Strong *trans* Activation of the Human Cytomegalovirus Major Immediate-Early Enhancer by p40^{tax} of Human T-Cell Leukemia Virus Type I via Two Repetitive Tax-Responsive Sequence Elements

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The immediate-early 1 and 2 gene locus of human cytomegalovirus (HCMV) that encodes *trans*-activator proteins with effects on both homologous and heterologous promoters is expressed under control of a complex enhancer/promoter regulatory region. This enhancer contains four types of repetitive sequence elements with 17, 18, 19, and 21 bp that bind cellular transcription factors. Although the HCMV enhancer acts as a powerful stimulator of transcription in most cell types examined, human T cells do not support strong activity. The present study demonstrates that the *tax* gene product of human T-cell leukemia virus type I *trans* activates the major enhancer of HCMV more than 60-fold in the T-cell line Jurkat. When a series of chloramphenicol acetyltransferase expression plasmids containing synthetic oligonucleotides with the 17-, 18-, 19-, or 21-bp motif upstream of a minimal immediate-early 1 and 2 gene promoter was tested, two of the four repeat motifs could be identified as Tax-responsive elements. Both the 18- and the 19-bp motifs were able to act as strong Tax-responsive elements even when they were present as single copies. Thus, in addition to interacting with human immunodeficiency virus, HCMV is able to interact with a second retrovirus of clinical importance.

Human cytomegalovirus (HCMV), a ubiquitous herpesvirus of worldwide occurrence, is capable of establishing lifelong persistent infections. Between 50 and 80% of the adult population in Central Europe and North America are seropositive for HCMV (43). Despite the high prevalence of HCMV infections, the virus is usually not pathogenic in healthy persons. In people with immunosuppression, however, HCMV can cause severe disease, resulting in clinical manifestations such as retinitis, hepatitis, or life-threatening interstitial pneumonia (25). One kind of paralysis of the immune system gaining importance is AIDS, which is caused by infection with human immunodeficiency virus type 1 or 2 (HIV-1 or HIV-2). HCMV has been described as an important pathogen in AIDS patients, although the discussion of whether cytomegalovirus infection is associated with a more rapid progression to AIDS is still going on (5, 14, 28, 32, 73). Interactions between HCMV and HIV on the molecular level, where reciprocal enhancement of viral gene expression can occur, have also been demonstrated (26, 60). In particular, proteins of the immediate-early 1 and 2 gene (IE-1/2) region of HCMV have been shown to *trans* activate HIV-1 enhancer/promoter sequences (4, 9, 22).

In this study, we describe the molecular interaction of HCMV with another retrovirus of clinical importance, human T-cell leukemia virus type I (HTLV-I). HTLV-I, which has also been proposed to play a cofactor role in accelerating clinical progression to AIDS in HIV-positive patients (24, 45, 47), is an etiologic agent for adult T-cell leukemia (48, 75) and is strongly associated with tropical spastic paraparesis (21, 29, 44). The genome of HTLV-I encodes a protein with a molecular mass of 40 kDa, termed p40^{tax}, that is able to *trans* activate both the homologous enhancer within the HTLV-I long terminal repeat (LTR) (18, 57, 62, 63) and several heterologous promoters (3, 34, 36, 53, 59). Activa-

tion of its own enhancer occurs via three imperfectly repeated sequence motifs, designated 21-bp repeats, that contain the sequence TGACG (46, 58). This sequence can act as a cyclic-AMP-responsive element (CRE) and has been identified in several cellular and viral promoters (52). However, it is not sufficient to confer Tax responsiveness; additional copies of the 21-bp repeat are required (20, 38). Stimulation by p40^{tax} of heterologous promoters such as the HIV I LTR (59) is mediated in most cases by a sequence element that binds transcription factor NF- κ B. This transcription factor acts as a cellular second messenger in communicating the effects of, for example, the protein kinase C stimulator tetradecanoyl phorbol acetate (TPA) to the cell nucleus and therefore plays an important role in the activation of resting T cells (33).

Initiation of HCMV gene expression occurs independently of prior viral protein synthesis and mainly under the control of cellular transcription factors. Expression of the IE-1/2 region (UL123-122) (15), which encodes proteins with important regulatory functions for progression of the viral lytic cycle (35, 67), is driven by an enhancer/promoter element with a cell-type-dependent strong constitutive activity (10, 71). This enhancer, termed major immediate-early enhancer, contains four types of repetitive sequence elements of 17, 18, 19, and 21 bp that act as binding sites for nuclear transcription factors and contribute differentially to enhancer function (65, 68). Specifically, the 18- and 19-bp motifs bind to proteins of the NF- κ -B and ATF/CREB families of transcription factors, respectively (54, 64). Whereas the transcription-stimulating properties of the HCMV enhancer are extremely strong in most cell types examined, lymphoid cells of T-cell origin do not support strong activity (27, 64). In these cells, however, enhancer functions can be stimulated by cellular signal transmission pathways involving protein kinases A and C (27, 40, 54, 64). Here, we demonstrate that the p40^{tax} gene product of HTLV-I acts as a powerful *trans* activator of

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the major enhancer of HCMV in the T-cell line Jurkat. Two types of repetitive-sequence elements, the 18- and 19-bp repeats, act as Tax-responsive elements. Since the two viruses have potentially identical target cells, a pathogenetic role for this molecular interaction might be possible.

MATERIALS AND METHODS

Plasmid constructions. Cloning reactions were performed according to standard procedures (2). Construction of chloramphenicol acetyltransferase (CAT) expression vector pRR55 containing HCMV enhancer/promoter sequences between -671 and +52 relative to the IE-1/2 transcription start has been described previously (19). Deletion plasmids pRR56/1, pRR56/2, pRR56/3, pRR56/4, and pRR56/5, which were created by *Bal* 31 shortening of HCMV IE-1/2 enhancer/promoter sequences, were obtained from R. Rüger (Erlangen, Germany) (31, 41, 64). CAT expression vectors pHTLVICAT and pRSVCAT are under the control of the HTLV-I LTR and the Rous sarcoma virus LTR, respectively. Tax expression vector pHISLESXdsPH (23), which contains the coding sequence for the p40^{tax} protein under the control of the murine SL3-3 promoter, was a kind gift of R. Grassmann (Erlangen, Germany). Expression plasmids wtctax and m7tax, which contain a genuine or mutated cDNA for Tax, respectively, were obtained from W. C. Greene (61). Tat expression vector pCT21, which contains the *tat* gene of HIV-I under the control of the HCMV IE-1/2 enhancer/promoter, was obtained from C. Aepinus (Erlangen, Germany). Insertion of oligonucleotides into CAT expression vector puPCAT containing HCMV IE-1/2 promoter sequences between -65 and +52 followed by polymerization of cloned oligonucleotides was performed as described previously (64). Briefly, oligonucleotides were synthesized by using a Cyclone DNA Synthesizer (Biosearch, San Rafael, Calif.) with *Sac*I-*Sal*I sticky ends and a *Xho*I recognition sequence overlapping the *Sac*I site. Since cleavage with *Sal*I and *Xho*I forms compatible ends, cloned oligonucleotides could be polymerized by cleavage of the appropriate vector, i.e., one with a unique *Hind*III cleavage site downstream of the CAT gene, with *Sac*I-*Hind*III and *Xho*I-*Hind*III, isolation of the corresponding fragments, and ligation. All plasmid constructs were confirmed by DNA sequence analysis with a commercially available T7 sequencing kit (Pharmacia, Freiburg, Germany).

Cell culture, transfections, and CAT assays. T-cell-derived Jurkat cells (55) were cultured in RPMI 1640 medium (GIBCO/BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS). Cells were split 1:5 every 3 days. Plasmid transfections were performed by using the DEAE-dextran procedure of Queen and Baltimore (50). Jurkat cells were washed three times with RPMI 1640 medium without FCS. Approximately 10^7 cells were then suspended in 3 ml of medium without FCS. After the addition of 5 μ g of CAT reporter plasmid, 5 to 10 μ g of effector plasmid (or as indicated in the Results), and 150 μ l of a solution containing 5 mg of DEAE-dextran (Sigma Chemical Co., Deisenhofen, Germany) per ml in 1 M Tris HCl (pH 7.4), cells were incubated for 1 h at 37°C. Cells were then washed with medium without FCS, suspended in 10 ml of complete medium, and grown for an additional 36 to 48 h. CAT assays were performed as described previously (19). Briefly, cellular extracts were prepared by freeze-thawing three times. After centrifugation to remove cell debris, protein concentrations of cell extracts were determined by using the Bradford assay (BioRad Laboratories, Munich, Germany). Extracts were analyzed for CAT activity by incubation in the

presence of 0.2 μ Ci of [¹⁴C]chloramphenicol and 0.4 mM acetyl coenzyme A. Percent conversion of chloramphenicol to acetylated forms was determined by ascending thin-layer chromatography and liquid scintillation counting of the spots cut from the plate. The results shown in Fig. 6 were obtained by quantitation of CAT enzyme levels with a commercially available CAT enzyme-linked immunosorbent assay (ELISA) (Boehringer, Mannheim, Germany). Each transfection was repeated at least three times, and representative results are given in this paper.

RNA isolation and RNase protection analysis. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (16) as described previously (66). RNA was analyzed for CAT-specific transcripts by quantitative RNase protection using a T7 RNA polymerase-generated antisense RNA probe (64). The probe was derived from plasmid pRR61, which contains a 1-kb *Hind*III-*Eco*RI fragment of plasmid pRR55 within the Bluescribe vector (Stratagene, Heidelberg, Germany). The probe, which was approximately 1 kb in size, covered about 250 nucleotides (nt) of the CAT gene and the IE-1/2 leader sequence as contained within plasmid pRR55. Therefore, this probe allowed the identification of correctly initiated transcripts from the HCMV IE-1/2 promoter as contained within plasmid pRR55 or puPCAT. RNA (40 μ g) was hybridized with the probe overnight at 37°C in 10 μ l of hybridization buffer containing 80% formamide, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid; pH 6.4)], 400 mM NaCl, and 1 mM EDTA. Single-stranded RNA was digested for 1 h at 37°C with 6 μ g of RNase A and 12 U of RNase T₁ (Boehringer) per ml in RNase buffer containing 300 mM NaCl, 5 mM EDTA, and 10 mM Tris HCl (pH 7.5). After ethanol precipitation, samples were analyzed on 6% (wt/vol) denaturing polyacrylamide gels. Correctly initiated transcripts from the HCMV IE-1/2 promoter resulted in a signal of 300 nt; transcripts from the Rous sarcoma virus LTR gave rise to a protected fragment of 250 nt.

RESULTS

Strong *trans* activation of the HCMV major immediate-early enhancer by the p40^{tax} protein of HTLV-I. The HCMV enhancer contains a repetitive-sequence element with homology to the binding site for transcription factor NF- κ B. This transcription factor has been shown to be involved in *trans* activation of several cellular and viral genes by the p40^{tax} protein of HTLV-I. Consequently, we asked whether the HCMV enhancer can be activated by p40^{tax}. To answer this question, transient expression assays with the human T-cell line Jurkat were performed. Plasmid pRR55, containing the HCMV IE-1/2 enhancer/promoter in front of the CAT gene as reporter, was cotransfected with 5 μ g of the Tax expression plasmid pHISLESXdsPH, and CAT activities were determined 48 h later. As shown in Fig. 1A, and B, lanes and bars 1 and 2, strong activation of HCMV enhancer functions could be observed in the presence of Tax. Stimulation of the HCMV enhancer was also found when a Jurkat cell line containing plasmid pRR55 stably integrated within the genome (64) was transfected with the Tax expression construct (data not shown). This effect was specific, as cotransfection with a plasmid containing the Rous sarcoma virus LTR in front of the CAT gene did not result in significantly elevated levels of CAT (Fig. 1A and B, lanes and bars 3 and 4). In contrast to the enhancer of HTLV-I, which was, as expected, strongly stimulated by Tax (Fig. 1A and B, lanes and bars 5 and 6), the HCMV enhancer had considerable constitutive activity in Jurkat cells without

FIG. 1. *trans* activation of the major enhancer of HCMV by $p40^{tax}$ of HTLV-I. Various CAT fusion constructs were transiently expressed in Jurkat cells with or without a Tax expression plasmid. (A) Representative autoradiogram showing induction of CAT activities by Tax. Lanes 1, 2, and 7 to 16, Jurkat cells were transfected with plasmid pRR55, which contains the HCMV enhancer/promoter upstream of the CAT gene; lanes 3 and 4, Jurkat cells were transfected with plasmid pRSVCAT, which contains the Rous sarcoma virus LTR upstream of the CAT gene; lanes 5 and 6, Jurkat cells were transfected with plasmid pHTLVICAT, which contains the HTLV-I LTR upstream of the CAT gene; lanes 2, 4, 6, and 12, cotransfection was performed with the Tax expression plasmid pHILESXdSPH (tax); lane 8, cotransfection was performed with the HIV-1 Tat expression plasmid pCT21 (tat); lane 10, cotransfection was performed with the vector used for expression of Tax (vec); lane 14, cotransfection was performed with the Tax expression plasmid wtctax (wtctax), which contains a *tax* cDNA; lane 16, cotransfection was performed with the Tax expression plasmid m7tax (m7tax), which contains a mutated *tax* cDNA. The CAT acetylation values of this experiment are shown beneath the autoradiogram. (B) Schematic diagram of CAT acetylation values. The graph represents the results of three independent experiments; standard deviations are indicated by bars. For nomenclature, see the legend for panel A.

trans activation (Fig. 1, lane and bar 1). As further controls, the vector used for expression of Tax or a plasmid which expressed the *trans*-activator Tat of HIV-1 was cotransfected with the HCMV enhancer-CAT construct. As shown in Fig. 1A and B, lanes and bars 7 to 10, no effect was observable. In addition, expression vector m7tax, which contains a mutated *tax* cDNA that had previously been shown to be *trans*-activation deficient (61), was not able to stimulate CAT activities from the HCMV IE-1/2 enhancer/promoter, whereas the wild-type cDNA expression vector wtctax was able to do so (Fig. 1A and B, lanes and bars 11 to 14).

To quantitate the effect of Tax on HCMV enhancer functions, we performed cotransfection experiments in a dose-dependent manner. Plasmid pRR55 was transfected either without *trans* activator or with the Tax expression plasmid pHILESXdSPH in a molar ratio of 1:1, 1:2, or 1:3.

FIG. 2. Quantitation of *trans* activation of HCMV enhancer/promoter by $p40^{tax}$. Cotransfection of plasmid pRR55, which contains the HCMV enhancer/promoter in front of the CAT gene, with the Tax expression plasmid pHILESXdSPH was performed in a dose-dependent manner. The diagram represents the mean CAT acetylation values from three independent experiments given as percent acetylation correlated with the molar ratio of reporter to transactivator. Standard deviations and fold activations are indicated.

HCMV enhancer activities were found to increase depending on the amount of cotransfected Tax expression plasmid (Fig. 2). About 60-fold *trans* activation was observed when a molar ratio of 1:3 was used (Fig. 2). Thus, the Tax protein is able to stimulate HCMV enhancer activities in a dose-dependent manner.

***trans* activation of HCMV enhancer by $p40^{tax}$ occurs on the RNA level.** RNase protection experiments were performed to ensure that activation of HCMV enhancer functions by the Tax protein occurs on the RNA level. Again, Jurkat cells were transfected either with plasmid pRR55, which contains the HCMV enhancer/promoter, or with plasmid pRSVCAT, which contains the Rous sarcoma virus LTR in front of the CAT gene, with or without the Tax expression plasmid. Total cellular RNA was harvested 36 h after transfection and analyzed for CAT-specific transcripts by quantitative RNase protection assay (Fig. 3). This experiment showed that the presence of Tax resulted in significantly increased amounts of correctly initiated transcripts (corresponding to the signal at 300 nt) from the HCMV IE-1/2 enhancer/promoter (Fig. 3, lanes 2 and 3), whereas transcript levels originating from the Rous sarcoma virus LTR were not altered (Fig. 3, lanes 4 and 5).

Two repetitive-sequence motifs of the HCMV enhancer act as Tax responsive elements. The HCMV enhancer contains four types of repetitive sequence elements, of 17, 18, 19, and 21 bp, that contribute differentially to the constitutive activity of this enhancer. In an initial attempt to localize Tax-responsive elements in the context of the HCMV IE-1/2 enhancer/promoter, a series of 5' deletion constructs containing various shortened fragments of the enhancer/promoter was cotransfected with Tax expression plasmid pHILESXdSPH, and CAT activities were determined 48 h later (Fig. 4). There was a correlation between the presence of repetitive-sequence elements and Tax response. Whereas plasmids pRR55 and pRR56/1 through pRR56/3 were *trans* activated by Tax, the removal of all repeat elements, as in plasmids pRR56/4 and pRR56/5, resulted in a loss of Tax responsiveness (Fig. 4). The degree of *trans* activation, however, varied between plasmids pRR55 and pRR56/1

FIG. 3. RNase protection analysis with total cellular RNA harvested from Jurkat cells after transient expression of plasmid pRR55 or plasmid pRSVCAT with or without the Tax expression plasmid pHISLESXdSPH. RNA was probed with a CAT-specific antisense transcript. Lane 1, molecular weight marker kilobase ladder; lanes 2 and 3, Jurkat cells were transfected with plasmid pRR55, which contains the HCMV enhancer/promoter in front of the CAT gene; lanes 4 and 5, Jurkat cells were transfected with plasmid pRSVCAT, which contains the Rous sarcoma virus (RSV) LTR in front of the CAT gene; lanes 3 and 5, cotransfection was performed with the Tax expression plasmid pHISLESXdSPH. Sizes (in nucleotides) of molecular size markers are shown on the left.

through pRR56/3. Interestingly, as plasmid pRR56/1 showed a response to Tax significantly higher than that of plasmid pRR55, the sequence between -671 and -526 seemed to exert a negative influence on Tax *trans* activation, whereas the constitutive activity of the enhancer was augmented (Fig. 4A, lanes 1 to 4 in panel B, and bars 1 to 4 in panel C). Because of the complex modular structure of the HCMV IE-1/2 enhancer/promoter, with repetitive-sequence elements that partly overlap each other, it was not possible to conclude from the analysis of 5' deletions any effects on distinct response elements. To test which of the repetitive enhancer motifs is involved in Tax *trans* activation, oligonucleotides corresponding in sequence to enhancer repeat elements were synthesized and inserted into the CAT expression vector puPCAT. This vector contains HCMV IE-1/2 promoter sequences between -65 and $+52$ relative to the IE-1/2 transcription start and allows a selective polymerization of inserted oligonucleotides upstream of the promoter, as already described for the 19- and 17-bp repeat motifs (64). In particular, it was important to clone the 18-bp element, as this motif has homology to binding sites for nuclear factor NF- κ B; therefore, it was the most probable candidate to act as Tax responsive element. After insertion of oligonucleotides into plasmid puPCAT, the motifs were dimerized and tetramerized. The nomenclature of the resulting plasmids and the oligonucleotide sequences of genuine and mutated enhancer motifs and of a CRE of the gonadotropin gene are listed in Table 1. CAT plasmids containing tetramers of HCMV enhancer motifs were then transiently

FIG. 4. *trans* activation of 5' deletion constructs of the HCMV IE-1/2 enhancer/promoter by p40^{tax}. (A) 5' deletion constructs of the IE-1/2 promoter region. Relative positions of repetitive sequence elements and transcription start of the IE-1/2 gene are shown at the top. The IE-1/2 enhancer/promoter sequences contained within each CAT expression plasmid are indicated by filled bars. Names of the 5' deletion plasmids are on the left. (B) Representative CAT assay of 5' deletion plasmids of the HCMV IE-1/2 enhancer/promoter in the presence or absence of the Tax expression plasmid pHISLESXdSPH. Lanes 1 and 2, plasmid pRR55; lanes 3 and 4, plasmid pRR56/1; lanes 5 and 6, plasmid pRR56/2; lanes 7 and 8, plasmid pRR56/3; lanes 9 and 10, plasmid pRR56/4; lanes 11 and 12, plasmid pRR56/5. In each lane, 5 μ g of plasmid was used for transfection of Jurkat cells. In lanes 2, 4, 6, 8, 10, and 12, cotransfection was performed with 5 μ g of the Tax expression plasmid. CAT acetylation values are shown at the bottom of the autoradiogram. (C) Schematic diagram of CAT acetylation values. Results are from three independent experiments; standard deviations are indicated by bars. Filled bars represent CAT acetylation values in the absence of p40^{tax}, and hatched bars show CAT acetylation values in the presence of p40^{tax}.

expressed in Jurkat cells with or without Tax expression plasmid pHISLESXdSPH. As an internal control for transfection variation, plasmid pRSVCAT, which did not respond upon Tax *trans* activation, was cotransfected with the various IE-1/2 promoter fusion genes. Total cellular RNA was harvested 36 h after transfection and analyzed for CAT-specific transcripts by using a riboprobe derived from plasmid pRR61 (see Materials and Methods). As this probe

TABLE 1. Sequences of oligonucleotides inserted in puPCAT and nomenclature of resulting plasmids

Motif	Oligonucleotide sequence			Plasmid designation		
				1 copy	2 copies	4 copies
19 bp	CGAG	CCCCATTGACGTCAATGGG	G	p19PCAT	p2-19PCAT	p4-19PCAT
	TCGAGCTC	GGGGTAACTGCAGTTACCC	CAGCT			
19(-1)	CGAG	CCCCATTGACG CAATGGG	G	p19(-1)PCAT	p2-19(-1)PCAT	p4-19(-1)PCAT
	TCGAGCTC	GGGGTAACTGC GTTACCC	CAGCT			
CRE-go	CGAG	AAATTGACGTCAATGGGTAA	G	pCREPCAT	p2-CREPCAT	
	TCGAGCTC	TTTAACTGCAGTACCCATT	CAGCT			
17 bp	CGAG	ACTTGGCAGTACATCAA	G	p17PCAT	p2-17PCAT	p4-17PCAT
	TCGAGCTC	TGAACCGTCATGTAGTT	CAGCT			
18 bp	CGAG	CCTAACGGGACTTTCCAA	G	p18PCAT	p2-18PCAT	p4-18PCAT
	TCGAGCTC	GGATTGCCCTGAAAGGTT	CAGCT			
21 bp	CGAG	ACGGTAAATGGCCGCCCTGGC	G	p21PCAT	p2-21PCAT	p4-21PCAT
	TCGAGCTC	TGCCATTACCGGGCGGACCG	CAGCT			

contains about 50 nt of IE-1 leader sequence, it gives rise to a signal of 250 nt with transcripts originating from the Rous sarcoma virus LTR and to a signal of approximately 300 nt which corresponds to correctly initiated transcripts from the HCMV IE-1/2 promoter. Whereas cloning vector puPCAT and plasmids p4-17PCAT and p4-21PCAT, which contain tetramers of the 17- and 21-bp motifs, respectively, did not respond significantly to Tax *trans* activation (Fig. 5A, lanes 9 and 10; data not shown), a strong increase in levels of CAT-encoding RNA was observable after cotransfection of plasmids p4-18PCAT and p4-19PCAT with Tax (Fig. 5A, lanes 3 to 6). When a mutated 19-bp motif in which one nucleotide from the center of the palindromic sequence had been deleted was tested (Table 1), no response to Tax could be found (Fig. 5A, lanes 7 and 8). Identical results were obtained when CAT activities were determined and when the Tax expression plasmid wtctax was used for cotransfection (Fig. 5B). In addition, expression vector m7tax, which contains the mutated *tax* cDNA, did not have any significant effects (Fig. 5B). Therefore, two of the repetitive motifs of the HCMV enhancer are able to mediate Tax *trans* activation, at least when they are tetramerized.

Single elements of the 18- or 19-bp repeat element are sufficient to mediate Tax responsiveness. To test whether single copies of the 18- and 19-bp repeat elements are sufficient for Tax *trans* activation, additional transient expression experiments were performed. Plasmids containing one, two, or four copies of the 18- or 19-bp repeat element (Table 1) were transfected alone or with Tax expression plasmid pHISLESXSPH into Jurkat cells. As shown in Fig. 6, plasmid p19PCAT, containing one 19-bp motif, and plasmid p18PCAT, containing one 18-bp motif, were *trans* activated 18- and 31-fold, respectively, by Tax. Thus, one single copy of these elements is able to confer strong Tax responsiveness. When plasmids containing several copies were used, an increase in both constitutive activity and activity after cotransfection with Tax was obvious (Fig. 6, p2-19PCAT, p4-19PCAT, p2-18PCAT, p4-18PCAT). As the 19-bp repeat element has been shown to function as a CRE (27, 64), we wanted to see whether Tax responsiveness is a general property of CRE motifs. For this reason, a construct containing the CRE of the human gonadotropin gene in one or two copies upstream of the IE-1/2 promoter (Table 1) was tested in parallel. No significant increase in levels of CAT could be found after cotransfection of these constructs with Tax (Fig. 6, pCREPCAT and p2-CREPCAT). Therefore, similar to the 21-bp elements of the HTLV-I LTR, the HCMV 19-bp motif can act as both CRE and Tax-responsive

element, whereas strong Tax responsiveness is not a general property of CREs.

DISCUSSION

In this study, we describe a molecular interaction of HCMV with HTLV-I, which encodes a strong *trans*-activator protein termed p40^{tax} (3, 18, 34, 36, 57, 62, 63). We demonstrate that this protein *trans* activates the HCMV major immediate-early enhancer. This enhancer is located upstream of the IE-1/2 transcription unit within the genome of HCMV. It controls the expression of IE-1 and -2 polypeptides that are required for efficient activation of promoters of the next temporal class (35, 67). Therefore, these proteins were suggested as playing a key role in progression of the HCMV lytic cycle. They are also able to stimulate expression from heterologous promoters such as the HIV-1 LTR (4, 9, 22). Consequently, they seem to be involved in the interaction of HCMV with HIV-1 (26, 60), at least at the molecular level. Thus, the presence or absence of IE-1 and -2 proteins as well as the levels of these proteins within a cell may be critical in determining whether virus replication of HCMV or other viruses proceeds. Activation of HCMV enhancer functions by p40^{tax} was rather strong and occurred in a dose-dependent manner. At a molar ratio of reporter to *trans*-activator plasmid of 1:3, >60-fold stimulation of HCMV enhancer activities was detected. Induction could also be found when RNA was analyzed, suggesting that the responsible mechanism is on the transcriptional level. Thus, although the constitutive activity of the HCMV enhancer is relatively low in human T cells (27, 40, 64), it can be induced considerably by the p40^{tax} gene product. Stimulation values obtained after cotransfection with tax even exceeded those that were reported earlier for cyclic-AMP, phytohemagglutinin, and/or TPA induction of the enhancer (27, 40, 54, 64).

To identify Tax responsive elements, a 5' deletion series of the HCMV IE-1/2 enhancer was tested for inducibility by Tax. This showed that the presence of repetitive-sequence elements is necessary for Tax induction, as deletion plasmids pRR56/4 and pRR56/5 did not respond efficiently. Since the analysis of 5' deletion constructs did not allow any conclusion on a distinct response element, the four repetitive-sequence motifs of the HCMV enhancer were cloned in one, two, and four copies upstream of a minimal HCMV IE-1/2 promoter. This approach revealed that two of the four types of motifs react upon cotransfection with Tax. The 18-bp motif shows homology to target sites for transcription factor NF- κ B, and it has been demonstrated previously that

FIG. 5. *trans* activation by $p40^{tax}$ of CAT fusion constructs containing tetramers of the 18- or 19-bp repeat element upstream of the IE-1/2 minimal promoter. (A) RNase protection analysis of total cellular RNA harvested from Jurkat cells transfected with various IE-1/2 promoter fusion genes and plasmid pRSVCAT (as internal control for transfection variation) either together or without Tax expression plasmid pHISLESXdsPH. Lane 1, riboprobe used for RNase protection analysis; lane 2, molecular size standard kilobase ladder; lanes 3 and 4, plasmid p4-19PCAT; lanes 5 and 6, plasmid p4-18PCAT; lanes 7 and 8, plasmid p4-19(-1)CAT; lanes 9 and 10, plasmid puPCAT. In each lane, 5 μ g of plasmid was used for transfection. In lanes 4, 6, 8, and 10, cotransfection was performed with 10 μ g of Tax expression plasmid pHISLESXdsPH. Arrows on the right indicate positions of signals that correspond to transcripts from the Rous sarcoma virus (RSV) LTR or the IE-1/2 promoter. Sizes (in nucleotides) of molecular size markers are on the left. (B) Representative CAT assay of various CAT fusion genes containing genuine or mutated tetramerized enhancer motifs in the presence or absence of genuine or mutated $p40^{tax}$. Lanes 1, 2, and 7, plasmid p4-19PCAT; lanes 3, 4, and 8, plasmid p4-19(-1)CAT; lanes 5, 6, and 9, plasmid p4-18PCAT; lanes 10 through 12, plasmid puPCAT. In each lane, 5 μ g of plasmid was used for transfection. In lanes 2, 4, 6, and 11, cotransfection was performed with 5 μ g of Tax expression plasmid wtTax; in lanes 7, 8, 9, and 12, cotransfection was performed with 5 μ g of Tax expression plasmid m7Tax (containing a mutated *tax* cDNA).

FIG. 6. Effects of various CAT expression constructs containing repeat elements of the HCMV enhancer or the CRE of the human gonadotropin gene in mediating *trans* activation by $p40^{tax}$. Jurkat cells were transfected with various CAT expression constructs (for detailed nomenclature and descriptions, see Table 1) with or without the Tax expression plasmid pHISLESXdsPH, and CAT levels were determined 48 h after transfection by using a CAT ELISA. Bars: ■, CAT levels in the absence of the Tax expression plasmid; ▨, CAT levels after cotransfection with the Tax expression plasmid. Data are means of three independent experiments; standard deviations are indicated by bars.

proteins with binding characteristics indistinguishable from those of NF- κ B interact with this sequence (54). In addition, the induction of a factor binding to the 18-bp repeat element could be observed in gel retardation assays when nuclear extracts from Tax-expressing cells were used (data not shown). As $p40^{tax}$ is known to induce NF- κ B (3, 34), it was not too surprising to find the 18-bp element to be reactive upon cotransfection with Tax. In contrast to earlier studies, however, in which several copies of the 18-bp element were reported to be necessary for efficient TPA induction in Jurkat cells (54), we found that one single copy is sufficient to confer strong Tax responsiveness on the otherwise non-responsive minimal IE-1 promoter. Whether this discrepancy is due to a more-efficient induction of NF- κ B by Tax than by TPA or to the use of different promoters in combination with the 18-bp element is at present not clear.

It was more surprising to identify a second Tax-responsive element within the HCMV enhancer, namely, the 19-bp repeat motif. This motif has a palindromic structure and was shown previously to be responsible for the strong constitutive activity of the HCMV enhancer in some cell types (19, 41, 69). The inner core of this element corresponds to the sequence TGACGTCA, which has been found in various hormone genes and can act as a CRE (52). The 19-bp motif itself acts as a very efficient CRE and confers cyclic-AMP inducibility on the HCMV enhancer (27, 64). We report now that the 19-bp motif can also act as a Tax-responsive element. This fact is not trivial, as it has been demonstrated that the CRE consensus sequence itself, as also contained within the homologous Tax-responsive element of the HTLV-I LTR, the 21-bp repeat, is not sufficient to confer Tax responsiveness (20, 38). In addition, several CRE or ATF sequences such as those of the somatostatin CRE, the vasoactive intestinal peptide CRE, the gonadotropin CRE,

FIG. 7. Alignment of the 21-bp repeat elements of the HTLV-I LTR and the 19-bp repeat motifs of the HCMV major immediate-early enhancer and comparison of sequences. Boxed sequences of the HTLV-I 21-bp repeats are conserved in the three repeats and were named domains A, B, and C. Boxed sequences of the HCMV 19-bp repeats indicate domain B, which is perfectly conserved compared with the HTLV-I 21-bp repeats. Nucleotides on stippled backgrounds are homologous between the HTLV-I 21-bp repeats and part of the HCMV 19-bp repeats and were found to be critical for Tax *trans* activation.

and the ATF site of the adenovirus E1aE promoter reacted not at all or showed a low response upon cotransfection with Tax (20, 30, 37, 38). The exact sequence requirements that determine whether a CRE reacts to Tax are not clear at present. In the case of the 21-bp repeat of HTLV-I, it has been reported that additional sequences that flank the CRE core are required for efficient induction by Tax (20). Comparison of the 21-bp repeats of the HTLV-I LTR revealed the existence of three conserved sequence blocks, termed domains A, B, and C (Fig. 7). Mutations within domain B, which corresponds to the CRE consensus, completely abolished *trans* activation, thus demonstrating that the CRE is absolutely required though not sufficient for Tax induction. Whereas domain C proved to be dispensable, mutations within domain A at positions 4 and 5 of the 21-bp repeat could also abrogate *trans* activation, suggesting a critical role for these nucleotide positions (20). When the sequences of the HTLV-I 21-bp repeats were compared with those of the HCMV 19-bp motifs, no extensive homology was detected except for a perfectly conserved domain B (Fig. 7). Three of the four 19-bp repeats had a conserved C corresponding to position 5 of the HTLV-I 21-bp motif, but all other nucleotides of domain A differed (Fig. 7). This may suggest an important role for this C in determining whether a CRE reacts to Tax. Further experiments will be necessary to test this hypothesis.

The mechanism of how p40^{tax} *trans* activates its own enhancer is poorly understood. Purified Tax protein does not bind specifically to DNA (6). The DNA-protein interactions detected on the 21-bp repeat motif of the HTLV-I LTR by DNase I footprinting or gel mobility shift assays are similar in the presence or absence of the Tax protein (1, 7, 42). Also, we were not able to detect a significant difference in the gel shift pattern when nuclear extracts from Jurkat cells were used with or without the Tax protein but with the 19-bp repeat element of the HCMV IE-1/2 enhancer as probe (data not shown). This could be due to the fact that the gel shift assay is not sensitive enough to detect a direct or indirect interaction of the Tax protein with DNA. Alternatively, the mechanism exerted by Tax could involve posttranscriptional modifications such as phosphorylation of proteins. Investi-

gations of proteins binding to the 21-bp repeats of HTLV-I detected ATF/CREB factors that interact with domain B and two additional proteins, termed HEB1 and HEB2, that interact with domains A and C or domain B, respectively (37, 70). A recent study suggested that p40^{tax} is able to bind to the 21-bp element, but this interaction was found to be indirect via the HEB1 protein (8). With respect to this result, it would be of interest to determine whether the HCMV 19-bp motif is also a specific target for this HEB1 protein. A selective binding of HEB1 to various CREs could also explain the differential responses of these sequences to Tax.

In contrast to the 21-bp repeat of HTLV-I (46, 58), the HCMV 19-bp motif was found to react efficiently upon Tax cotransfection when the motif was present as a single copy upstream of the minimal IE-1 promoter. The 21-bp motif also does not act as an efficient CRE in Jurkat cells (38, 70), whereas the 19-bp repeat does. The constitutive activities of multimerized 19-bp motifs were also higher than those of the 21-bp repeat. Thus, although these sequences are convergent in responding to p40^{tax}, they have different properties that seem to be determined by the sequences flanking the CRE core (17).

The described molecular interaction of HCMV with HTLV-I was observed in a human T-cell line. T cells are the target for infection with HTLV-I. The role of T cells for replication of HCMV is not clearly established. It has been shown that human T cells can harbor HCMV, but the frequency of positive cells was found to be rather low (11, 51, 56). Also, immediate-early gene expression could be demonstrated in only a small percentage of cells (56), but T-cell stimulation resulted in an increase of positive signals (39). In vitro, an interaction of both viruses within the same cell is possible, as it has been reported that HUT102 cells can be efficiently infected with HCMV (74). This cell line has been isolated from a patient with cutaneous T-cell lymphoma and is endogenously infected by HTLV-I (48). Therefore, since the *tax* gene product of HTLV-I activates human T cells, it may be able to stimulate HCMV immediate-early gene expression during natural infection. This ability may correlate with clinical observations that HCMV is an important pathogen in patients suffering from HTLV-I-associated adult T-cell lymphoma (12, 13, 72). Opportunistic infections in these patients were not due to chemotherapeutic immunosuppression, since the infections occurred before the onset of chemotherapy (12). In addition, opportunistic infections are uncommon in other cutaneous T-cell lymphomas that are not associated with HTLV-I (49). Therefore, although the biological significance of the described molecular interaction between HCMV and HTLV-I is not definitely clear, the observation that both viruses have T cells as a common target for infection may suggest a role for the interaction in pathogenesis.

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