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Phytohemagglutinin-Induced Activity of Cyclic AMP (cAMP) Response Elements from Cytomegalovirus Is Reduced by Cyclosporine and Synergistically Enhanced by cAMP

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The 19-base-pair enhancer repeat of the human cytomegalovirus immediate-early 1 gene mediates cyclic AMP- and phytohemagglutinin-induced expression in Jurkat T cells. Synergistic activity was observed in the presence of both drugs, suggesting a convergence of the protein kinase A and C pathways on this transcription element. In addition, the immunosuppressive drug cyclosporine strongly reduced the ability of the 19-base-pair repeat to activate gene expression in phytohemagglutinin-stimulated T cells.

T lymphocytes may serve as a reservoir for human cytomegalovirus (HCMV) during latency, and it has been suggested that T-cell activation plays a role in stimulating HCMV gene expression (3, 31, 33). The regulation of the first viral genes expressed, the immediate-early (IE) genes (40, 41), is in part controlled by host-cell-encoded transcription factors which interact with repeated and unique sequence motifs in the promoter-enhancer region (15, 17). Specifically, the 18- and 19-base-pair (bp) repeats bind to transcription factors (15) and contribute to the enhancer activity in vivo (7, 14, 22, 32, 37) and in vitro (17). The sequence GG GACTTTCC, which is found within the 18-bp repeat, binds to the transcription factor NF-κB and enhances gene expression in B cells (34) and in phorbolester–lectin-activated T cells (29, 30). The 19-bp repeat, which contains the cyclic AMP (cAMP) response element (CRE) consensus sequence TGACGTCA (26, 27), is present in all CMVs (2, 10, 25, 38), suggesting that it plays a vital role in IE gene expression. Since the 19-bp repeat mediates cAMP (protein kinase A)- and phytohemagglutinin (PHA; protein kinase C)-induced gene expression (22), we asked if the two signaling pathways share a route of activation or converge to synergistically increase gene expression from the IE1 promoter in T cells. Furthermore, we investigated whether the immunosuppressive agent cyclosporine (CsA) could block the PHA-activated expression.

Synergistic activation of the 19-bp enhancer repeat by PHA and cAMP. The mitogenic lectin PHA seems to require a functional T-cell-receptor complex for its effects on T-cell activation (28) and is thought to mimic antigenic stimulation of T cells (8). Upon stimulation of Jurkat T cells with PHA, expression from the IE1 enhancer increased about eightfold (Fig. 1). To identify enhancer elements mediating the PHA effect, oligonucleotides containing the 18- and 19-bp repeats of the HCMV IE1 region (2) were ligated to an IE1 core promoter (−55 to +7) in front of the bacterial chloramphenicol acetyltransferase (CAT) gene and analyzed. Footprinting studies suggested that this sequence is recognized only by promoter-binding proteins (16), which thereby excluded, to the best of our knowledge, possible interactions between the elements to be analyzed and additional transcription factors. One and six copies of the 19-bp repeat induced expression from the IE1 promoter in PHA-activated Jurkat cells 3- and 23-fold, respectively (Fig. 1). As expected, expression from the IE1 enhancer and the 19-bp repeats in Jurkat cells was also activated by cAMP. The IE1 core promoters linked to one and six copies of the 19-bp repeat were activated 6- and 13-fold, respectively, and the IE1 enhancer was activated about 4-fold (Fig. 2). Since the 19-bp repeat is a target for both cAMP and PHA stimulation, we asked whether the two drugs function identically. If both agents share a route of activation, we would not expect additive effects upon stimulation with saturable amounts of PHA and cAMP. In contrast, if the two drugs utilize different pathways for activation, an additive biological effect would be expected. PHA and cAMP had a more than additive effect on the core promoter containing one or six copies of the 19-bp repeats (Fig. 2). In particular, the six copies of the 19-bp repeat were activated about 26- and 14-fold by PHA and cAMP, respectively, and about 180-fold in the presence of both drugs. This suggests that the two activation pathways converge on this single transcription element. When the IE1 enhancer was tested, an additive effect of cAMP and PHA was observed (Fig. 2). Since the IE1 enhancer contains an array of transcription elements (e.g., Sp1-like, κB, NF1-like, and CRE) whose activities could be positively or negatively modulated by either signaling pathway, the additive effect probably reflects the combined activities of all elements. The synergistic effect of the T-cell mitogen PHA and the second messenger cAMP on the 19-bp repeat underscores the importance of these signals in the activation of the IE1 enhancer and possibly in the reactivation of CMV in T lymphocytes. The modulation of gene expression through the convergence of dual pathways on a single element may be a common theme (23).

The octameric CRE binding site (TGACGTCA) differs from the heptameric AP1 binding site (TGACTCA) by only a single base, and it is possible that the PHA-induced activity is mediated by AP1 proteins (1). However, when the functional octameric core of the 19-bp repeat was converted into a heptameric AP1 binding site, induction by PHA and cAMP was lost (Fig. 2) and protein binding was not detected (Fig. 3B). Thus, the CRE sequence within the 19-bp repeat binds to proteins other than AP1.

Since κB elements can mediate PHA-induced transcriptional stimulation from several promoters, including the

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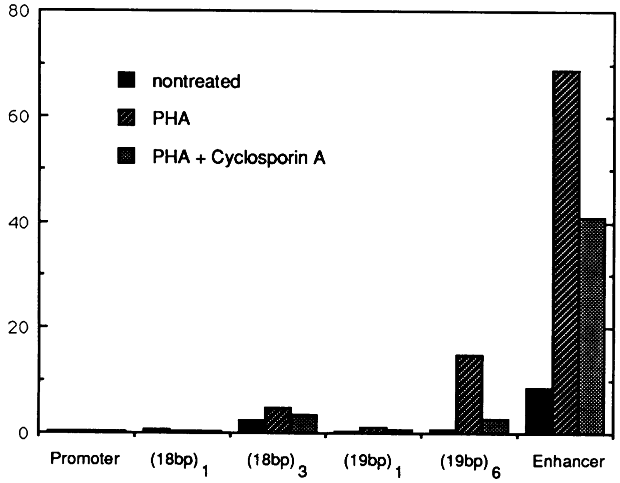

FIG. 1. Activities of HCMV transcriptional elements in Jurkat cells treated with 0.7 μg of PHA (Sigma Chemical Co.) per ml and 1 μg of CsA (Sandoz) per ml 18 h before cell harvest. The drugs and constructs are shown on the abscissa, and relative CAT activities are shown on the ordinate. The activity of the core promoter in nonactivated Jurkat cells is set at 0.4. The drug-induced activities of the promoter or of the $(18bp)_1$, $(18bp)_3$, $(19bp)_1$, $(19bp)_6$, and enhancer plasmids are related thereto. To generate the expression plasmids, oligonucleotides (from −428 to −411 for the 18-bp repeat and from −469 to −451 for the 19-bp repeat of the HCMV IE1 region [2]) were cloned immediately upstream of the HCMV IE1 core promoter (−55 to +7) in front of the structural CAT gene from pA10CAT2 (19). The enhancer (−524 to +7) plasmid was obtained from Jay Nelson, San Diego, Calif. For each transfection, 5 μg of plasmid DNA was incubated with 5×10^6 to 10×10^6 cells for 15 min in 2 ml of serum-free Dulbecco modified Eagle medium containing 250 μg of DEAE-dextran per ml, 0.1 mM chloroquindiphosphate, and 50 mM Tris hydrochloride at pH 7.3 at 37°C in a shaking water bath. The cells were then washed twice with medium and incubated in RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml under 5% CO_2 and at 37°C for 40 h. CAT assays were done by standard methods (19). Each experiment was done three times.

simian CMV (29, 30, 32), but fail to do so in the context of the HCMV IE1 (Fig. 1) (22) and IL-2Rα (9) promoters, we suggest that contextual sequences are critical for their activity. However, we cannot rule out the possibility that the 18-bp repeats are PHA inducible within their native environment in the IE1 enhancer.

Protein binding to the 19-bp repeat. We attempted to identify proteins which may mediate PHA- and cAMP-induced gene expression by analyzing the abilities of nuclear proteins from Jurkat cells grown in the presence of the two drugs to bind to the 19-bp repeat. Nuclear proteins from nontreated and cAMP-treated cells formed one predominant (Fig. 3A, complex d) and three minor (a through c) complexes with a single copy of the 19-bp repeat (Fig. 3A, lanes 1 and 3). PHA treatment resulted in a decrease in complex a and an increase in complex b (Fig. 3A, lane 2), which was even more pronounced in extract from PHA-cAMP-stimulated cells (Fig. 3A, lane 4). This extract also contained protein activity that formed a novel complex e (Fig. 3A, lane 4). Competition assays suggested that the complexes are sequence specific (data not shown). PHA inducibility of the Jurkat cells was monitored by analyzing κB binding activity. As expected (29), novel binding activity to the 18-bp repeat (κB site) was detected after PHA treatment (Fig. 3C, two arrows). To exclude the possibility that the 19-bp-repeat

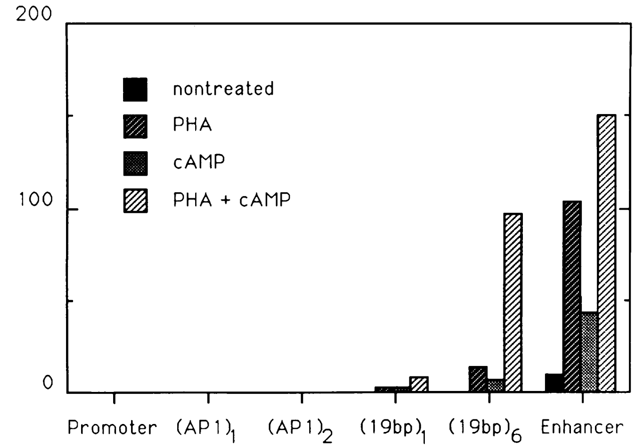

FIG. 2. Activation of HCMV transcription elements in Jurkat cells treated with 1 μg of PHA per ml and 1 mM 8-bromo cAMP (Sigma). The plasmids used are shown on the abscissa. The activity of the IE1 core promoter was not altered by PHA and cAMP and was arbitrarily set at 0.4. The activities of the plasmids containing one or two AP1 sites one or six 19-bp repeats, or of the entire enhancer are presented as fold increases over the corresponding activity of the core promoter. To generate the plasmids $(AP1)_1$ and $(AP1)_2$, an oligonucleotide containing the AP1 site (AGATCTT GACTCAAGGCCT) was cloned upstream of the IE1 core promoter in front of the structural CAT gene. Each experiment was done three times.

binding activity was related to AP1, we tested AP1 binding sites which differed from the functional CRE core in the 19-bp repeat by a single nucleotide (Fig. 2). No binding was detected in extract from unstimulated or PHA-stimulated Jurkat cells (Fig. 3B), suggesting that these cells do not contain AP1 activity.

Since CREs are recognized by dimeric forms of transcription factors (20, 26) containing protein kinase A and C target sequences (18), two models, both of which are compatible with our binding data, could account for the synergistic action of PHA and cAMP. Covalent modification of the preexisting CREB protein (42) by protein kinase A or C could result in the transcriptional stimulation observed with either cAMP or PHA alone. Phosphorylation with both protein kinases could result in a more potent transcription factor. Alternatively, cAMP and PHA could act through different transcription factors, and the cooperative binding of heterodimers to CREs would lead to enhanced transcription.

CsA-reduced PHA-induced gene expression. Clinical observations suggest that the rate of HCMV infection in organ transplant patients has declined since the introduction of CsA as an immunosuppressive agent (4, 5). Since CsA affects the initial mitogen-induced phase of T-cell activation (35) and suppresses the activation of several T-cell genes on the transcriptional level (8, 11–13, 24, 36, 39, 43), we studied its ability to block PHA-activated expression from the IE1 enhancer and the 19-bp repeat. CsA did not affect the basal activity of any of the constructs tested (data not shown). However, the PHA-induced activity of the IE1 enhancer was reduced by 45% when the two drugs were administered simultaneously (Fig. 1). Activation of the 18-bp repeat, which in the context of the $(18bp)_3$ plasmid responds only marginally to PHA, was also in part suppressed by CsA. Again, we cannot rule out the possibility that the 18-bp repeats play a larger role in the context of the whole

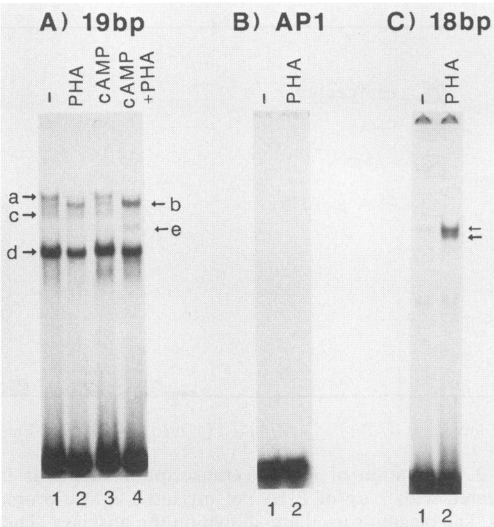

FIG. 3. Binding of T-cell nuclear proteins to the 18- and 19-bp enhancer repeats and to an AP1 binding site (as shown in Fig. 2). (A) Gel retardation of protein-DNA complexes on the 19-bp repeat. Lanes show nuclear extracts from untreated cells (lane 1) and from cells treated with 1 μg of PHA per ml for 18 h before harvest (lane 2), 1 mM 8-bromo cAMP for 18 h before harvest (lane 3), or both drugs (lane 4). (B) Gel retardation of protein-DNA complexes on an AP1 consensus site with nuclear extracts from untreated (lane 1) and PHA-treated (lane 2) Jurkat cells. (C) Gel retardation of protein-DNA complexes on the 18-bp repeat with nuclear extracts from untreated (lane 1) and PHA-treated (lane 2) Jurkat cells. For each gel shift experiment, 0.1 ng of a radioactively labeled 50-bp fragment containing the indicated specific sequences within the same polylinker context was incubated with 1 μg of nuclear protein from differentially treated Jurkat cells in 25 μl of a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH at pH 7.9, 40 mM NaCl, 40 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 1 μg of poly(dI-dC) (Pharmacia) as described previously (21). The resulting protein-DNA complexes were separated in a 4% polyacrylamide gel containing 50 mM Tris, 380 mM glycine, and 2 mM EDTA as gel and running buffer as described previously (21). Nuclear extracts were prepared as described previously (6, 21) from 5×10^8 cells grown in the presence or absence of drugs as indicated.

enhancer. The PHA-induced activities of one and six copies of the 19-bp repeats were reduced in the presence of CsA by 50 and 80%, respectively (Fig. 1). PHA-induced protein binding to the 19-bp repeat did not change upon treatment with CsA (data not shown). Although the mode of action of CsA is not known, it can be suggested that CsA interferes with a PHA-induced pathway that activates transcription from the IE1 enhancer through the 19-bp repeat. Similarly, CsA abolished the activity of the T-cell-inducible element NF-AT (13), which has been found in the interleukin-2 gene and has no sequence similarity to the 19-bp repeat. This suggests that CsA may inhibit the function of a proximal member of the signal transmission cascade leading from the antigen receptor to the nucleus.

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