Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 666-673

TAX-INDUCED HTLV-I LTR TRANSCRIPTIONAL ACTIVATION IS MODULATED BY PHOSPHORYLATION

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Received October 12, 1994

Summary. We studied the effect of protein phosphatase and kinase inhibitors on Tax-mediated transcription of constructs carrying the reporter gene chloramphenicol acetyl transferase under the control of either the full-length LTR of HTLV-I or three copies of the tax-responsive 21-bp repeats. We observed that treatment with okadaic acid, which inhibits the serine/threonine protein phosphatases type 1 and 2A, reduced HTLV-I LTR transcriptional activation in MT2 and K562 cells; on the contrary, the enhancer activity of the 21-bp sequences was significantly increased in both cell lines; treatment with the protein kinase C inhibitor H-7 blocked Tax-mediated transcription of both constructs. We also found that treatment with sodium orthovanadate, a tyrosine phosphatase inhibitor, reduced Tax-mediated activation of both plasmids. These findings indicated that specific serine/threonine phosphorylation events are required for Tax-mediated HTLV-I LTR activation and also suggested that phosphorylation at tyrosine residues is involved in this process.

Human T-lymphotropic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL) (1, 2), and the inflammatory/degenerative neurological disorder known as tropical spastic paraparesis or HTLV-I-associated myelopathy (TSP/HAM) (3, 4). HTLV-I transcription is strongly dependent upon the viral Tax protein, which acts through three imperfectly conserved 21 bp motifs located in the LTR (5, 6). Tax-mediated transcription of other viral promoters and several cellular genes involved in cell growth and differentiation has also been described (7). As Tax does not directly interact with DNA, activation of the large number of genes by the viral protein is likely due to its capacity to interact with host cellular factors and increase their DNA-binding activity (8-11). Mutational analyses indicated that the critical region of the Tax-responsive 21 bp repeats is a CREB/ATF

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0006-291X/94 $5.00

binding site (12, 13), and it was shown that the activity of CREB family proteins strictly depends upon phosphorylation at the serine residues (14). However, Champion-Arnaud and coworkers (15) found that phosphotyrosine phosphatase expression significantly reduced the activation of transcription endowed by AP-1 and CREB binding sites, suggesting that phosphorylation at tyrosine residues is also important.

We studied the influence of phosphorylation on the transcriptional activity of HTLV-I Tax protein by analyzing the effect of different phosphatase inhibitors. We found that HTLV-I Tax-induced transcription is positively regulated by inhibitors of serine/threonine protein phosphatases, while the inhibition of tyrosine protein phosphatases down-regulates Tax transactivation activity.

MATERIALS AND METHODS

Plasmids. The CAT reporter construct LTR-CAT (pU3R1; 16), and the Tax-expressing plasmid pTAX (pLcXL; 6) were kindly provided by Drs. W. A. Haseltine (Dana-Farber Cancer Inst., Harvard, Boston, MA), and G. Pavlakis (NCI-Frederick Cancer Res., Frederick, MD), respectively. The 3X21-CAT construct was obtained by inserting three copies of the synthetic oligonucleotide 5'-AGC TTA AGG CTC TGA CGT CTC CCC CCG-3', representing the 21 bp motif, into the *Hind*-III/*Bam*-HI sites of the plasmid BL-CAT2 containing the Herpes simplex virus Tk promoter (17).

Cell culture, transfection and treatment. HTLV-I-infected MT2 cells, and the erythroleukemic K562 cell line were transiently transfected by the DEAE-Dextran method (16).To uniform the efficiency of transfection in a given experiment, at the end of transfection the cells were pooled, split $5x10^6$ cells/ flask, and incubated at 37°C; K562 cells were cultured in the presence of 1% FCS to avoid low basal activation of the plasmids. Okadaic acid (OA) (Sigma), sodium orthovanadate (Vanadate) (Sigma), H-7 (Sigma), and PMA (Sigma) were added at different time after transfection, as described in the legends. At the end of treatment, the cells were collected, rinsed twice with phosphate-buffered saline (PBS), and lysed by three freeze-thaw cycles in 50 µl of 0.25 M Tris HCl,pH 7.8; the lysates were clarified by centrifugation at 13,000g for 10 min. Total protein concentration was determined with a Protein Assay Kit (Bio-Rad).

CAT assay. Aliquots of extracted proteins, corresponding to 5 µg (MT2 cells) or 20-30 µg (K562 cells) of total protein, were assayed for CAT activity as previously described (18). Chloramphenicol and its derivatives were extracted with 1 ml ethyl acetate; the organic phase (900 µl) was dried down in a Speed Vac (Savant Inst.), resuspended in 20 µl of ethyl acetate, spotted onto a thin layer chromatography plates (Merck), and run in chloroform:methanol (95:5). The percent conversion of chloramphenicol (Cm) into its acetylated forms (Ac Cm) was determined by scintillation counting of the appropriate areas of the chromatography plate.

RNA extraction and Northern blot. RNA was isolated by the hot-phenol method. Five µg of total RNA were separated by electrophoresis on 1.2% agarose formaldehyde gels, and then transferred to Hybond-N membranes (Amersham, Bukinghamshire, England). The membranes were hybridized with an actin probe, at 42°C in 50% formamide and then washed for 1 h in 0.1 X SSC (1 X SSC: 0.15 M sodium chloride, 15 mM sodium citrate), and 0.1% sodium dodecyl sulfate (SDS) at 65 °C. RNA loading and transfer to membrane were checked by examination of ribosomal RNA under ultraviolet (UV) light .

RESULTS

Effect of protein phosphatase inhibition on HTLV-I LTR transcription. To analyze the effect of okadaic acid (OA) and sodium orthovanadate (Vanadate) on the promoter activity

of HTLV-I LTR, MT2 cells, that constitutively express Tax protein, were transiently transfected with the LTR-CAT plasmid, which contains the full-length HTLV-I LTR (16). We also transiently cotransfected K562 cells with the LTR-CAT construct and a plasmid expressing the viral Tax product (pTAX) (6).

OA is known to inhibit the serine/threonine phosphoprotein phosphatases types 1 (PP1) and 2A (PP-2A), and it has no effect on protein kinase C (PKC) (19, 20); furthermore, OA treatment stimulates the expression of certain cellular and viral genes (21, 22), and it has been repoted that OA treatment enhances CREB phosphorylation (23). Vanadate is a tyrosine phosphatase inhibitor whose specificity is not known. We found that, following OA treatment, Tax-induced pLTR-CAT transcription was decreased in both MT2 and K562 cells, albeit to different extents (Fig. 1, panel A). Vanadate treatment produced similar results, and a dose-dependent inhibition of CAT synthesis was observed in both cell lines (Fig. 1, panel B) suggesting that proteins phosphorylated at the tyrosine residues might be also involved in Tax-mediated HTLV-I transcriptional activation.

As treatment with protein phosphatase inhibitors brings about an enhanced phosphorylation of many protein kinase substrates, we tested whether a combined treatment of OA or Vanadate with phorbol 12-myristate 13-acetate (PMA) would influence the transcription of pLTR-CAT in K562 cells. Simultaneous treatment of K562 cells with OA and PMA inhibited the HTLV-I LTR response to phorbol ester (Fig. 1, panel A), but only at the highest OA dose (0.15 μM) used. These data suggested that OA treatment might activate negative regulatory factor(s) which counteracts both Tax and PMA activities. On the contrary, vanadate did not induce a significant inhibition in PMA-treated K562 cells (Fig. 1, panel B).

Effect of phosphatase inhibitors on Tax-responsive 21-bp repeat-mediated transcription. To determine whether the above observed negative effect of OA and Vanadate treatment on HTLV-I LTR transcriptional activity involved Tax-responsive elements, we constructed a plasmid (3X21-CAT), carrying three copies of the 21-bp elements. This plasmid, which was unresponsive to PMA activation (data not shown), was transfected into MT2 cells or into K562 cells in association with a Tax-expressing plasmid. OA treatment enhanced transcription of the 3X21-CAT construct in both cell lines (Fig. 2, panel A), thus demonstrating that the previously observed inhibition (see Fig. 1, panel A) was specific for pLTR-CAT, and suggesting that an increased phosphorylation of specific cellular transcriptional factors might exert an inhibitory effect on sequences other than the 21-bp motifs. OA did not enhance the basal transcription level of p3X21-CAT in the absence of Tax protein (fig. 2, panel A), thus indicating that phosphorylated cellular factors may rely on the presence of the viral protein for their transcriptional activity. In contrast, Vanadate treatment inhibited p3x21-CAT transcription in both cell lines (Fig. 2, panel B); this result supports the hypothesis that tyrosine phosphatases might be involved in Tax-mediated HTLV-I transcriptional activation.

Effect of protein kinase C (PKC) inhibition on HTLV-I transcriptional activation. Since OA was shown to indirectly potentiate the action of kinases, we studied whether PKC blockage

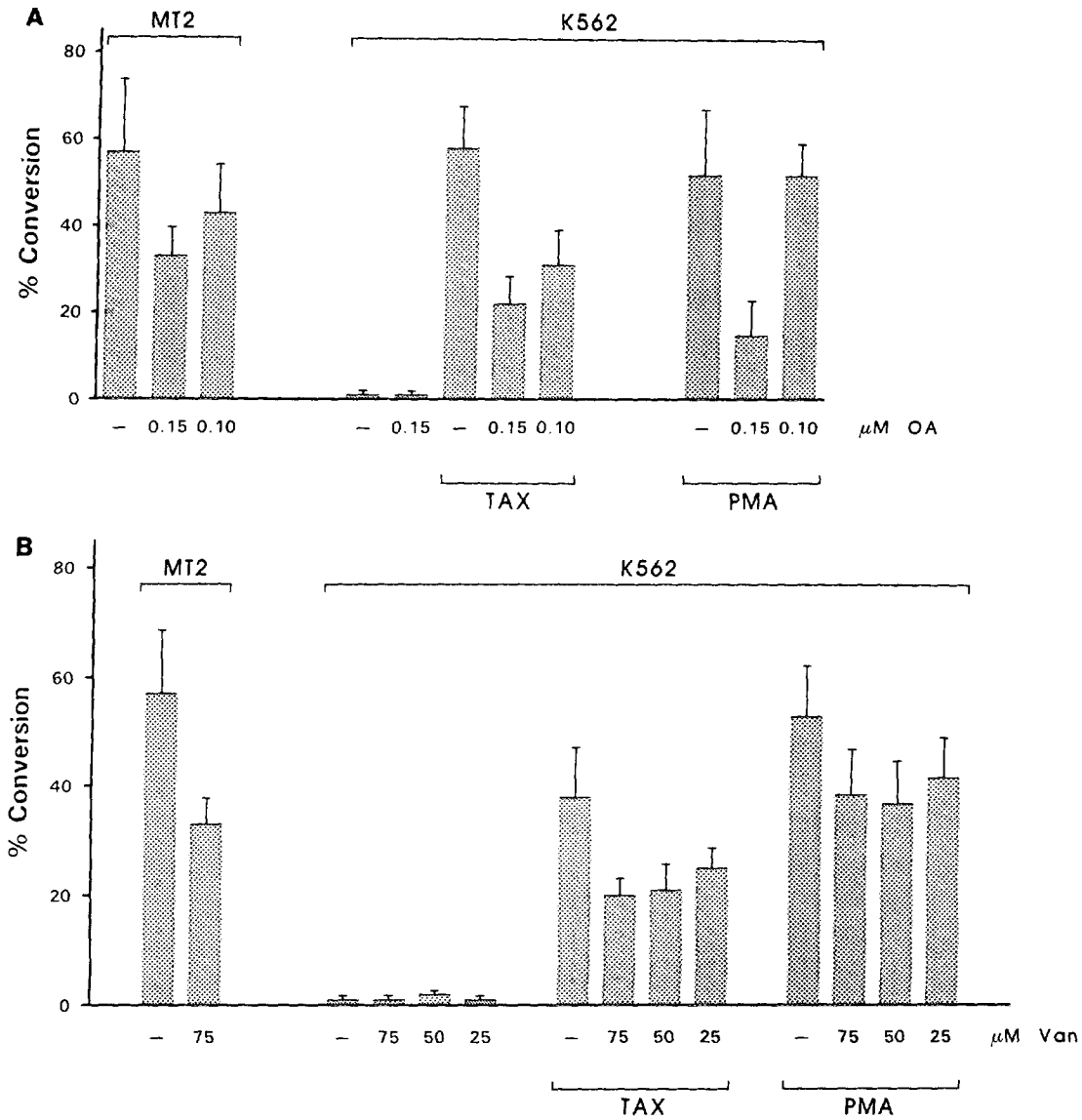

Figure 1 Effect of okadaic acid (OA) and sodium orthovanadate (Vanadate) on HTLV-I LTR promoter activity. MT2 and K562 cells were transfected with the reporter construct LTR-CAT, 16-18 hours later collected and processed for protein extraction and CAT assay. OA (**panel A**) or Vanadate (**panel B**) were added 3 (MT2 cells) or 6 (K562 cells) hours after transfection, while PMA treatment (10 μg/ml) was done immediately after transfection. The conversion percentages are reported as the mean±SD of at least three independent experiments

would influence Tax-mediated transcription of HTLV-I. K562 and MT2 cells were transfected as described above, and then treated with the kinase inhibitor H-7. We found that H-7 not only inhibited PMA-mediated HTLV-I transcription, but also Tax-mediated

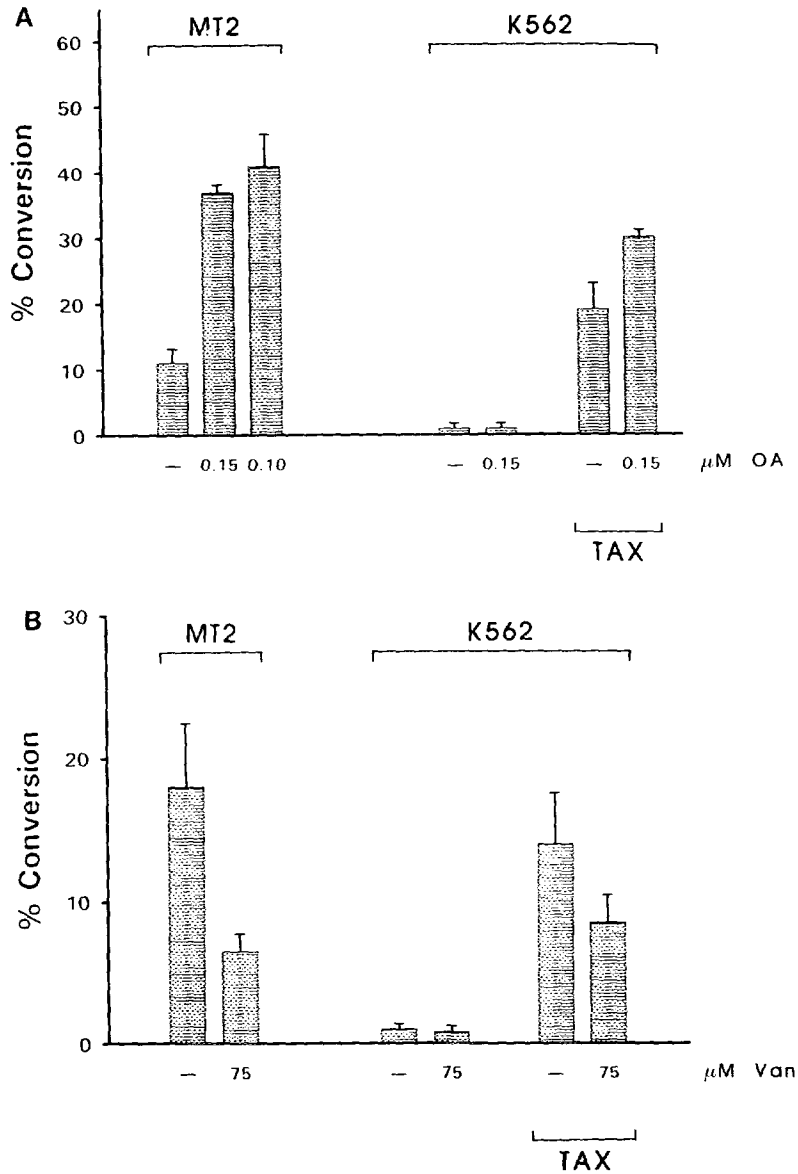

Figure 2. Effect of okadaic acid (OA) and sodium orthovanadate (Vanadate) on Tax-responsive sequences. MT2 and K562 cells were transfected with p3X21-CAT, treated with OA (**panel A**) or Vanadate (**panel B**) and processed as described in Fig. 1. The conversion percentages are reported as the mean±SD of at least three independent experiments.

activation (Fig. 3, panels A and B). This inhibitory effect was observed not only using pLTR-CAT but also on p3X21-CAT, and strongly suggested that Tax might interact with cellular factors involved on PKC phosphorylation pathway. To evaluate whether the observed transcription block was due to the compound toxicity, RNA was extracted from MT2 cells that had been incubated in the presence of scalar doses of H-7; the analysis of the

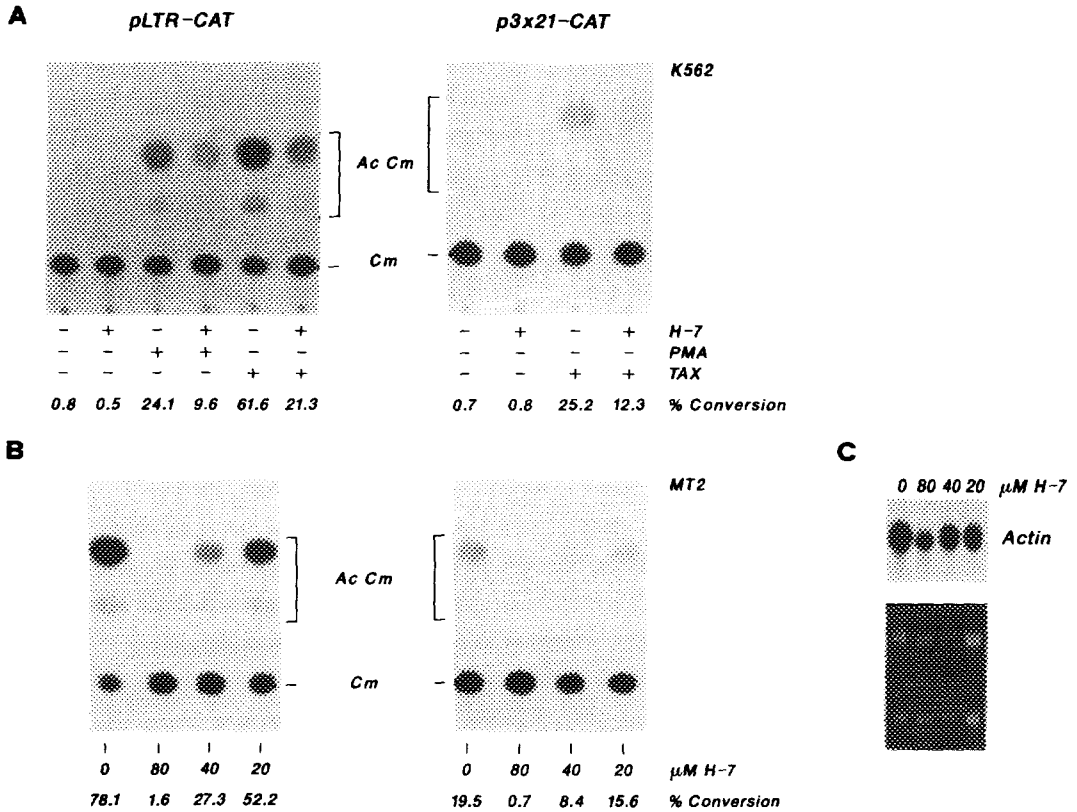

Figure 3 Effect of H-7 treatment on LTR and 21-bp repeats transcriptional activity. **Panel A**: K562 cells were transfected with pLTR-CAT or p3X21-CAT alone or in association with pTax. H-7 (40 μM) was added immediately after transfection, while PMA (10 μg/ml) was added 30 min later. One experiment, representative of three, is shown. **Panel B**: MT2 cells were transfected with pLTR-CAT or p3X21-CAT and then treated with scalar doses of H-7. One experiment, representative of three, is shown. **Panel C**: Five μg of total mRNA from MT2 cells, which had been treated for 16-18 hours with scalar doses of H-7, was hybridized with an actin probe; examination of ribosomal RNA under UV light was done to assess the uniformity of RNA loading.

actin gene synthesis showed that the mRNA level in treated cells was comparable to that of control untreated cells (Fig.3, panel C), thus confirming the specificity of the CAT synthesis block.

DISCUSSION

HTLV-I transcription involves the composite activity of both viral (Tax) and cellular transcription factors (reviewed in 24). The present study demonstrated that HTLV-I Tax-mediated activation is dependent on the signaling pathways affected by OA. Indeed, the block of protein phosphatases type 1 and 2A , that indirectly induced an increase in total serine/threonine phosphorylated protein, positively regulated the transcription of the

construct carrying the Tax-responsive enhancer (21 bp motif). These data were also supported by the finding that the PKC inhibitor H-7 blocked Tax-mediated activation of LTR-CAT and 3X21-CAT constructs. While serine/threonine phosphorylation by itself positively regulates c-jun (25), and HIV-1 transcription (22; personal observation), we found that a mere increase in the phosphorylation of cellular proteins was not sufficient to augment the basal transcription of p3x21-CAT, and Tax expression was strictly required. When the transcription was under the control of the entire HTLV-I LTR, we found that OA treatment induced a decrease in CAT synthesis. It is conceivable that negative regulatory cellular factor(s), which is phosphorylated at the serine/threonine residues, might interact with some components of the HTLV-I LTR sequences, other than the 21-bp motifs (26). Tax itself is a phosphoprotein, and is phosphorylated at the serine residues (27); therefore, changes on Tax phosphorylation might explain the observed transcriptional regulation. In fact, Fontes *et al.* (28) reported that treatment with OA and PMA increased Tax phosphorylation. It is possible that phosphorylation modifies Tax localization or its ability to interact with previously described cellular transcriptional factors (24). However, the role of Tax phosphorylation in virus-induced pathogenicity is still undefined, even though it was reported that bacterial synthesized Tax, which is consequently non-phosphorylated, was able to interact *in vitro* with CREB proteins that bind to the HTLV-I 21 bp repeat (8).

Our findings also demonstrated that inhibition of the protein tyrosine phosphatases negatively influenced Tax-mediated HTLV-I LTR transcription, thus suggesting that phosphorylation/dephosphorylation at the tyrosine residues might also be relevant to Tax-mediated activation. Since this inhibition can be reproduced by using the p3X21-CAT, it seems reasonable to suggest that the 21-bp repeats might be the target of the observed transcriptional block. An alternative explanation for this phenomenon might be that the level of serine/threonine phosphorylation of the cellular factors involved in Tax-mediated activation might be governed by other proteins which are influenced by modifications at the tyrosine residues.

ACKNOWLEDGMENTS

We thank Mrs.P. Segato for assistance in preparing this manuscript, and Dr. M.L. Calabro' for the critical reading of the manuscript.
This work was supported in part by grants from Associazione Italiana Ricerca sul Cancro (AIRC), Istituto Superiore di Sanità-Progetto AIDS, Ministero per l'Università e la Ricerca Scientifica e Tecnologica.
MP is supported by a fellowship from the Italian Association against Leukemia (AIL).

REFERENCES

1. Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D., Gallo, R.C. (1980) Proc. Natl. Acad. Sci. USA 77, 7415-7419
2. Hoshida, M., Miyoshi, I., Hinuma, A. (1982) Proc. Natl. Acad. Sci. USA 79, 2031-2035
3. Gessain, A., Barin, F., Vernant, J.C., Gout, O., Maurs, L., Calender, A., De The, G. (1985) Lancet 2, 407-410

4. Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitini, H., Igata, A., Matsumoto, M., Tara, M. (1986) Lancet 1, 1031-1032
5. Rosen, C.A., Sodroski, J.G., Haseltine, W.A. (1985) Proc. Natl. Acad. Sci. USA 82, 6502-6506
6. Paskalis, H., Felber, B.K., Pavlakis, G.N. (1986) Proc. Natl. Acad. Sci. USA 83, 6558-6562
7. Yoshida, M. (1993) Trends Microbiol 1, 131-135
8. Zhao, L.J., Giam, C.Z. (1992) Proc. Natl. Acad. Sci. USA 89, 7070-7074
9. Suzuki, T., Fujisawa, J.I., Toita, M., Yoshida, M. (1993) Proc. Natl. Acad. Sci. USA 90, 610-614
10. Wagner, S., Green, M.R. (1993) Science 262, 395-399
11. Paca-Uccaralertkun, S., Zhao, L.J., Adya, N., Cross, J.V., Cullen, B.R., Boros, I.M., Giam, C.Z. (1994) Mol. Cell. Biol. 14, 456-462
12. Giam, C.Z., Xu, Y.L. (1989) J Biol. Chem. 264, 15236-15241
13. Fujisawa, J.-I., Toita, M., Yoshida, M. (1989) J. Virol. 63, 3234-3239
14. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H., Montminy, M.R. (1988) Nature 334, 494-498
15. Champion-Arnaud, Gesnel, M.C., Foulkes, N., Ronsin, C., Sassone-Corsi, P., Breathnach, R. (1991) Oncogene 6, 1203-1209
16. Sodroski, J.G., Rosen, C.A., Haseltine, W.A. (1984) Science 225, 381-385
17. Luckow, B., Schutz, G. (1987) Nucl. Acids Res. 15, 5490
18. Saggioro, D., Forino, M., Chieco-Bianchi, L. (1991) Virology 182, 68-75
19. Bialojan, C., Takai, A. (1988) Biochem. J. 256, 283-290
20. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., Sugimura, T. (1988) Proc. Natl. Acad. Sci. USA 85, 1768-1771
21. Rieckmann, P., Thèvenin, C., Kehrl, J.H. (1992) Biochem. Biophys. Res. Commun. 187, 51-57
22. Thèvenin, C., Kim, S.J., Rieckmann, P., Fujiki, H., Norcoss, M.A., Sporn, M.B., Fauci, A.S., Kehrl, J.H. (1990) New Biol. 2, 793-800
23. Hagiwara, M., Alberts, A., Brindele, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., Montminy, M. (1992) Cell 70, 105-113
24. Lindholm, P.F., Kashanchi, F., Brady, J.N. (1993) Semin. Virol. 4, 53-60
25. Adunyah, S.E., Unlap, T.M., Franklin, C.C., Kraft, A.S. (1992) J. Cell. Physiol. 151, 415-426
26. Xu, X., Brown, D.A., Kitajima, I., Bilakovics, J., Fey, L.W., Nerenberg, M.I. (1994) Mol. Cell. Biol. 14, 5371-5383
27. Nyunoya, H., Akagi, T., Ogura, T., Maeda, S., Shimotohno, K. (1988) Virology 167, 538-544
28. Fontes, J.D., Strawhecker, J.M., Bills, N.D., Lewis, R.E., Hinrichs, S.H. (1993) J. Virol. 67, 4436-4441