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Phorbol Ester Stimulates Expression of the Human Tryptophanyl-tRNA Synthetase Gene

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Abstract—The effect of the phorbol ester phorbol 12-myristate 13-acetate (PMA) on expression of the human interferon (IFN)-inducible tryptophanyl-tRNA synthetase (WRS) gene was studied. PMA caused an increase in the basal and IFNγ-induced WRS protein content in HeLa and HEK293 cultured cells. Besides, PMA upregulated WRS mRNA level in HeLa cells. Since PMA is known as a selective activator of protein kinase C (PKC) and is widely used to study the PKC-related pathways, these results show possible PKC involvement in regulation of the WRS gene expression. PKC inhibition by staurosporine (10 and 100 nM) had no effect on either basal or IFNγ-induced expression of WRS in either cell line. Consequently, PKC is not an indispensable element in WRS induction by IFNγ. Rather, PKC may activate WRS gene expression only by a distinct pathway.

Key words: tryptophanyl-tRNA synthetase, phorbol 12-myristate 13-acetate, interferon, Ap_3A, protein kinase C

Human tryptophanyl-tRNA synthetase (WRS) [1-3], in contrast to other known aminoacyl-tRNA synthetases, is an interferon (IFN)-inducible enzyme [4-6]. It is known that along with its major function of cognate tRNA aminoacylation, aminoacyl-tRNA synthetases possess a range of non-canonical functions [3]. One of these functions is unique for WRS: it is directly involved in 5′,5‴-diadenosine triphosphate (Ap_3A) synthesis being unable to catalyze 5′,5‴-diadenosine tetraphosphate (Ap_4A) synthesis [7], the latter being readily produced by other aminoacyl-tRNA synthetases [3, 8]. It has been suggested that elevation of Ap_3A/Ap_4A ratio may be a major biological consequence of WRS induction by IFN and may be essential for intracellular processes related to the antitumor and antiviral activity of IFN [2, 3, 8]. As shown later, Ap_3A accumulates in human cultured cells after IFN treatment [9]. At least *in vitro* Ap_3A may prime [10-12] a 2′-5′-oligoadenylate (2-5A) system (this IFN-regulated RNA decay pathway provides innate immunity against viral infections). The WRS gene expression is inducible by IFNγ and IFNα in various cell lines [13]. In addition, human WRS was reported to be upregulated by polyinosinic-polycytidylic acid [13], during the late stages of mononuclear phagocyte maturation [14], and by erythropoietin in human endothelial cells [15].

The phorbol ester, phorbol 12-myristate 13-acetate (PMA), is known as a specific activator of protein kinase C (PKC) [16]. PMA is widely used to study PKC-related pathways (e.g. [17, 18]). PMA has been shown to potentiate 2-5A synthetase (2-5AS) induction by IFN [17-20] and to increase the Ap_3A concentration in cultured cells [21]. Therefore, this suggests that WRS content is somehow linked with the PKC activity. In this work we studied the PMA effect on WRS gene expression. We also studied

Abbreviations: PMA) phorbol 12-myristate 13-acetate; IFN) interferon; WRS) tryptophanyl-tRNA synthetase (EC 6.1.1.2); PKC) protein kinase C (EC 2.7.1.-); Ap_3A) A5′ppp5‴A, 5′,5‴-diadenosine triphosphate; Ap_4A) A5′pppp5‴A, 5′,5‴-diadenosine tetraphosphate; 2-5A) 2′-5′-oligoadenylate; 2-5AS) 2′-5′-oligoadenylate synthetase (EC 2.7.7.-); EPRS) glutamyl-prolyl-tRNA synthetase (EC 6.1.1.17); MRS) methionyl-tRNA synthetase (EC 6.1.1.10); QRS) glutamyl-tRNA synthetase (EC 6.1.1.18); GAPDH) glyceraldehyde-phosphate dehydrogenase; IDO) indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase, TDO (EC 1.13.11.11); RNase L) latent ribonuclease (EC 3.1.26.-); TGFβ) transforming growth factor β.

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the effect of staurosporine, a well-known PKC inhibitor, on WRS induction by IFNγ.

MATERIALS AND METHODS

Human epithelial HeLa and human embryonic kidney HEK293 cells were obtained from the ATCC and maintained as monolayers in DMEM medium supplemented with 10% fetal calf serum and antibiotics (all from Gibco BRL, USA). Cells were treated with 100 IU/ml of human recombinant IFNγ (Gibco BRL). PMA (Sigma, USA) and staurosporine (Calbiochem, USA) were added 15 min before the addition of IFNγ. The activity of both reagents was confirmed experimentally (data not shown).

Rabbit antisera against individual proteins were kindly provided by S. Park, Center for ARS Network (South Korea). Polyclonal antibodies against human WRS were purified from rabbit antiserum using protein A-agarose column chromatography. Protein concentrations in cell lysates were measured as described by Bradford [22] using chicken ovalbumin as a reference.

The content of WRS and other aminoacyl-tRNA synthetases including EPRS, MRS, and QRS in cell lysates were assayed by Western blotting with appropriate antibodies or antisera. Equal amounts of extracted proteins (10 μg) were subjected to SDS-PAGE [23] in 8% gels and electroblotted to Immobilon membrane (Millipore, USA). The membranes were blocked with 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.7, 0.14 M NaCl, 1% Tween 20, and then were incubated with primary antibodies against respective proteins for 1 h at room temperature. Following washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG, then washed, and the blot was developed with enhanced chemiluminescence (Bio-Rad, USA) using the X-ray film (Fuji). Immunoreactive bands were quantified with a Molecular Dynamics 300A (USA) computing densitometer. All experiments were run in triplicate.

Total RNA was isolated from control and PMA-treated HeLa cells and analyzed by Northern hybridization. Briefly, 15 μg of total RNA was fractionated on 1.0% agarose-formaldehyde gels and blotted to Hybond N nylon membrane (Amersham Pharmacia Biotech, UK). The membrane was hybridized using ^{32}P-labeled specific cDNA probes for human WRS. The equality of mRNA sample loading was controlled using rehybridization with radiolabeled cDNA probes for human GAPDH. All cDNA probes were body-labeled using a random primer labeling kit (Amersham Pharmacia Biotech) in the presence of [α-^{32}P]dATP. Plasmid with cloned WRS cDNA [24] was a generous gift from Dr. L. Frolova (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). Hybridization signals were quantified with a Packard Cyclone phosphor imager (USA).

RESULTS

1. PMA enhances basal and IFNγ-induced WRS gene expression. We measured the WRS protein content in two human cell lines (epithelial HeLa and embryonic kidney HEK293) treated with various combinations of PMA and IFNγ for 16 h or remaining untreated for the same time.

Cell treatment with 100 IU/ml of IFNγ was accompanied by increase in the WRS concentration, 5.5-fold in HeLa cells compared with the untreated cells (Fig. 1), whereas the content of EPRS, MRS, and QRS remained unchanged. Moreover, IFNγ elevated 2.9-fold the WRS content in HEK293 cells (Fig. 1). Not all cell lines show WRS induction by IFNγ [13]. Here, we have shown for the first time that IFNγ induces WRS in the embryonic kidney HEK293 cells; our results confirm earlier data on IFNγ action on WRS content in HeLa cells [6, 13, 25, 26].

PMA without IFNγ enhanced WRS expression 2- and 1.6-fold in HeLa and HEK293 cells, respectively (Fig. 1). We observed no reliable changes in the content of EPRS, MRS, and QRS in response to PMA (Fig. 1).

PMA potentiated WRS induction by IFNγ in both cell lines, while the contents of EPRS, MRS, and QRS remained unaffected (Fig. 1). After 16-h incubation with IFNγ and PMA, the WRS content increased 2-fold in HeLa and 2.6-fold in HEK293 cells over the cells treated with IFNγ alone. The maximal PMA effect on WRS content was observed at 16 h of treatment (data are shown only for 16 h).

To test whether WRS protein changes reflected corresponding changes in the WRS mRNA levels, the Northern blot analysis of total RNA from HeLa cells was carried out after various time intervals of cell treatment with PMA (Fig. 2). WRS mRNA level reached the maximum after 4-8 h of treatment (13-fold increase as compared with untreated cells after 8 h) and dropped down to the normal level after 16-h treatment. Thus, PMA activates WRS gene transcription. The level of WRS mRNA induction was considerably higher than the level of WRS protein induction by PMA.

2. Effect of staurosporine on WRS expression. We assayed the effect of the well-known protein kinase inhibitor staurosporine on WRS expression level with and without IFNγ induction. Staurosporine at concentrations 10 or 100 nM affected neither the basal WRS nor the IFNγ-induced WRS level in HeLa cells (Fig. 3). However, at high concentration (500 nM) staurosporine completely abolished the WRS induction by IFNγ (Fig. 3). We observed the same effect also in HEK293 cells (Fig. 1).

The contents of all assayed aminoacyl-tRNA synthetases, including WRS in the absence of IFN, were diminished by 500 nM staurosporine (Fig. 3), suggesting suppression of the initial step of protein biosynthesis, i.e., suppression of aminoacylation of tRNAs of various speci-

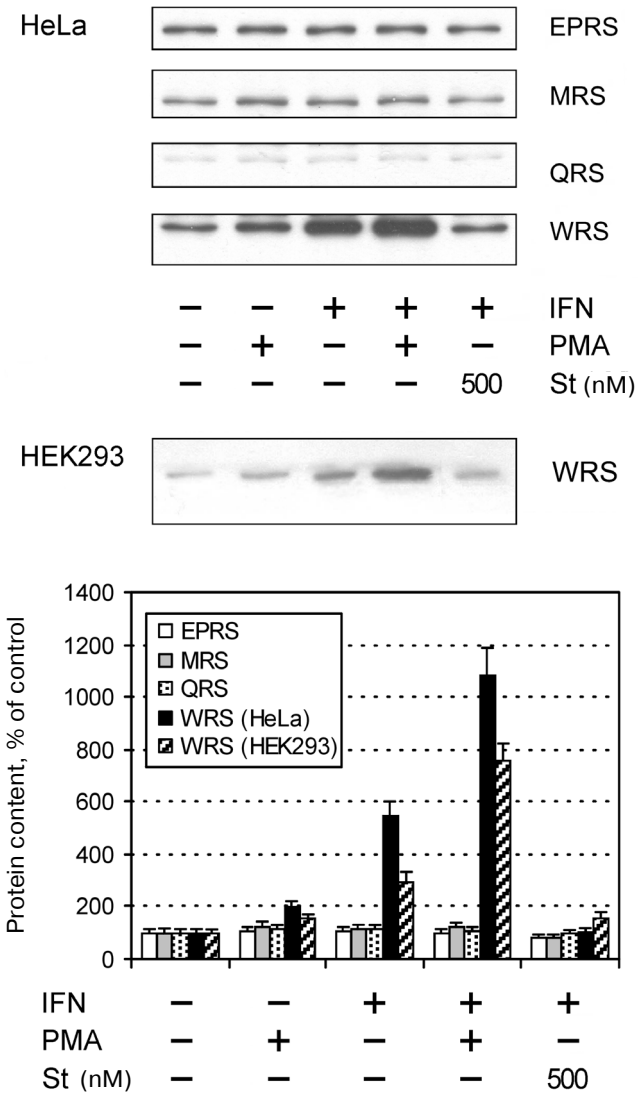

Fig. 1. Effects of IFNγ, PMA, and staurosporine on WRS content in human cells. Western blot analysis of WRS, EPRS, MRS, and QRS content in HeLa and WRS content in HEK293 cells after treatment with 0.1 μM PMA, 100 IU/ml IFNγ, and staurosporine (St) in various combinations for 16 h. Histogram of protein expression levels after indicated treatments. Spots from Western blot X-ray films reflecting protein bands were quantified using a densitometer and relative densities were normalized to that in untreated cells.

ficities by this inhibitor. This effect was insignificant after 16-h treatment (Fig. 1), and appeared evident after 21-h treatment (Fig. 3). The complete block of WRS inducibility by IFNγ by high staurosporine concentration took place as early as 4 h after simultaneous treatment with IFN and staurosporine (data not shown).

DISCUSSION

Here we have shown that PMA stimulates WRS gene expression (Figs. 1 and 2). Consequently, it becomes clear that shown earlier elevation of the Ap_3A content in human cultured cells in response to PMA [21] most probably is caused by elevated expression of WRS, which is able to synthesize Ap_3A. Since the content of other aminoacyl-tRNA synthetases responsible mainly for the Ap_4A synthesis showed no considerable change in response to PMA (Fig. 1), PMA presumably elevates also the Ap_3A/Ap_4A ratio in cells.

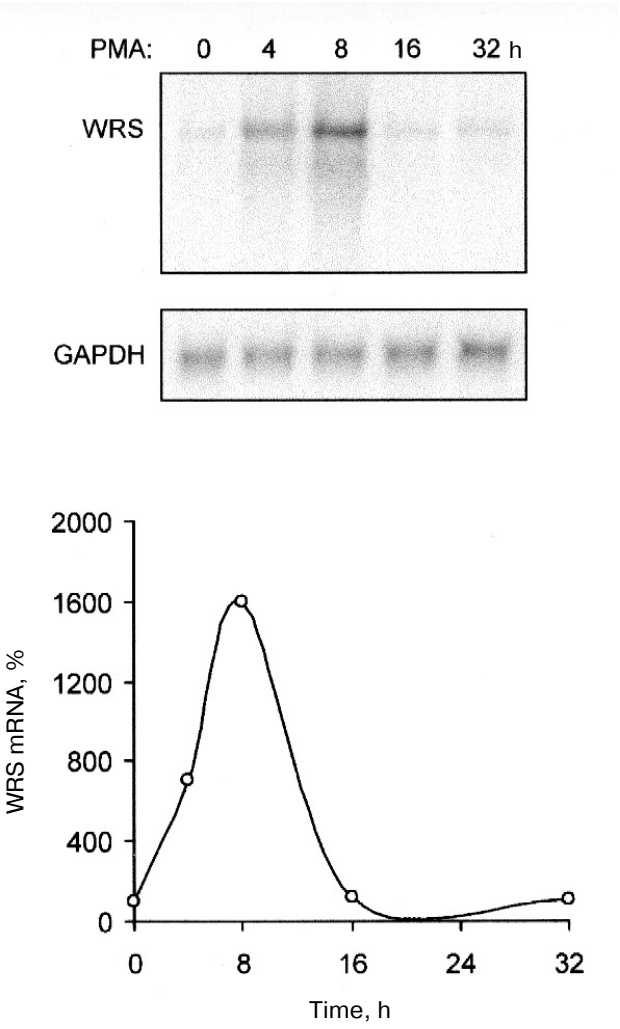

Fig. 2. WRS mRNA expression in PMA-treated HeLa cells. Cells were treated with 0.1 μM PMA for the indicated time intervals. Total RNA was isolated and WRS mRNA content was analyzed by Northern blot hybridization with ^{32}P-labeled WRS cDNA probe. After the filter exposition, the filter was washed and rehybridized for measurement of GAPDH mRNA levels. Quantifications were done by a phosphor imager, and the resulting values for WRS mRNA were normalized relative to values obtained for GAPDH mRNA. Each point is the mean of double determination.

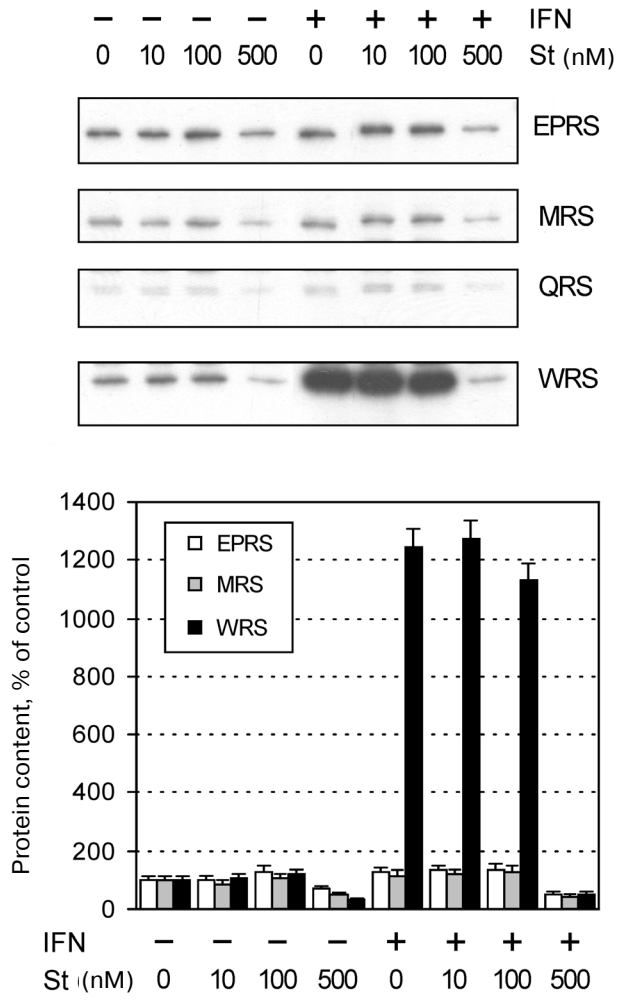

Fig. 3. Effect of staurosporine on basal and IFNγ-induced WRS expression in HeLa cells. Cells were treated for 21 h with staurosporine (St) in indicated concentrations, with or without 100 IU/ml IFNγ. Western blot analysis was used to detect WRS, EPRS, MRS, and QRS in cell lysates. Histogram of proteins contents after indicated treatments.

Since PMA is known as a selective activator of PKC mimicking the action of the unsaturated diacylglycerol [16], we suggest that the WRS gene expression is activated by PKC. Blocking of the PMA activity in the presence of a highly specific PKC inhibitor could prove that the effect of PMA is in fact mediated by PKC. Staurosporine has rather wide specificity toward protein kinases, so other more specific inhibitors should be used in further studies. Staurosporine at 10-100 nM concentrations provides effective PKC inhibition in cultured cells [27]. The fact that even 100 nM staurosporine does not block WRS gene induction by IFNγ (Fig. 3) implies that PKC is not an indispensable element in WRS induction by IFNγ. Rather, PKC may activate WRS gene expression only by another distinct pathway.

Selectivity of protein kinase inhibition by staurosporine decreases with increase of staurosporine concentration: at high concentrations it acquires the ability to inhibit a variety of protein kinases. As staurosporine inhibited IFNγ-induced WRS gene expression only at very high concentration (500 nM) (Fig. 3), we assume that this effect is not accounted for by PKC inhibition. Rather, 500 nM staurosporine acts through a PKC-independent pathway.

The 5′-flanking region of the WRS gene contains three GAS elements, two ISRE, and two Y-boxes [25, 28]. It was shown that in cell lysates proximal GAS of WRS promoter binds STAT1 protein in response to IFNγ [29] and STAT1 and STAT5 in response to prolonged PMA treatment [30]. Later studies showed that binding of STATs to the proximal GAS of WRS gene promoter is insufficient or insignificant for its transcription activation in response to IFNγ or α [13, 25]. The 0.4-kb region of WRS promoter, required for the maximal transcriptional activation of WRS gene promoter by IFNγ, contains two ISREs, the proximal GAS, and one Y-box [25]. ISREs of WRS gene promoter may be involved in stimulation of its transcription activation by PMA shown in this work (Figs. 1 and 2) similarly to the data obtained for 2-5AS: the induction of 2-5AS gene by IFNα is potentiated by PMA through the activation of ISREs of its promoter [19].

Shown here WRS mRNA accumulation in response to PMA occurred much earlier (WRS mRNA level reached maximum after 4-8 h of cell treatment) (Fig. 2) than previously reported [30] STAT1 and STAT5 binding with the proximal GAS element of WRS promoter in response to PMA (after 18, 24, and 48 h of whole cell lysate treatment). Furthermore, STAT binding to GAS elements is insufficient for transcription activation of WRS gene in response to IFNγ [13, 25]. Nevertheless, STAT binding to proximal GAS of WRS gene promoter may play a role in WRS transcription activation by PMA.

The activating effect of PMA on WRS expression distinguishes WRS from the expression of some other IFN-inducible proteins which were shown to be down-regulated by PMA and its effector PKC. For example, it was shown that PKC suppresses the IFNγ-induced expression of apoptotic protein Fas and HLA-DR member of major histocompatibility complex [31].

At the same time, our results relate WRS with such IFN-inducible enzymes as indoleamine 2,3-dioxygenase (IDO) and 2-5AS. It is known that WRS and IDO, the key enzyme of tryptophan degradation, are co-induced by IFNγ and by IFNα, both in the same cell lines [13]. TGFβ selectively inhibits both WRS and IDO IFNγ-stimulated expression in human fibroblasts [32]. Similarly to what is shown in this work for WRS, PMA potentiates IDO induction by IFNγ [33, 34]. It was also shown that PMA enhances 2-fold the 2-5AS induction by IFNα [17-20] in human cells, including HL-60 and HeLa [17]. It was shown that this effect is mediated by PKC [18].

In conclusion, our findings suggest that expression of WRS, 2-5AS, and IDO is co-activated by PMA. The biological significance of this coregulation remains obscure, but the obvious fact that WRS and IDO are using tryptophan as a substrate (and also as a cofactor for WRS), and WRS and 2-5AS most probably are related to Ap_3A metabolism, suggests a functional relationship between these enzymes.

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REFERENCES

1. Kisselev, L. (1993) *Biochimie*, **75**, 1027-1039.
2. Kisselev, L., Frolova, L., and Haenni, A.-L. (1993) *Trends Biochem. Sci.*, **18**, 263-267.
3. Kisselev, L. L., and Wolfson, A. D. (1994) *Progr. Nucleic Acids Res. Mol. Biol.*, **48**, 83-142.
4. Fleckner, J., Rasmussen, H. H., and Justesen, J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 11520-11524.
5. Rubin, B. Y., Anderson, S. L., Xing, L., Powell, R. J., and Tate, W. P. (1991) *J. Biol. Chem.*, **266**, 24245-24248.
6. Buwitt, U., Flohr, T., and Bottger, E. C. (1992) *EMBO J.*, **11**, 489-496.
7. Merkulova, T., Kovaleva, G., and Kisselev, L. (1994) *FEBS Lett.*, **350**, 287-290.
8. Kisselev, L. L., Justesen, J., Wolfson, A. D., and Frolova, L. Y. (1998) *FEBS Lett.*, **427**, 157-163.
9. Vartanian, A., Narovlyansky, A., Amchenkova, A., Turpaev, K., and Kisselev, L. (1996) *FEBS Lett.*, **381**, 32-34.
10. Turpaev, K., Hartmann, R., Kisselev, L., and Justesen, J. (1997) *FEBS Lett.*, **408**, 177-181.
11. Turpaev, K., Hartmann, R., and Justesen, J. (1999) *FEBS Lett.*, **457**, 9-12.
12. McLennan, A. G. (2000) *Pharmacol. Ther.*, **87**, 73-89.
13. Fleckner, J., Martensen, P. M., Tolstrup, A. B., Kjeldgaard, N. O., and Justesen, J. (1995) *Cytokine*, **7**, 70-77.
14. Krause, S. W., Rehli, M., Kreutz, M., Schwarzfischer, L., Paulauskis, J. D., and Andreesen, R. (1996) *J. Leukoc. Biol.*, **60**, 540-545.
15. Fodinger, M., Fritsche-Polanz, R., Buchmayer, H., Skoupy, S., Sengoelge, G., Horl, W. H., and Sunder-Plassmann, G. (2000) *J. Invest. Med.*, **48**, 137-149.
16. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) *J. Biol. Chem.*, **257**, 7847-7851.
17. Chang, Ch.-Ch., Borelli, T. J., Williams, B. R. G., and Wu, J. M. (1992) *Eur. J. Biochem.*, **207**, 297-304.
18. Yu, F., and Floyd-Smith, G. (1997) *Exp. Cell Res.*, **234**, 240-248.
19. Yu, F., Wang, Q., and Floyd-Smith, G. (1999) *Gene*, **237**, 177-184.
20. Yu, F., and Floyd-Smith, G. (1999) *Cytokine*, **11**, 744-750.
21. Vartanian, A., Prudovsky, I., Suzuki, H., Dal Pra, I., and Kisselev, L. (1997) *FEBS Lett.*, **415**, 160-162.
22. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
23. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
24. Frolova, L. Yu., Grigorieva, A. Yu., Sudomoina, M. A., and Kisselev, L. L. (1991) *Gene*, **109**, 291-296.
25. Tolstrup, A. B., Bejder, A., Fleckner, J., and Justesen, J. (1995) *J. Biol. Chem.*, **270**, 397-403.
26. Shaw, A. C., Rossel Larsen, M., Roepstorff, P., Justesen, J., Christiansen, G., and Birkelund, S. (1999) *Electrophoresis*, **20**, 984-993.
27. Tamaoki, T. (1991) *Meth. Enzymol.*, **201**, 340-347.
28. Frolova, L. Y., Grigorieva, A. Y., Sudomoina, M. A., and Kisselev, L. L. (1993) *Gene*, **128**, 237-245.
29. Strehlow, I., Seegert, D., Frick, P., Bange, F.-C., Schindler, C., Bottger, E. C., and Decker, T. (1993) *J. Biol. Chem.*, **268**, 16590-16595.
30. Eilers, A., Baccarini, M., Horn, F., Hipskind, R. A., Schindler, C., and Decker, T. (1994) *Mol. Cell. Biol.*, **14**, 1364-1373.
31. De Saint Jean, M., Brignole, F., Feldmann, G., Goguel, A., and Baudouin, C. (1999) *Invest. Ophthalmol. Vis. Sci.*, **40**, 2199-2212.
32. Yuan, W., Collado-Hidalgo, A., Yufit, T., Taylor, M., and Varga, J. (1998) *J. Cell. Physiol.*, **177**, 174-186.
33. Edelstein, M. P., Ozaki, Y., and Duch, D. S. (1989) *J. Immunol.*, **143**, 2969-2973.
34. Holmes, E. W. (1998) *J. Interferon Cytokine Res.*, **18**, 509-520.