranded RNA was degraded using RNase-A. The protected double stranded RNA fragments were separated on a 1.0 percent agarose/ formaldehyde gel, and visualized by autoradiography.

Northern Blot Analysis. TCDD-exposed and control cultures were lysed in guanidinium isocyanate, and total cytoplasmic RNA was collected. Ten-micrograms of each sample was separated by electrophoresis on a 1% agarose/formaldehyde gel (19). To ensure that equivalent amounts of RNA were applied to the gels, duplicate parallel lanes of cellular RNA from control and TCDD-exposed cultures were subjected to electrophoresis and stained with ethidium bromide. The 18S band was visualized by illumination with ultraviolet light. The RNA was transferred to a Biotrans + nylon membrane (ICN, Irvine, CA) and immobilized by ultraviolet irradiation using a GS Genelinker (BioRad). A random priming reaction was used to generate an [α-^{32}P]-d-CTP labeled probe from the

1.25 kb cDNA GAPDH plasmid insert (20,21). The nylon membrane was hybridized for 18 h with the probe at 62 °C in 6X SSPE, 5x Denhardt's, 0.1 mg/ml single stranded DNA, 0.5 % sodium dodecyl sulfate. The labeled GAPDH was visualized by autoradiography. After the blot was stripped of the probe by immersion in 0.1 x SSPE and 0.1 percent SDS at 100 °C, the nylon was stained with 0.02 percent methylene blue in 0.3 M sodium acetate pH 5.5 to determine that equal transfer of RNA had occurred among the samples. Relative densities were assessed using a Model GS-670 imaging densitometer and Molecular Analyst Software (BioRad).

Western Blot Analysis. Cells exposed to TCDD or DMSO for 8 days were collected and lysed in 50 mM Tris-HCl, 150 mM NaCl, 1 percent Nonidet P-40, pH 8.0. Protein was fractionated using ammonium sulfate (0.75 saturation) (22), and dialyzed against water for 16 hours. Equal volumes, weights and percentages of the ammonium sulfate-precipitated cellular proteins were separated on a 15 percent polyacrylamide gel in the presence of sodium dodecyl sulfate (23). Proteins were transferred to 0.22 micron nitrocellulose in a BioRad Transblot electrotransfer apparatus, and blocked with 3 percent bovine serum albumin in saline, containing Tris, pH 8.0. After incubation for 4 hours at 20 °C with mouse monoclonal anti-human GAPDH antibodies (40.10.09, generous gift of M. Sirover, Fels Institute) in 3 percent bovine serum albumin. Bbands were visualized using alkaline phosphatase conjugated sheep anti-mouse IgG (Cappel, Durham, NC), and nitroblue tetrazolium and bromochloroindolyl-phosphate (Sigma) (24).

Enzymatic Analyses. Total cellular GAPDH activity was measured by monitoring the oxidation of 0.1 mM β-NADH catalyzed by cell lysates previously concentrated by precipitation with ammonium sulfate (0.75 saturation) in 83 mM triethanolamine, 3 mM cysteine, 6.7 mM phosphoglycerate, 2 mM magnesium sulfate, 1.1 mM ATP and 5 units phosphoglyceric phosphokinase at 340 nm in a spectrophotometer (22). For nuclear UDG, cells were collected and lysed on ice in 10 mM Tris-Cl pH 7.9, 10 mM NaCl, 5 mM $MgCl_2$, 250 mM sucrose, and the protease inhibitors, leupeptin (2 mg/ml), phenylmethylsulfonylfluoride, N-tosyl-L-lysine chloromethyl ketone, N-tosyl-L-phenylalanine chloromethyl ketone (0.02 mg/ml). KCl was added to a final concentration of 100 mM and the nuclei were collected by centrifugation at 500 x g. Nuclear proteins were collected by lysis in 10 mM Tris-Cl pH 7.9, containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ and 250 mM sucrose. DNA was sheared using a syringe. Cellular UDG activity was assessed in cells that had been sonicated (two times at 60 W, for 15 s) on ice in 20 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 10 percent glycerol. Cell debris was precipitated and the supernatant was fractionated with 75 percent ammonium sulfate. The protein was collected by centrifugation at 28,600 x g, suspended in 1 ml of the sonication buffer, and dialyzed overnight in the sonication buffer containing 100mM NaCl. UDG activity was assessed using a poly-deoxyadenylate-[^{3}H]-deoxyuridylate (dA-[^{3}H]dU) substrate, synthesized by nick translation using DNA polymerase I (21) and a poly-deoxyadenylate-deoxythymidylate template (2). Nuclear extracts were incubated with 1 μg poly(dA-[^{3}H]dU) (2) at 37 °C for 30 min. The reaction was terminated using ethyl alcohol, NaCl and bovine serum albumin. Macromolecules were precipitated with ice-cold ethyl alcohol and collected by centrifugation at 23,000 x g. The supernatant, containing ethanol soluble nucleotides, was counted in a liquid scintillation counter. UDG activity in TCDD and DMSO-exposed cells was measured by determining the release of ethanol-soluble [^{3}H]deoxyuridine.

Protein Estimation. and the protein concentration was estimated using a modification of the Lowry assay (25).

Results

In SCC-12F keratinocyte cultures exposed to TCDD, we observed an increase in GAPDH transcription, which correlated with increased duration of exposure to the dioxin TCDD (Figure 1). Because serum status is an important parameter that influences the relative induction of a number of proteins (26), we examined whether the presence of serum played any role in the observed increase in transcription. Cells from cultures exposed to TCDD for 5 days in the absence of serum showed a five-fold increase in GAPDH transcription, however, when cultures were exposed to TCDD for 5 days, in the presence of serum for four of those days, a 13-fold increase in transcription was observed (Figure

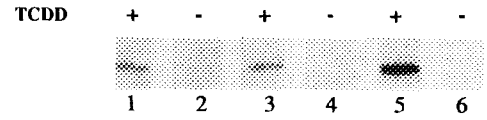

Figure 1. Transcription of GAPDH in SCC-12F cultured human keratinocytes exposed to TCDD. When exposed to 100 nM TCDD, GAPDH transcription was elevated: five-fold after five days in serum-free medium (lanes 1 and 2); 13-fold after five days when exposed four days in serum-containing medium and one additional day in serum-free medium (lanes 3 and 4); 23-fold at eight days when treated for four days in serum-containing medium followed by four additional days in serum-free medium (lanes 5 and 6).

1). After eight days of exposure to TCDD, the first four of which were in the presence of serum, a 23-fold increase in GAPDH transcription was observed.

Because we observed a time-dependent increase in transcription of GAPDH, we examined whether changes in the steady-state levels of GAPDH message also occurred. The steady-state level of GAPDH mRNA increased 250 percent after four days and 420 percent after eight days exposure to TCDD (Figure 2). To determine whether the increase in transcription rate and steady-state levels of mRNA resulted in increased production of the protein gene product, we analyzed GAPDH protein by western immunoblotting. A proportional increase of approximately 400 percent in protein levels was observed after eight days exposure to TCDD (Figure 3). This level of protein is in general agreement with the increase in mRNA under the same exposure conditions. Similarly, after prolonged exposure to TCDD (eight days) the total cellular GAPDH oxidoreductase activity increased by 500 percent, a level that is consistent with the observed increase in mRNA and protein (Figure 4). The specific activity remained relatively constant (1.4 ± 0.34 *versus* 2.2 ± 0.16 mmol mg^{-1} $min.^{-1}$ in cytosol from control and TCDD-exposed cells, respectively). These data indicate the observed increase in enzymatic activity results from an increase in the GAPDH protein product, rather than an alteration in the GAPDH gene.

To determine whether the action of TCDD on GAPDH was activity-specific, we examined whether TCDD affected the DNA repair function of the enzyme in keratinocyte cultures. Excision-repair

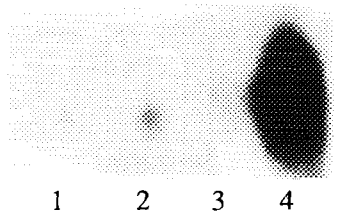

Figure 2. Steady state GAPDH mRNA levels in cultured human keratinocytes by TCDD. Lane 1. RNA from DMSO- exposed cells; **Lane 2.** RNA from TCDD-exposed cells. To determine the specificity of RNase cleavage of the GAPDH mRNA, we used two controls. Either t-RNA was substituted for human cytoplasmic RNA during the hybridization (**Lane 3**) or, the RNase-A was omitted from the assay (**Lane 4**). Relative densities were determined using a BioRad GS-670 imaging densitometer and Molecular Analyst software (BioRad). Relative areas were 9.87 and 23.69 arbitrary units for lanes 1 and 2, respectively. A similar experiment was carried out using total cytoplasmic RNA from cultures exposed to TCDD for eight days. Relative areas were 3.69 and 15.44 for RNA from DMSO and TCDD-exposed cultures, respectively (not shown).

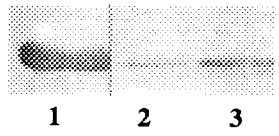

Figure 3. Expression of GAPDH protein after prolonged exposure to TCDD. Cells exposed to DMSO (lane 2) or TCDD (lane 3) for 8 days were collected and lysed in 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 8.0; proteins were subjected to electrophoresis on polyacrylamide in the presence of sodium dodecyl sulfate followed by Western immunoblot analysis. Commercial GAPDH (Sigma, St. Louis, MO) is shown in lane 1.

of an exogenous tritiated-poly-deoxyuracil oligomer decreased by greater than 50 percent in the nuclear fraction of the cells (Figure 4). Extra-nuclear cellular uracil DNA glycosylase activity, however, was negligible (3.87 ± 0.9 *vs.* 3.1 ± 0.3 cpm per ng protein in total cell extracts from control and TCDD-exposed cultures, respectively), indicating little if any leakage of UDP from nucleus to the cytoplasm. There are several possible mechanisms that could account for a reduced excision- repair capability in nuclei of cells exposed to TCDD. It is possible that TCDD exposure results in the induction of a shift in cellular redox status because of a dioxin-modulated shift in equilibrium of the subunit structure of the enzyme from a monomer that catalyzes DNA repair activity to the tetrameric redox active form of the enzyme.

Discussion

Transcription, mRNA levels, protein product, and GAPDH activity in human keratinocyte cultures exposed to TCDD are significantly increased. It is possible that the observed dioxin-modulated induction of GAPDH reflects a shift in intermediary metabolism to aerobic glycolysis, similar to that commonly observed in carcinogenesis (27,28). Aerobic glycolysis, called the Warburg effect, in tumor cells is a process by which more glucose is converted to lactic acid under aerobic conditions than in untransformed cells (29). It is possible that TCDD functions in the tumorigenic process by altering the normal metabolic state of the cell through deregulation of one or more key redox or glycolytic enzymes. It is not clear whether deregulation of GAPDH is a direct result of exposure to TCDD or secondary response occurring as a result of altered expression of growth factors, a consequence of TCDD-modulated induction of autocrine factors. For example, TCDD induces transforming growth factor alpha, a peptide that binds to the epidermal growth factor receptor of the cells (15), which could thereby stimulate aerobic glycolysis by increasing the proliferation rate of the cells (30).

Although it may be due to random occurrences, the sequence for the consensus dioxin-response element (DRE) is contained within the genes for several glycolytic/redox enzymes, including human lactate dehydrogenase, glucose-6-phosphate dehydrogenase, aldolase and GAPDH (31). These redox enzymes are essential regulatory enzymes that control cellular redox status and metabolic pools. It has been proposed that redox regulation may be a general mechanism for control of transcription factors (32). Experimentally, TCDD has been shown to induce other redox enzymes, including aldehyde dehydrogenase-3 in rat liver and NADPH:Quinone oxidoreductase in human Hep G2 cells, derived from liver (33).

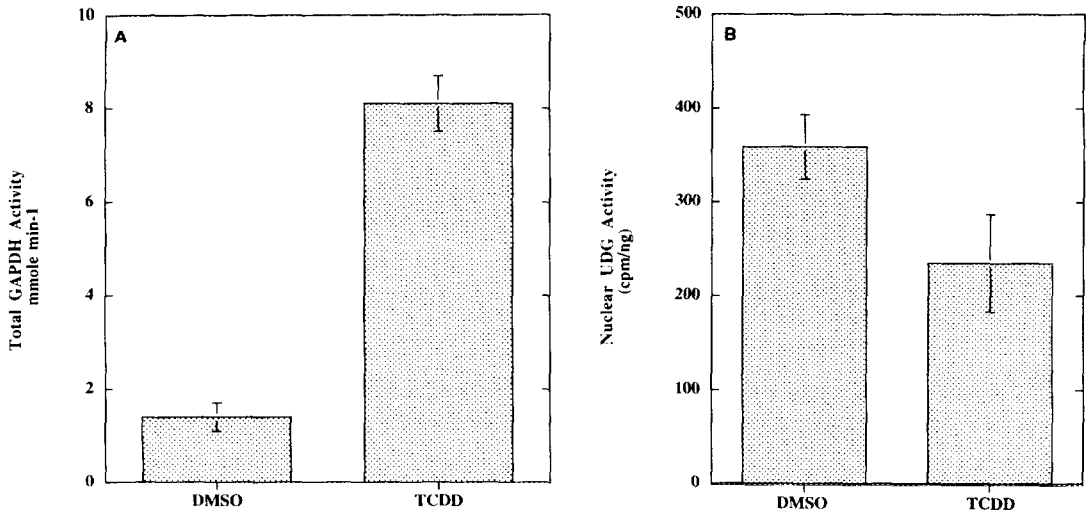

Figure 4. Total cellular GAPDH oxido-reductase and UDG-catalyzed DNA repair activity in nuclear extracts from keratinocytes exposed to TCDD. Panel A. Total cellular GAPDH activity was measured spectrophotometrically as described in MATERIALS AND METHODS. **Panel B**. UDG-catalyzed DNA-repair was assessed in nuclei from keratinocytes exposed to TCDD using a poly-deoxyadenylate-[^{3}H]-deoxyuridylate (dA-[^{3}H]dU) substrate and a poly-deoxyadenylate deoxythymidylate template as described in MATERIALS AND METHODS. UDG activity of TCDD and DMSO-exposed cells was measured by determining the release of ethanol-soluble [^{3}H]deoxyuridine.

Our data suggest the possibility that the *Ah* receptor could influence transcription of key regulatory glycolytic/redox enzymes through the DRE. TCDD may elicit one manifestation of the tumor phenotype, increased aerobic glycolysis, through a non-mutagenic mechanism. Whether enhanced glycolysis precedes or follows deregulation of cellular proliferation is a fundamental question in tumor biology. Examination of the regulation of the enzymes of intermediary metabolism by TCDD may help elucidate the role of glycolytic enzymes in tumor promotion and progression. Shift of redox status in cells after exposure to dioxins may be an important mechanism of dioxin-modulated toxicity and tumor promotion, and is currently under further study in our laboratory.

Acknowledgments

We thank J. Rheinwald for the gift of SCC-12F cells; L. Nutter for the gift of plasmids containing the 1.25 Kb GAPDH insert; M. Sirover for the gift of mouse monoclonal anti-human GAPDH antibodies (40.10.09). This work was supported by a grant from the NIH, ES-02866 (W. A. T.).

References

1. Racker, E. (1976) A New Look at Mechanisms in Bioenergetics, Academic Press, New York, NY, 197 pp.
2. Sirover, M. (1979) Cancer Res. 39: 2090-2095.
3. Poland, A., and Knutson, J. C. (1982) Annu. Rev. Pharmacol. Toxicol. 22:517-554.
4. Pitot, H. C., Goldsworthy, T., Campbell, H. A., and Poland, A. (1980) Cancer Res. 40: 3616-3620.
5. Poland, A., Palen, D., and Glover, E. (1982) Nature (London) 300: 271-273.
6. Fingerhut, M. A., Halperin, W. E., Marlow, D. A., Piacitelli, L. A., Honchar, P. A., Sweeney, M. G., Griefe, A. L., Dill, P. A., Steenland, A. B. K., and Surunda, A. J. (1991) N. Engl. J. Med. (Boston) 324: 212-218.

7. Bertazzi, P. A., Pesatori, A. C., Consonni, D., Tironi, A., Landi, M. T., and Zocchetti, C. (1993) Epidemiology 4: 398-406.
8. Collins, J. J., Strauss, M. E., Levinskas, G. J., and Conner, P. R. (1993) Epidemiology 4: 7-13.
9. Lilienfeld, D. E., and Gallo, M. A. (1989) Epidem. Revs. 11: 28-58.
10. Elferink, C. J., and Whitlock, J. P., Jr. (1990) J. Biol. Chem. 265: 5718-5721.
11. Reyes, H., Reisz-Porzasz, S., and Hankinson, O. (1992) Science (Washington, DC) 256: 1193-1195.
12. Dolwick, K. M., Schmidt, J. V., Carver, L. A., Swanson, H. I., and Bradfield, C. A. (1994) Mol. Pharmacol. 44: 911-917.
13. Whitlock, J. P. (1994) Trends Endocrinol. Metab. 5: 183-188.
14. Jones, K. W., and Whitlock, J. P., Jr. (1990) Mol. Cell. Biol. 10: 5098-5105.
15. Choi, E. J., Toscano, D. G., Ryan, J. A., Riedel, N., and Toscano, W. A., Jr. (1991) J. Biol. Chem. 266: 9591-9597.
16. Sutter, J. T., Guzman, K., Dold, K. M., and Greenlee, W. F. (1991) Science (Washington, DC) 254: 415-418.
17. Gaido, K. W., Maness, S. C., Leonard, L. S., and Greenlee, W. F. (1992) J. Biol. Chem. 267: 24591-24595.
18. Rheinwald, J. G. (1989) in Cell Growth and Division: A Practical Approach (Baserga, R., ed), pp. 81-94, IRL Press, New York, NY.
19. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struch, K. (eds) (1990) Current Protocols in Molecular Biology. 2 vols., Greene-Wiley, New York.
20. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132: 6-13.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 3 vols., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
22. Krebs, E. G. (1955) Methods in Enzymol. Colowick, and Kaplan, N. O. eds. vol 1 pp 407-415, Academic Press, New York, NY.
23. Laemmli, U. K. (1970) Nature (London) 227: 680-685.
24. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Peterson, G. L. (1977) Anal. Biochem. 83: 346-356.
26. Kupper, T. S. (1993) in Clinical Applications of Cytokines: Role in Pathogenesis, Diagnosis, and Therapy (Oppenheim, J. J., Rossio, J. L., and Gearing, A. J. H., eds), pp. 251-255, Oxford University Press, London.
27. Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K., and Sakiyama, S. (1987) Cancer Res. 47: 5616-5619.
28. Bhatia, P., Taylor, W. R., Greenberg, A. H., and Wright, J. A. (1994) Anal. Biochem. 216: 223-226.
29. Racker, E. (1972) J. Biol. Chem. 247: 8198-8200.
30. Diamond, I., Legg, A., Schneider, J. A., and Rozengurt, E. (1978) J. Biol. Chem. 253: 866-871.
31. Fishbein, L. (1990) Adv. Modern Environ. Toxicol. 17, 25-68.
32. Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E., and Curran, T. (1992) EMBO J. 11: 3323-3335.
33. Asman, D. C., Takimoto, K., Pitot, H. C., Dunn, T. J., and Lindahl, R. (1993) J. Biol. Chem. 268: 12530-12537.