trans Activation of the Thymidylate Synthase Promoter of Herpesvirus Saimiri

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Received 30 April 1990/Accepted 20 July 1990

Herpesvirus saimiri has been shown to possess a thymidylate synthase (TS) gene that is unusual in its transcriptional regulation. Although TS is believed to be required for viral DNA synthesis, the TS-specific 2.5-kb mRNA was found most abundantly during the late phases of asynchronous virus replication in permissive cultures. To study the kinetics of gene activation, the TS promoter and regulatory sequences were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene. No CAT expression or transcripts were found after transfection of fusion genes into permissive owl monkey kidney (OMK) cells. However, the promoter was strongly activated when CAT plasmids were cotransfected with intact herpesvirus saimiri virion DNA or were transferred to OMK cells that were lytically infected with herpesvirus saimiri or a related herpesvirus, herpesvirus ateles. CAT was expressed at reduced levels in cultures when viral DNA replication was inhibited by phosphonoacetic acid; this indicates that the gene is activated during the delayed-early phase. However, the highest amounts of mRNA were present in the late period of replication. Deletion analyses localized essential response elements for *trans* activation in the promoter upstream region between nucleotides -311 and -56; they consisted of related tandem repeats and perfect palindromes. A sequence with two overlapping palindromes of 16 and 18 bp was found to be a major target for activation of the herpesvirus saimiri TS promoter. These palindromes did not have any significant homologies with known sequences of herpesviruses or cellular DNA; the 18-bp palindrome had, however, a certain structural similarity with a conserved sequence of the E2-responsive *cis* sequence that is required for transcription regulation of early papillomavirus genes.

Herpesvirus saimiri, a virus of squirrel monkeys (*Saimiri sciureus*) that belongs to the gamma 2 subgroup of herpesviruses, induces malignant lymphoproliferative diseases in various New World primates and is capable of transforming T lymphocytes in vitro. Tumor cell lines and in vitro-transformed cells contain multiple copies of the viral genome in episomal form (29, 56, 65). Besides a characteristic set of five small nuclear RNAs (32, 43, 64), a single polyadenylated viral 2.5-kb transcript was found in transformed cells (30) that could be mapped to the right half of the *Kpn*I D fragment of herpesvirus saimiri strain 11 protein-coding DNA (L-DNA) (5). The open reading frame of this mRNA codes for a polypeptide of 33.5 kDa that has extensive sequence homology with the thymidylate synthase (TS; 5,10-methylenetetrahydrofolate:dUMP C-methyl-transferase; EC 2.1.1.45) (5) coding sequences of various bacteria and eucaryotic cells. The viral TS gene product was shown to act as a functional enzyme (26). TS genes were also detected in herpesvirus ateles, a related tumor virus of the gammaherpesvirus subgroup (53) and in the human varicella-zoster virus (VZV) (63); however, no TS activity has been found among other herpesviruses.

The structure and regulation of the herpesvirus saimiri TS gene are unusual in many regards. The mRNA has a long leader sequence of 1,207 nucleotides (nt) with 22 ATG codons, each followed by a translational stop signal. Upstream reading frames are generally assumed to downregulate gene expression (42, 50). The gene appeared to be weakly transcribed in permissive owl monkey kidney (OMK) cell cultures when protein synthesis was blocked with cycloheximide prior to virus infection (4). The same

type of mRNA was most abundantly found late in replication of herpesvirus saimiri or herpesvirus ateles (5, 53). Because of the asynchronous replication of the viruses in OMK cells and in all other known permissive systems (49), it remained unclear whether the remarkably late TS mRNA accumulation is the consequence of unusual stability of the mRNA or a reflection of the strength of the TS promoter. Here we describe the regulation of the herpesvirus saimiri TS gene that is increasingly expressed from delayed-early to late times of virus infection and identify a genomic upstream region with a tripartite palindrome as a response element for activation in *trans*.

MATERIALS AND METHODS

Virus and cell culture. The OMK cell line 637 (12) was used for propagation of herpesvirus saimiri strains 11 (17), SMHI (38), OMI (28), S295C (39), 484-77 and 488-77 (15), and attenuated strain S4 (14) and herpesvirus ateles strain 810 (40) by standard procedures. Transfection with intact herpesvirus saimiri virion DNA was done as described before (19, 22), followed by a 20% (wt/vol) glycerol shock. Phosphonoacetic acid (PAA; ICN Pharmaceuticals, Inc., Life Sciences Group, Plainview, N.Y.) was used to inhibit viral DNA replication at a final concentration of 200 to 400 µg/ml; it was added at the time of virus infection or transfection of virion DNA (24, 45). Virus particles were purified from infected cell cultures by velocity sedimentation in sucrose gradients (20).

Plasmid cloning, oligonucleotide synthesis, and sequencing. Cloning of DNA fragments was done by standard protocols (35). A 786-bp *Hind*III fragment containing the entire TS promoter (5) was cloned into the *Hind*III site of pSV0CAT (21) to generate pSV0CAT786. A series of promoter deletions were constructed in pSV0CAT786 by restriction enzyme digestion and subsequent shortening with exonuclease *Bal*31

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from an *Xba*I site or a *Kpn*I site (35) and with exonuclease III from an *Nhe*I site (25). The deletion (Δ A) was made by digestion of pSV0CAT786 with *Asp*718, followed by digestion of the cohesive ends with mung bean nuclease and religation.

Oligonucleotides were polymerized by the phosphoramidite procedure with a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.) and purified by polyacrylamide gel electrophoresis and ion-exchange chromatography through DEAE-Sephadex G-25 columns (Pharmacia, Uppsala, Sweden). The oligonucleotides were hybridized to create double-stranded palindromes and ligated into the *Sma*I site of plasmid pUC18. Sequences of cloned oligonucleotides and of all deletion mutants were determined by double-stranded DNA sequencing by standard methods (55).

DNA transfection and CAT assays. All transfection experiments were carried out by the calcium phosphate precipitation method with approximately 80% subconfluent monolayers of OMK cells (22). The cells were usually infected with herpesvirus saimiri or herpesvirus ateles 1 day prior to transfection. DNA transfer was performed with 10 μ g of supercoiled plasmid DNA. All transfection experiments were repeated at least twice with separate plasmid preparations.

To monitor gene expression by chloramphenicol acetyltransferase (CAT) assays, cells were transfected with plasmids containing fusion genes and incubated for 48 or 72 h. Cell extracts were assayed for CAT activity (21). One-tenth of each probe was used for DNA isolation to monitor the quality of transfection by Southern blot hybridization. The protein content in each extract was measured with the protein assay of Bio-Rad Laboratories (Munich). Enzymatic reactions were carried out at 37°C with 200 μ g of protein extract, 0.1 to 0.3 μ Ci of [14 C]chloramphenicol (New England Nuclear Corp., Boston, Mass.), 4 mM acetyl coenzyme A in 0.2 M Tris hydrochloride (pH 7.8). The reaction was stopped after 20, 40, and 60 min by extraction with 1 ml of ethyl acetate. The organic phase was dried and spotted on silica gel thin-layer chromatography plates. The chromatography was performed in chloroform-methanol (95:5). The plates were autoradiographed on Kodak XAR5 films. Acetylation was determined quantitatively by scraping the silica gel off the glass plates and counting in a scintillation counter.

Transcription analyses. Plasmid DNA was transfected into herpesvirus saimiri-infected OMK cells; after 3 days, cytoplasmic RNA was prepared by a published procedure (10). Nuclease S1 protection assays were performed to determine the transcription initiation site of the TS promoter in CAT fusion genes (3). CAT transcripts were quantitated by RNase protection assays. Total cellular RNA of transfected OMK cells was isolated (11), and the RNA was analyzed for CAT-specific transcripts with an RNA probe generated by T7 RNA polymerase. Labeled RNA was synthesized by using plasmid pRR61 (58), which contains a CAT-specific sequence downstream of the T7 promoter in the Bluescribe vector (Vector Cloning Systems, San Diego, Calif.). Total-cell RNA (40 μ g) was hybridized with the probe for at least 12 h 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 (31). Digestion of single-stranded RNA was performed at 37°C for 1 h with 6 μ g of RNase A and 8 to 15 U of RNase T₁ per ml. The protected RNA duplexes were resolved on 6% (wt/vol) denaturing polyacrylamide gels.

Computer analyses of DNA sequences. A program package from the University of Wisconsin Genetics Computer Group (Madison, Wis.) (16) was used that was implemented on Vax 11/780 and MicroVax 3500 computers. The data bases used

were GenBank release 60.0 of June 1989, EMBL release 19.0 of May 1989, and NBRF (nucleic acids) release 35.0 of May 1989.

RESULTS

Virus-specific activation and temporal regulation of TS transcription. To determine whether transcription of the herpesvirus saimiri TS gene is constitutive or regulated by viral proteins, a TS-CAT fusion gene was constructed by placing the 786-bp *Hind*III fragment with the TS promoter into the *Hind*III cleavage site of the promoter-negative plasmid pSV0CAT (21). The resulting plasmid, pSV0CAT786 (Fig. 1), was transfected into uninfected OMK cells and into cultures that had been infected with herpesvirus saimiri or herpesvirus ateles the previous day. The fusion gene appeared to be strongly activated within 2 or 3 days in all virus-infected cultures, while no measurable CAT conversion was seen in mock-infected OMK cells (Fig. 2). Virus-specific activation was seen with herpesvirus saimiri strains of all known subgroups, including strains 11 and OMI (subgroup A) (17, 28), strain SMHI (subgroup B) (38, 62), strains 484-77 and 488-77 (subgroup C) (15), and the non-cogenic variant S4 (14). The same patterns of virus-specific *trans* activation of the herpesvirus saimiri TS promoter were seen with the monkey kidney cell lines CV1 and COS and when HeLa cells were used (data not shown).

It had been shown previously for herpes simplex virus type 1 (HSV-1) that a structural protein stimulates immediate-early (IE) gene expression (2, 47, 48); thus, UV-inactivated herpesvirus saimiri was added to OMK cell cultures prior to transfection of pSV0CAT786. *trans* activation decreased with decreasing titers of infectious virus, confirming that the activity of the TS-CAT fusion gene depends on virus replication (data not shown). When intact virion DNA was cotransfected with the TS-CAT fusion gene, the TS promoter was fully stimulated. Nuclease S1 analysis showed that the TS promoter was correctly used after transient expression. There was only one initiation site of transcription at nt 641 in the 786-bp TS promoter construct, confirming earlier primer extension studies (5) (data not shown).

Studying the kinetics of herpesvirus saimiri transcription and protein synthesis has been difficult because a multiplicity of infection of ≥ 1 was difficult to obtain; the start of replication in the most-permissive cells has always remained asynchronous (49). Thus, lytic infection of all cells is reached only after several replication cycles, when all stages of gene expression may be present at the same time.

To analyze TS regulation during a defined early phase, we transferred infectious virion DNA to OMK cells in the presence of 200 to 400 μ g of PAA per ml for 2 or 3 days. There was no difference when 200 or 400 μ g/ml was used. To study late replication, OMK cells were transfected with virion DNA and incubated without PAA for the same time period. Early and late conditions were checked by Northern (RNA) blot analyses of the known late gene which encodes the 143-kDa matrix protein of herpesvirus saimiri (8). Progeny herpesvirus saimiri were not expected to be synthesized within 3 days because the culture supernatant was free of infectious virus and no cytopathic changes were observed.

Cotransfection of the TS promoter-CAT fusion gene with virion DNA resulted in significant activation in the presence of PAA. Even higher enzyme activities were reached when replication proceeded to the late phase (Fig. 3 and 4). RNase I protection assays indicated that CAT activity and the amount of transcripts from pSV0CAT786 correlated (data

FIG. 1. Map position of the TS gene in the herpesvirus saimiri genome and structure of the TS promoter-CAT fusion gene in plasmid pSVOCAT786. The virion DNA of herpesvirus saimiri strain 11 consists of a 112-kb L-DNA segment flanked at both ends by tandem repetitions (H-DNA). A 786-bp *Hind*III fragment containing the TS promoter and upstream regulatory sequences was inserted into the unique *Hind*III site of the plasmid pSVOCAT (21).

not shown). This indicated that, as may be expected for an enzyme of nucleotide synthesis, TS gene expression commences before DNA replication; however, the TS mRNA appeared to accumulate to the highest concentrations in the late phase of replication.

Regulatory region upstream of the TS promoter. To define the promoter and regulator sequences necessary for activation by herpesvirus saimiri gene products, a series of deletion mutants were constructed by restriction enzyme digestions and by progressive shortening with exonucleases III or *Bal*31 (Fig. 5). The resulting fusion genes were tested for transient CAT expression in lytically infected cells.

Deletions upstream of nt -291 did not appreciably decrease *trans* activation; they showed a moderately silencing effect when they were preserved in the *cis* position. CAT activity with mutants $\Delta 1$ and $\Delta 2$, lacking these sequences, was increased compared with activity with the wild-type plasmid pSVOCAT786. CAT activity was increased 138-fold with plasmid pSVOCAT786, 191-fold with mutant $\Delta 1$, and 180.5-fold with $\Delta 2$ in infected cells compared with the activity of pSVOCAT786 in uninfected cells (Table 1). This upstream region (nt -346 to -641), which contains an imperfect 30-bp palindrome (nt -467 to -499), had a slight inhibitory influence when it was fused with the promoters of the human cytomegalovirus (HCMV) major IE, HSV-1

thymidine kinase (*tk*), or simian virus 40 (SV40) early T antigen gene (data not shown).

The major target sequences for activation of the herpesvirus saimiri TS gene were localized downstream of nt -311 (Fig. 5, Table 1). When sequences between nt -311 and -56 were deleted, as in mutants $\Delta 5$ and $\Delta 3/4$, TS promoter activity was drastically reduced in lytically infected cells. When the transcription initiation site was cut out, CAT expression was abolished. Table 1 and Fig. 6 show the kinetics of CAT assays from all promoter mutants. Activation of pSVOCAT786 in virus-infected cells was compared with that in uninfected OMK cells. The *Xba*I-*Nhe*I fragment, which contains important transactivator sequences of the herpesvirus saimiri TS promoter, did not confer this activity on four heterologous genes, the HCMV major IE, SV40 early, or HSV-1 *tk* gene or the human T-cell leukemia virus type 2 long terminal repeat (data not shown).

The levels of CAT mRNA were measured by RNase protection with approximately equimolar amounts of expression plasmid and an excess of radioactive RNA probe. This was to confirm that enzyme activities were a measure of the steady-state levels of CAT-specific RNA (Fig. 7). The amount of protected RNA was proportional to the CAT activities (Table 2). RNA was not detected when pSVOCAT786 was transferred into uninfected OMK cells.

FIG. 2. *trans* activation of the TS promoter in herpesvirus saimiri-infected OMK cells. Cultures were infected 1 day after plating, and transfection was carried out with 10 μ g of DNA from plasmid pSV0CAT786. Lane 1, Transfection control (plasmid pRR55 [18] containing the constitutive HCMV enhancer/promoter in front of CAT transfected into OMK cells). Lane 2, Activation of transfected plasmid pSV0CAT786 at simultaneous virus infection, harvested after 2 days. Lane 3, Activation of herpesvirus saimiri TS promoter. Cells were infected 1 day after plating. Transfection of CAT expression plasmids was done the following day, and the cells were harvested 2 days later. Lane 4, Activation of herpesvirus saimiri TS promoter by herpesvirus ateles. Infection was done 1 day after plating, transfection at the second day, harvesting at the fourth day. Lane 5, Transfer of TS promoter-CAT fusion gene into mock-infected cells (2 days of incubation). Cm, chloramphenicol; 1AcCm, chloramphenicol-1-acetate; 3AcCm, chloramphenicol-3-acetate; 1,3AcCm, chloramphenicol-1,3-acetate.

Palindromic regulatory element. The nucleotide sequence containing the essential response elements for *trans* activation of the herpesvirus saimiri TS promoter is shown in Fig. 8. A canonical binding site for transcription factor AP-1 was located at nt -158 to -150 (6, 7, 33, 41, 51). The same sequence element was found in the herpesvirus ateles TS upstream region at an equivalent position. A remarkable AT cluster of 34 bp was positioned between nt -230 and -264. Most striking were three perfect symmetrical palindromes of 16, 18, and 24 bp. The palindromes of 16 and 18 bp were overlapping, forming a tripartite structure and resulting in a 10-bp tandem repetition (Fig. 8). One half of the related 24-bp palindrome was repeated twice, yielding a threefold 10-bp tandem reiteration.

Partial destruction of the tripartite complex of two overlapping 16- and 18-bp palindromes resulted in more than 60% loss of activity upon transfection into herpesvirus saimiri-transfected OMK cells (Table 1, mutant Δ 3). When the 24-bp palindrome sequence was also removed, CAT activity was not diminished further (Table 1, mutant Xba5'). This suggests that the tripartite palindrome between nt -272 and -297 is a relevant element for *trans* activation in virus-infected cells. It led us to alter this structure by removing 4 bp at a *KpnI* cleavage site in its center. CAT activity from the resulting mutant, Δ A, corresponded to about a fourth of

FIG. 3. Activation of the herpesvirus saimiri TS promoter during the early and late phases of virus replication. OMK cells were transfected with 10 μ g of DNA from plasmid pSV0CAT786 and cotransfected with 10 μ g of virion DNA in the presence of PAA (200 μ g/ml). Lane pSV0CAT786, TS-CAT plasmid transferred to mock-infected cells. Lane virion DNA, Cotransfection of TS-CAT plasmid with 10 μ g of virion DNA without inhibitor to allow progression into the late phase of replication. Lane PAA inhibition, Cotransfection of plasmid pSV0CAT786 with virion DNA in the presence of PAA. Lane OMK, OMK cells as a negative control. For abbreviations, see Fig. 2 legend.

that from the plasmid pSV0CAT786. The promoter of Δ A was stimulated in herpesvirus saimiri-infected cells by a factor of 32 compared with uninfected OMK cells; the wild-type promoter-upstream sequences were activated by a factor of 138 through virus infection. This indicates that the complex tripartite palindrome is a functionally important

FIG. 4. Time kinetics of TS promoter activation (measured by CAT conversion) during the early and late phases of virus replication. Time kinetics was determined for the CAT assay shown in Fig. 3. CAT conversion was measured after 20, 40, and 60 min of enzyme reaction. Virion DNA, Cotransfection without inhibitor, incubation for 2 days to reach the late phase. Virion DNA + PAA, Cotransfection in the presence of PAA (200 μ g/ml). pSV0CAT786, TS-CAT plasmid on OMK cells without virion DNA. CAM, Chloramphenicol.

FIG. 5. Map of the herpesvirus saimiri TS promoter upstream sequences in the TS-CAT fusion plasmid pSV0CAT786 and location of deletions in the mutants. The numbers refer to the distance from the cap site (+1). The TATA-box is indicated; vector pBR322 sequences are shown as dashed lines. Lytically infected OMK cells were transfected with the TS-CAT plasmids (10 μ g) and assayed for CAT enzyme activity.

target sequence for virus-specific *trans* activation, although other elements must exist that confer inducibility by viral proteins.

Another approach to defining the functional relevance of the 16- and 18-bp palindromes was done by competition

assays with cloned synthetic oligonucleotides. The plasmid pSV0CAT786 (10 μ g) was cotransfected with plasmid pGL18, which contains one copy of the 18-bp palindrome, with plasmid pGL24, which has one copy of the 24-bp palindrome, and with pGL30, which has the 30-bp palindrome found in the moderately silencing upstream region.

Cotransfection experiments with pGL18 at a 20-fold molar

TABLE 1. Activity of TS promoter and deletion mutants

Plasmid	Mutation ^a	% CAT acetylation		Induction in infected cells at 20 min ^b (fold)
		Uninfected cells, 20 min	Infected cells 20 min 60 min	
pSV0CAT786	Wild type	0.5	69 92	138
Δ1	pBR322/-346	0.3	95.5 99.2	191
Δ2	pBR322/-291	0.5	90.3 97.3	180.5
Δ3	-346/-286	0.4	27 87	54
Δ5	-311/-75	0.3	8 40	16
Δ7	-89/+49	0.5	0.5 1	1
Xba3'	-361/-321	0.8	73 95.6	146
Xba5'	-443/-158	0.5	22 76.8	44
Asp2/2	-585/-105	0.6	16 53.8	32
Asp1/6	-385/-93	0.6	12 49.4	24
Asp3/4	-346/-56	0.4	7.4 21	14.8
Asp2/4	-585/-56	0.8	7 18.7	14
Asp2/1	-480/-2	0.4	0.7 2	1.4
E6	-141/-41	0.3	3 ND ^c	6
ΔN	pBR322/-91	0.4	1.5 ND	3
ΔS	pBR322/-346/+50/+145	0.8	86 ND	172
ΔA	4-base deletion	0.5	16 ND	32

^a Coordinates of deletions (Fig. 5).

^b Induction relative to basal activity of pSV0CAT786 in uninfected cells.

^c ND, Not done.

% acetylated CAM

FIG. 6. Time kinetics of TS promoter activation in five mutants. The TS-CAT fusion genes Δ1, Δ3, Xba5', Asp1/6, and Asp2/4 were transfected into lytically infected OMK cells; pSV0CAT786 shows the result of transfection of wild-type plasmid into mock-infected cells. Average percentages of acetylation for three independent assays after enzyme reactions of 20, 40, and 60 min were determined with standard deviations. CAM, Chloramphenicol.

FIG. 7. RNase protection analysis of RNA from transfected OMK cells. TS promoter-CAT fusion genes and deletion mutants were transferred into infected OMK cultures. RNA was isolated 2 or 3 days after transfection. Total cellular RNA was analyzed for CAT-specific transcripts. Lane 1, RNA from OMK cells; lane 2, RNA from infected OMK cells; lane 3, RNA from infected OMK cells transfected with $\Delta 1$; lane 4, RNA from infected OMK cells transfected with pSV0CAT786; lane 5, RNA from infected OMK cells transfected with Xba5'; lane 6, RNA from infected OMK cells transfected with $\Delta 2$; lane 7, RNA from infected OMK cells transfected with Asp1/6; lane 8, RNA from infected OMK cells transfected with $\Delta 5$; lane 9, RNA from infected OMK cells transfected with Asp3/4; lane 10, RNA from infected OMK cells transfected with Asp2/4; lane 11, pSV0CAT786 transfected into uninfected cells; R, riboprobe; M, size markers (pBR322 cleaved with *MspI*) (in base pairs). The CAT mRNA is indicated. For nomenclature and description of the mutants, see Fig. 5 and Table 1.

excess resulted in 50% reduction of CAT expression (Fig. 9). Parallel experiments with the 24-bp and 30-bp palindromes showed no reduction in CAT activity. Cotransfection with plasmid pSV0CAT786 and pUC18 as a control showed that the reduction was not the result of nonspecific competition by the addition of excessive amounts of DNA. It seems that *trans*-acting proteins may be sequestered by an excess of an oligonucleotide with specific binding properties.

Computer searches did not hint at any homology of the herpesvirus saimiri TS promoter palindromes to sequences in the other herpesviruses with known primary structure (HSV-1, VZV, Epstein-Barr virus [EBV], and HCMV) or to

known cellular transcription factor binding sites. However, a similarity was found to a sequence in the noncoding region of papillomaviruses. The 18-bp palindromes resembled the consensus sequences ACCGN₄CGGT and ACCN₆GGT (23, 57) in its flanking sequences. However, the spacing was different and resulted in ACCGN₆CGGT. The palindromes in papillomaviruses are the binding sites for the early proteins of E2. These are transcriptional activators or, as fragments, inhibitors that are important in the regulation of papillomavirus gene expression (36, 59).

DISCUSSION

The structure and expression of the TS gene of herpesvirus saimiri is enigmatic in many regards. Besides herpesvirus ateles, another gamma 2 herpesvirus (53), and the unrelated VZV (63), a number of other herpesviruses do not possess a TS gene. Transcripts of TS genes of primate gammaherpesviruses are accumulated late in virus replication, though it might be expected that an enzyme of nucleotide metabolism should be present prior to DNA replication. We analyzed the temporal regulation of TS transcription, defining the conditions of delayed-early and late replication by cotransfections with virion DNA into permissive OMK cells. Transient expression of TS promoter-CAT fusion genes and quantitation by nuclease protection indicated that the TS gene is expressed during late replication conditions, but reduced amounts of transcripts were also found when DNA replication was inhibited. Thus, the TS gene of herpesvirus saimiri resembles the kinetics of $\beta\gamma$ genes (γ_1) of HSV (54). Similar expression control was found for the 65-kDa lower-matrix

TABLE 2. Expression of TS-CAT fusion genes: correlation between CAT activity and RNA levels as determined by RNase protection

Plasmid	Induction of CAT activity (fold) by virus infection ^a	Protected RNA (cpm) after infection ^b
$\Delta 1$	191	1,200
pSV0CAT786	138	930.7
Xba5'	44	278.5
$\Delta 2$	180.5	1,050.3
Asp1/6	24	138.2
$\Delta 5$	16	90.2
Asp3/4	14.8	60.0
Asp2/4	14	60.0

^a Relative to transfection in uninfected cells.

^b CAT RNA hybrids were cut out of the RNase protection gel shown in Fig. 7. Counts in the RNA were counted in a scintillation counter.

FIG. 8. Nucleotide sequence of the upstream region of the TS promoter from the 5' *Xba*I site (–346) to the *Nhe*I site (–91) relative to the cap site (+1). The palindromes are indicated by large shaded arrows, the repeats are boxed, and the long AT stretch is indicated by black dots. The borders of the deletions in pSV0CAT786, Δ1, Δ2, *Xba*5', and Asp1/6 are shown by vertical arrows. Deletion ΔA, with a 4-base (GTAC) deletion, is indicated by a bracket above the 16- and 18-palindrome.

phosphoprotein (pp65) of HCMV, in which lower levels of transcripts are expressed prior to DNA replication, but high amounts of mRNA are formed in the late phase after the onset of viral DNA replication (13). Determination of the kinetics of herpesvirus saimiri gene expression has always been hampered by asynchronous virus replication (49); later

phases of virus replication coincide with the appearance of newly infected permissive cells through secondary infection and continuously increasing gene dosage. Thus, we defined the early and late phases of virus replication by transfection of intact virion DNA in the presence or absence of PAA. The complete inhibition of DNA replication was verified by Northern blot screening for the late transcript of the 143-kDa matrix phosphoprotein (8). Newly formed infectious virus was not detected in the cell cultures within the 48 to 60 h after transfection defining the late phase of replication. This excluded the possibility that the described TS expression kinetics between the early and late phase were influenced by possible regulatory effects of externally added virion components. Other experiments have shown that the TS promoter is not stimulated by UV-inactivated herpesvirus saimiri.

To identify *cis*-acting sequences required for TS gene regulation, a series of CAT fusion genes were constructed with deletions in the TS promoter upstream region. A control region was defined between nt –56 and –311, encompassing a group of 16-, 18-, and 24-nt palindromes and tandem repeats, a pyrimidine nucleotide stretch with 34 bp, and a canonical AP-1 consensus sequence (Fig. 8). Loss of the potential AP-1 binding site did not strongly diminish gene activation in infected cells. In addition to binding sites for known protein factors, AT clusters have been found to be upstream elements of constitutive gene expression (9, 60), creating active chromatin windows for entry of the transcriptional machinery. Future studies will show whether the polypyrimidine stretches of the herpesvirus saimiri TS gene are functionally important. Deletion of the tripartite palindrome complex (overlapping 16- and 18-bp palindromes) or removal of 4 bp from its center caused an approximately three- to fourfold decrease in activation of the TS promoter by virus infection, and *trans* activation can be reduced by a molar excess of a homologous synthetic oligonucleotide. This suggests that the palindromic complex acts as a binding site for virus-encoded activating proteins, virus-induced cellular transcription factors, or viral/cellular proteins displacing a repressor. The tripartite palindrome is a strong element for virus-mediated gene activation; however, other

FIG. 9. Reduction of TS promoter activity in transient expression by in vivo competition with cloned synthetic oligonucleotide representing the 18-bp palindrome. OMK cells were cotransfected with 10 μ g of the TS promoter-CAT fusion plasmid pSV0CAT786 and 200 μ g of plasmid pGL18, pGL24, or pGL30. Plasmid pGL18 had one copy of the 18-bp palindrome, pGL24 had one copy of the 24-bp palindrome of the upstream *trans*-activating region, and pGL30 had one copy of the 30-bp palindrome in the moderately silencing region. Lane 1, Transfection of plasmid pSV0CAT786 into mock-infected OMK cells; lanes 2 to 5, cotransfection into infected OMK cells of pSV0CAT786 with 200 μ g of plasmid pUC18 (lane 2), pGL18 (lane 3), pGL24 (lane 4), or pGL30 (lane 5). The fold induction of pSV0CAT786 expression is shown at the bottom.

regulatory elements must exist in the promoter region. When the TS regulatory upstream sequences were juxtaposed to heterologous promoters, virus-specific induction was not induced in permissive cells, suggesting that additional elements essential for herpesvirus saimiri *trans* activation are located farther downstream.

Sequence motifs with perfect dyad symmetry that control gene expression in *cis* have been found in bacterial and, in some cases, eucaryotic genes, for example, the 14-bp consensus sequence of *Drosophila* heat shock genes (46) and the various cyclic AMP response elements (18). As far as it is known, each half site of a dyad element is the binding site for a monomer of the respective dimeric proteins (27). In the case of the tripartite palindromic structure of the herpesvirus saimiri TS gene, in which two perfect dyad symmetries overlap, a model may be drawn in which two different dimeric proteins, activator or repressor, can interfere with each other in DNA binding. The 18-bp palindrome (ACCGN₆CGGT) has homologies to the consensus sequences ACCGN₆CGGT and ACCN₆GGT, which are the binding sites for the early transactivator protein E2 in the noncoding regions of all papillomavirus genomes that have been sequenced to date.

A few examples of sequence-specific DNA binding by virus-coded proteins are known. A nuclear antigen of EBV, EBV nuclear antigen 1 (EBNA-1), binds to a dyad symmetry element in the origin of plasmid replication (ori-P) (52, 61). Computer searches did not find any similarities between the 16-, 18-, and 24-bp palindromes of primate gammaherpesviruses (herpesvirus saimiri and herpesvirus ateles) and the lymphotropic EBV or any other known herpesvirus DNA sequences. This includes the VZV TS upstream sequence, which is entirely different. The sequence element TAATGA RAT, where R is a purine, and an octamer motif similar to that of OTF-1, which is an ubiquitous cellular octamer transcription factor, are characteristic of upstream sequences in HSV-1 IE (α) promoters (1, 34). They bind nuclear factor III and are a target for the transcription-activating HSV-1 virion component α -TIF (37). Such sequences are not included in the TS upstream region of herpesvirus saimiri, and we did not find induction of TS *trans* activation by herpesvirus saimiri virion proteins.

The nature of TS gene expression-stimulating proteins is not known. Early experiments have shown that transient expression of a presumed IE gene of herpesvirus saimiri with sequence homology to the open reading frame of EBV transactivator BMLF-1 (44) stimulates TS transcription (G. Lang, unpublished). In summary, the specific feature of herpesvirus saimiri TS regulation does not find a precedent among other known herpesvirus genes and is an example of the extreme variability of herpesviruses at the level of transcription control; a single gene, the TS gene of primate gammaherpesviruses, may have developed its own transcription regulation mechanisms, using so far unknown signal transducing pathways to target particular DNA sequences.

ACKNOWLEDGMENTS

This work was supported by Deutsche Forschungsgemeinschaft, Forschergruppe "DNA-Viren des Hämatopoetischen Systems."

We thank B. Biesinger for critical reading and comments and Jens Albrecht for help in the computer searches.

LITERATURE CITED

1. Aprhys, C. M. J., D. M. Ciuffo, E. A. O'Neill, T. J. Kelly, and G. S. Hayward. 1989. Overlapping octamer and TAATGARAT motifs in the VF65 response elements in herpes simplex virus immediate-early promoters represent independent binding sites for cellular nuclear factor III. *J. Virol.* **63**:2798-2812.
2. Batterson, W., and B. Roizman. 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. *J. Virol.* **46**:371-377.
3. Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**:721-732.
4. Bodemer, W., E. Knust, S. Angermüller, and B. Fleckenstein. 1984. Immediate-early transcription of herpesvirus saimiri. *J. Virol.* **51**:452-457.
5. Bodemer, W., H.-H. Niller, N. Nitsche, B. Scholz, and B. Fleckenstein. 1986. Organization of the thymidylate synthase gene of herpesvirus saimiri. *J. Virol.* **60**:114-123.
6. Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian. 1987. Human proto-oncogene *c-jun* encodes a DNA-binding protein with structural and functional properties of transcription factor AP-1. *Science* **238**:1386-1392.
7. Bos, T. J., D. Bohmann, H. Tsuchie, R. Tjian, and P. K. Vogt. 1988. *v-jun* encodes a nuclear protein with enhancer binding properties of AP-1. *Cell* **52**:705-712.
8. Cameron, K. R., T. Stamminger, M. Craxton, W. Bodemer, R. W. Honess, and B. Fleckenstein. 1987. The 160,000- M_r virion protein encoded at the right end of the herpesvirus saimiri genome is homologous to the 140,000- M_r membrane antigen encoded at the left end of the Epstein-Barr virus genome. *J. Virol.* **61**:2063-2070.
9. Chen, W., S. Tabor, and K. Struhl. 1987. Distinguishing between mechanisms of eukaryotic transcriptional activation with bacteriophage T7 RNA polymerase. *Cell* **50**:1047-1055.
10. Chirgwin, J. M., A. E. Przybala, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
11. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
12. Daniel, M. D., D. Silva, and N. Ma. 1976. Establishment of owl monkey kidney 210 cell line for virological studies. *In Vitro* **12**:290-294.
13. Depto, A. S., and R. M. Stenberg. 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. *J. Virol.* **63**:1232-1238.
14. Desrosiers, R. C., R. L. Burghoff, A. Bakker, and J. Kamine. 1984. Construction of replication-competent herpesvirus saimiri deletion mutants. *J. Virol.* **49**:343-348.
15. Desrosiers, R. C., and L. A. Falk. 1982. Herpesvirus saimiri strain variability. *J. Virol.* **43**:352-356.
16. Devereux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
17. Falk, L., L. G. Wolfe, and F. Deinhardt. 1972. Isolation of herpesvirus saimiri from blood of squirrel monkeys (*Saimiri sciureus*). *J. Natl. Cancer Inst.* **48**:1499-1505.
18. Fickenscher, H., T. Stamminger, R. Rüger, and B. Fleckenstein. 1989. The role of a repetitive palindromic sequence element in the human cytomegalovirus major immediate-early enhancer. *J. Gen. Virol.* **70**:107-123.
19. Fleckenstein, B., G. W. Bornkamm, and H. Ludwig. 1975. Repetitive sequences in complete and defective genomes of herpesvirus saimiri. *J. Virol.* **15**:398-406.
20. Fleckenstein, B., and H. Wolf. 1974. Purification and properties of herpesvirus saimiri DNA. *Virology* **58**:55-64.
21. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.
22. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
23. Haugen, T. H., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek. 1987. *Trans*-activation of an upstream early promoter of bovine papilloma virus-1 by a product of viral E2 gene. *EMBO J.* **6**:145-152.
24. Hell, W., S. Modrow, and H. Wolf. 1985. Mapping of herpesvirus saimiri proteins on the viral genome: proteins dependent and

- not dependent on viral DNA synthesis. *J. Virol.* **56**:414–418.
25. Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**:351–359.
 26. Honess, R. W., W. Bodemer, K. R. Cameron, H.-H. Niller, B. Fleckenstein, and R. E. Randall. 1986. The A+T-rich genome of herpesvirus saimiri contains a highly conserved gene for thymidylate synthase. *Proc. Natl. Acad. Sci. USA* **83**:3604–3608.
 27. Hope, I. A., and K. Struhl. 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. *EMBO J.* **6**:2781–2784.
 28. Hunt, R. D., F. G. Garcia, H. H. Barahona, N. W. King, C. E. O. Fraser, and L. V. Melendez. 1973. Spontaneous herpesvirus saimiri lymphoma in an owl monkey. *J. Infect. Dis.* **127**:723–725.
 29. Kaschka-Dierich, C., F. J. Werner, I. Bauer, and B. Fleckenstein. 1982. Structure of nonintegrated, circular herpesvirus saimiri and herpesvirus ateles genomes in tumor cell lines and in vitro-transformed cells. *J. Virol.* **44**:295–310.
 30. Knust, E., W. Dietrich, B. Fleckenstein, and W. Bodemer. 1983. Virus-specific transcription of a herpesvirus saimiri-transformed lymphoid tumor cell line. *J. Virol.* **48**:377–383.
 31. Krieg, P. A., and D. A. Melton. 1987. In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* **155**:397–414.
 32. Lee, S. I., S. C. S. Murthy, J. J. Trimble, R. C. Desrosiers, and J. A. Steitz. 1988. Four novel U RNAs are encoded by a herpesvirus. *Cell* **54**:599–607.
 33. Lee, W., P. Mitchell, and R. Tjian. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**:741–752.
 34. Mackem, S., and B. Roizman. 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. *J. Virol.* **44**:939–949.
 35. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 36. McBride, A. A., J. C. Byrne, and P. M. Howley. 1989. E2 polypeptide encoded by bovine papillomavirus type 1 forms dimers through the common carboxyl-terminal domain: transactivation is mediated by the conserved amino-terminal domain. *Proc. Natl. Acad. Sci. USA* **86**:510–514.
 37. McKnight, J. L. C., T. M. Kristie, and B. Roizman. 1987. Binding of the virion protein mediating α gene induction in herpes simplex virus 1-infected cells to its *cis* site requires cellular proteins. *Proc. Natl. Acad. Sci. USA* **84**:7061–7065.
 38. Medveczky, P., E. Szomolanyi, R. C. Desrosiers, and C. Mulder. 1984. Classification of herpesvirus saimiri into three groups based on extreme variation in a DNA region required for oncogenicity. *J. Virol.* **52**:938–944.
 39. Melendez, L. V., M. D. Daniel, R. D. Hunt, and F. G. Garcia. 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (*Saimiri sciureus*). *Lab. Anim. Care* **18**:374–381.
 40. Melendez, L. V., R. D. Hunt, N. W. King, H. H. Barahona, M. D. Daniel, C. E. O. Fraser, and F. G. Garcia. 1972. Herpesvirus ateles, a new lymphoma virus of monkeys. *Nature (London) New Biol.* **235**:182–184.
 41. Mermod, N., T. J. Williams, and R. Tjian. 1988. Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription *in vitro*. *Nature (London)* **332**:557–560.
 42. Müller, P. P., B. M. Jackson, P. F. Miller, and A. G. Hinnebusch. 1988. The first and fourth upstream open reading frames in *GCN4* mRNA have similar initiation efficiencies but respond differently in translational control to changes in length and sequence. *Mol. Cell. Biol.* **8**:5439–5447.
 43. Murthy, S., J. Kamine, and R. C. Desrosiers. 1986. Viral-encoded small RNAs in herpesvirus saimiri-induced tumors. *EMBO J.* **5**:1625–1632.
 44. Nicholas, J., U. A. Gompels, M. A. Craxton, and R. W. Honess. 1988. Conservation of sequence and function between the product of the 52-kilodalton immediate-early gene of herpesvirus saimiri and the BMLF1-encoded transcriptional effector (EB2) of Epstein-Barr virus. *J. Virol.* **62**:3250–3257.
 45. O'Hare, P., and R. W. Honess. 1983. Identification of a subset of herpesvirus saimiri polypeptides synthesized in the absence of virus DNA replication. *J. Virol.* **46**:279–283.
 46. Parker, C. S., and J. Topol. 1984. A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. *Cell* **37**:273–283.
 47. Post, L. E., S. Mackem, and B. Roizman. 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. *Cell* **24**:555–565.
 48. Preston, C. M., M. C. Frame, and M. E. M. Campbell. 1988. A complex formed between cell components and a HSV structural polypeptide binds to a viral immediate-early gene regulatory DNA sequence. *Cell* **52**:425–434.
 49. Randall, R. E., R. W. Honess, and P. O'Hare. 1983. Proteins specified by herpesvirus saimiri: identification and properties of virus-specific polypeptides in productively infected cells. *J. Gen. Virol.* **64**:19–35.
 50. Rao, C. D., M. Pech, K. C. Robbins, and S. A. Aaronson. 1988. The 5' untranslated sequence of the *c-sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. *Mol. Cell. Biol.* **8**:284–292.
 51. Rauscher, F. J., L. C. Sambucetti, T. Curran, R. J. Distel, and B. M. Spiegelman. 1988. Common DNA-binding site for *fos* protein complexes and transcription factor AP-1. *Cell* **52**:471–480.
 52. Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward. 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell* **42**:859–868.
 53. Richter, J., I. Puchtler, and B. Fleckenstein. 1988. Thymidylate synthase gene of herpesvirus ateles. *J. Virol.* **62**:3530–3535.
 54. Roizman, B., and A. E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795–1841. In B. N. Fields and D. M. Knipe (ed.), *Virology*, 2nd ed., vol. 2. Raven Press, New York.
 55. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 56. Schirm, S., I. Müller, R. C. Desrosiers, and B. Fleckenstein. 1984. Herpesvirus saimiri DNA in lymphoid cell line established by *in vitro* transformation. *J. Virol.* **49**:938–946.
 57. Sowden, M., S. Harrison, R. Ashfield, A. J. Kingsman, and S. M. Kingsman. 1989. Multiple cooperative interactions constrain BPV-1 E2-dependent activation of transcription. *Nucleic Acids Res.* **17**:2959–2972.
 58. Stamminger, T., H. Fickenscher, and B. Fleckenstein. 1990. Cell type-specific induction of the major immediate-early enhancer of human cytomegalovirus by cyclic AMP. *J. Gen. Virol.* **71**:105–113.
 59. Stenlund, A., and M. R. Botchan. 1990. The E2 *trans*-activator acts as a repressor by interfering with a cellular transcription factor. *Genes Dev.* **4**:123–136.
 60. Struhl, K. 1985. Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. *Proc. Natl. Acad. Sci. USA* **82**:8419–8423.
 61. Sugden, B., and N. Warren. 1989. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.* **63**:2644–2649.
 62. Szomolanyi, E., P. Medveczky, and C. Mulder. 1987. *In vitro* immortalization of marmoset cells with three subgroups of herpesvirus saimiri. *J. Virol.* **61**:3485–3490.
 63. Thompson, R., R. W. Honess, L. Taylor, J. Morran, and A. J. Davison. 1987. Varicella-zoster virus specifies a thymidylate synthetase. *J. Gen. Virol.* **68**:1449–1455.
 64. Wassarman, D. A., S. I. Lee, and J. A. Steitz. 1989. Nucleotide sequence of HSUR 5 RNA from herpesvirus saimiri. *Nucleic Acids Res.* **17**:1258.
 65. Werner, F.-J., G. W. Bornkamm, and B. Fleckenstein. 1977. Episomal viral DNA in a herpesvirus saimiri-transformed lymphoid cell line. *J. Virol.* **22**:794–803.