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Thymidylate Synthase Gene of Herpesvirus Ateles

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The putative thymidylate synthase (TS) gene of herpesvirus ateles, a T-lymphotropic tumor virus of New World primates, has a single large open reading frame encoding a polypeptide of 32.9 kilodaltons. The gene is transcribed into an unspliced 2.4-kilobase mRNA that is abundantly expressed late in virus replication. The AT-rich 5′ untranslated leader sequence of TS mRNA in herpesvirus ateles-infected cells is remarkable in length (1,184 nucleotides), containing 29 minicistrons; this may indicate a role in translation regulation.

Herpesvirus ateles is a common agent of spider monkeys (Ateles spp). It apparently is not pathogenic in the natural host (14, 32). However, the virus causes T-cell lymphomas in various New World primates (22, 33) and is capable of in vitro transformation of peripheral blood T lymphocytes of tamarin marmoset monkeys (12, 13). Thus, herpesvirus ateles is similar in some respect to another T-lymphotropic virus, herpesvirus saimiri, which is taken as the prototype of the γ2 subgroup of herpesviruses (20). The viruses share general principles of genome structure. A unique region of AT-rich DNA (L-DNA, about 110 kilobases [kb]) is flanked at both ends by multiple tandem repeats of noncoding high-GC DNA (H-DNA) (2, 7, 15). A single viral genome region was found to be transcribed in tumor-derived, herpesvirus saimiri-transformed lymphoid cells (28); computer screening of nucleotide sequence (3) and of protein databanks (11) led to the observation that this gene encodes thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase; EC 2.1.1.45) (21). The TS gene of herpesvirus saimiri is unusual in its structure and regulation (5). It is expressed abundantly into a nonspliced transcript of 2,190 nucleotides late during virus replication. The single, large open reading frame (ORF) of herpesvirus saimiri TS mRNA which encodes a 33.5-kilodalton polypeptide shares extensive sequence homologies with TSs of human cells and various procaryotes; however, the 5′ leader and the 3′ untranslated sequence of herpesvirus saimiri TS mRNA are not homologous with other known TS genes. A TS gene was also found in varicella-zoster virus (VZV; 40), a herpesvirus of the α2 subgroup with AT-rich DNA (9), although a number of other herpesviruses do not possess TS genes (21). Also, the VZV gene is unrelated in the nucleotides flanking the ORF for TS, compared with the respective herpesvirus saimiri sequences. Thus, we searched for a TS gene in herpesvirus ateles, a second γ2 herpesvirus, in order to determine the evolutionary conservation of the protein-coding ORF and flanking regulatory sequences as a possible measure of their functional importance.

A cleavage map of the entire L region (110 kb; 15) from herpesvirus ateles strain 810 (33) was constructed for a series of infrequently cutting restriction endonucleases (Fig. 1). To this end, purified virion DNA was simultaneously cleaved by two or three enzymes in various combinations; the fragments were blotted to nitrocellulose filters and hybridized with a series of labeled *Eco*RI fragments from herpesvirus ateles L-DNA cloned (31) in the plasmid vector pACYC184 (8) or pUC8 (42). Southern blots with a series of *Eco*RI fragments from herpesvirus saimiri strain 11 indicated an overall colinear arrangement of homologous sequences in the L-DNAs of herpesvirus ateles and herpesvirus saimiri (data not shown). Two plasmid clones with *Eco*RI fragments of herpesvirus ateles L-DNA, designated pIP4 (4.0 kb of inserted DNA) and pIP6 (3.0 kb of inserted DNA) were found to hybridize weakly with a cloned *Hin*dIII fragment of 921 nucleotides representing the C-terminal part of the herpesvirus saimiri TS gene (5). The two plasmids pIP4 and pIP6 contained adjacent herpesvirus ateles DNA fragments mapping between L-DNA coordinates 0.80 to 0.84 (Fig. 1). The *Hin*dIII cleavage maps were aligned by relaxed Southern blot hybridization with the four *Hin*dIII fragments of herpesvirus saimiri L-DNA known to contain the entire TS gene (5) (Fig. 1); the hybridization conditions were set at 37°C in 50 mM sodium phosphate buffer (pH 6.5) containing 40 or 50% (vol/vol) formamide, 0.75 M NaCl, 0.075 M sodium citrate, 5× Denhardt solution (10), and 0.25 mg of yeast RNA per ml. The nucleotide sequence (Fig. 2A) of the putative TS gene region from herpesvirus ateles was determined by the dideoxy-chain termination method (39) with recombinant bacteriophage derivatives M13mp18 and M13mp19 (45) or by double-stranded DNA sequencing of exonuclease III- and S1 nuclease-shortened fragments (19) in the plasmid vector pSP64 (34). A single reading frame was found that could encode a polypeptide of 290 amino acids, about the size of the known eucaryotic TSs (Fig. 2B). The mRNA transcribed from this gene of herpesvirus ateles was tentatively mapped by a series of Northern blots, as described before (23), by using radioactive probes from all restriction fragments generated by *Hin*dIII, *Hin*dIII-*Eco*RI, or *Hin*dIII-*Kpn*I. The RNA was isolated from cell cultures at late stages of viral replication (about day 8) by a guanidinium-isothiocyanate procedure (17) with minor modifications (28). A single late RNA of 2.4 kb was identified with each of the cloned probes (with 452, 271, 1,321, and 386 nucleotides) but not with the adjacent fragments. The RNA from herpesvirus ateles-infected owl monkey kidney (OMK) cells was slightly smaller than the 2.5-kb mRNA that had been proven to encode the TS of herpesvirus saimiri (Fig. 3). This corresponds to the location of recognizable signals for transcription initiation and termination in the nucleotide sequence of the herpesvirus ateles TS region (Fig. 2A). A single possible TATA consensus sequence was located between nucleotides 264 and 269, and the single sequence motif for polyadenylation, AATAAA, was located immediately

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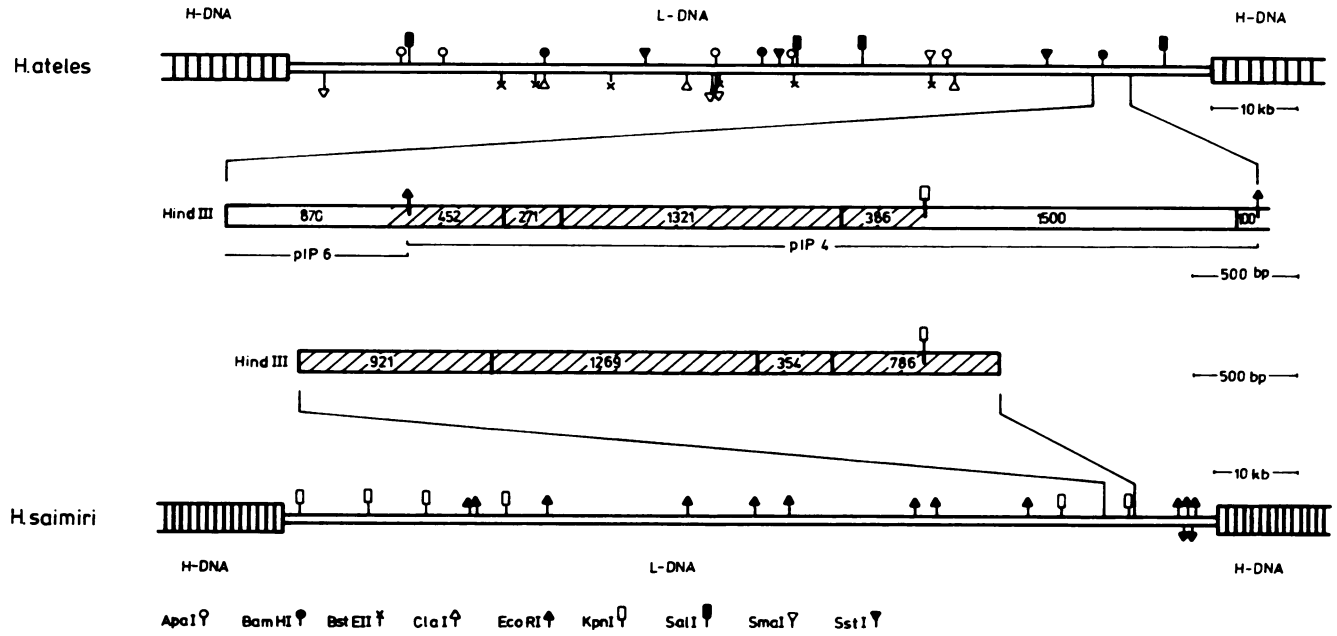

FIG. 1. Comparative cleavage maps of M genomes from herpesvirus ateles (strain 810) and herpesvirus saimiri (strain 11) and genome arrangements of the respective TS genes. Symbol: ▨, DNA segments whose nucleotide sequences were determined. Numbers indicate base pairs (bp).

following the translational stop codon TAA between nucleotides 2353 and 2358 (Fig. 2A). Since the putative transcription initiation and termination sites share significant homology with colinear sequences of herpesvirus saimiri (see below) (5), the cap site was tentatively placed at nucleotide 293 and, by following known rules (4), the probable polyadenylation addition site was placed at nucleotide 2371 (Fig. 2A). This implies a highly unusual structure for the TS mRNA of herpesvirus ateles, with a very long leader (1,184 nucleotides) upstream of the TS reading frame and a 3′ untranslated sequence essentially missing.

The TS genes of herpesvirus ateles and herpesvirus saimiri were compared first in a dot matrix analysis of the nucleotide sequences by a VAX 11/780 computer (Digital Equipment), applying a program package from the University of Wisconsin Genetics Computer Group. A high sequence conservation was confined precisely to the ORF for the TS polypeptides of both viruses (Fig. 4). The derived polypeptides were identical in 83.8% of the respective amino acids, although the TS polypeptide of herpesvirus ateles was shorter by four amino acids at the N terminus than the equivalent protein of herpesvirus saimiri. The ORF of herpesvirus ateles was 62.7% homologous with the amino acid sequence of VZV, 68.6% homologous with that of mouse cells, and 69.6% homologous with that of human cells; there was no insertion or deletion of a single amino acid residue over the entire lengths of the homologous sequences, emphasizing the exceptional conservation of this protein in nature.

Significant nucleotide sequence homologies were seen in the upstream promoter regions of herpesvirus ateles and herpesvirus saimiri and around the transcription initiation sites, up to about 50 nucleotides into the 5′ untranslated region of the genes. In the majority, however, the 5′ untranslated sequences of TS genes from herpesvirus ateles and herpesvirus saimiri did not reveal any recognizable sequence homology, although they are well conserved with regard to their lengths (Fig. 4). The 5′ untranslated region of herpesvirus ateles TS gene has 1,184 nucleotides (versus 1,207 in herpesvirus saimiri). Also, a very high average AT content (71.9% for herpesvirus ateles, 75.7% for herpesvirus saimiri) is characteristic for the 5′ untranslated DNA regions. The respective herpesvirus ateles sequence contains 29 ATG codons, each shortly followed by a translational stop codon in frame. Thus, the sequence between the transcription initiation site and the beginning of the open translational frame for herpesvirus ateles TS is interrupted by 21 minicistrons. With regard to the rules of efficient translational initiation (30), many of those ATGs are not typical start signals for peptide synthesis. The hypothetical peptides from those short reading frames did not reveal any sequence homology with similar ORFs of herpesvirus saimiri, since they are not in colinear positions (Fig. 2C). Analysis of the codon usage of the short reading frames with the computer program TESTCODE (16) also indicated that those are probably not protein-encoding sequences (data not shown).

The cell cycle-dependent expression of TS genes in human (1) and mouse (25) cells appears mainly controlled by post-transcriptional events. The human TS mRNA has a triple GC-rich tandem repeat immediately upstream of the ATG initiator codon; the deletion of such repeats increases translation efficiency (27). The TS gene of VZV, as judged from the primary sequence and arrangement of neighboring reading frames, seems not to have a leader sequence with upstream ATG codons or other unusual structural features (9).

Eucaryotic genes have, in general, short 5′ untranslated sequences, mostly in the size range of 20 to 100 nucleotides (29). Upstream ATG codons are rare and, if present, are often inefficient translational starts. Most exceptions are found among proto-oncogenes. The c-*sis* gene, which encodes the B polypeptide chain of platelet-derived growth factor, has a GC-rich 5′ leader sequence with three ATG codons (36); a portion of the first exon in the c-*sis* proto-oncogene with the 5′ nucleotide region inhibits synthesis of

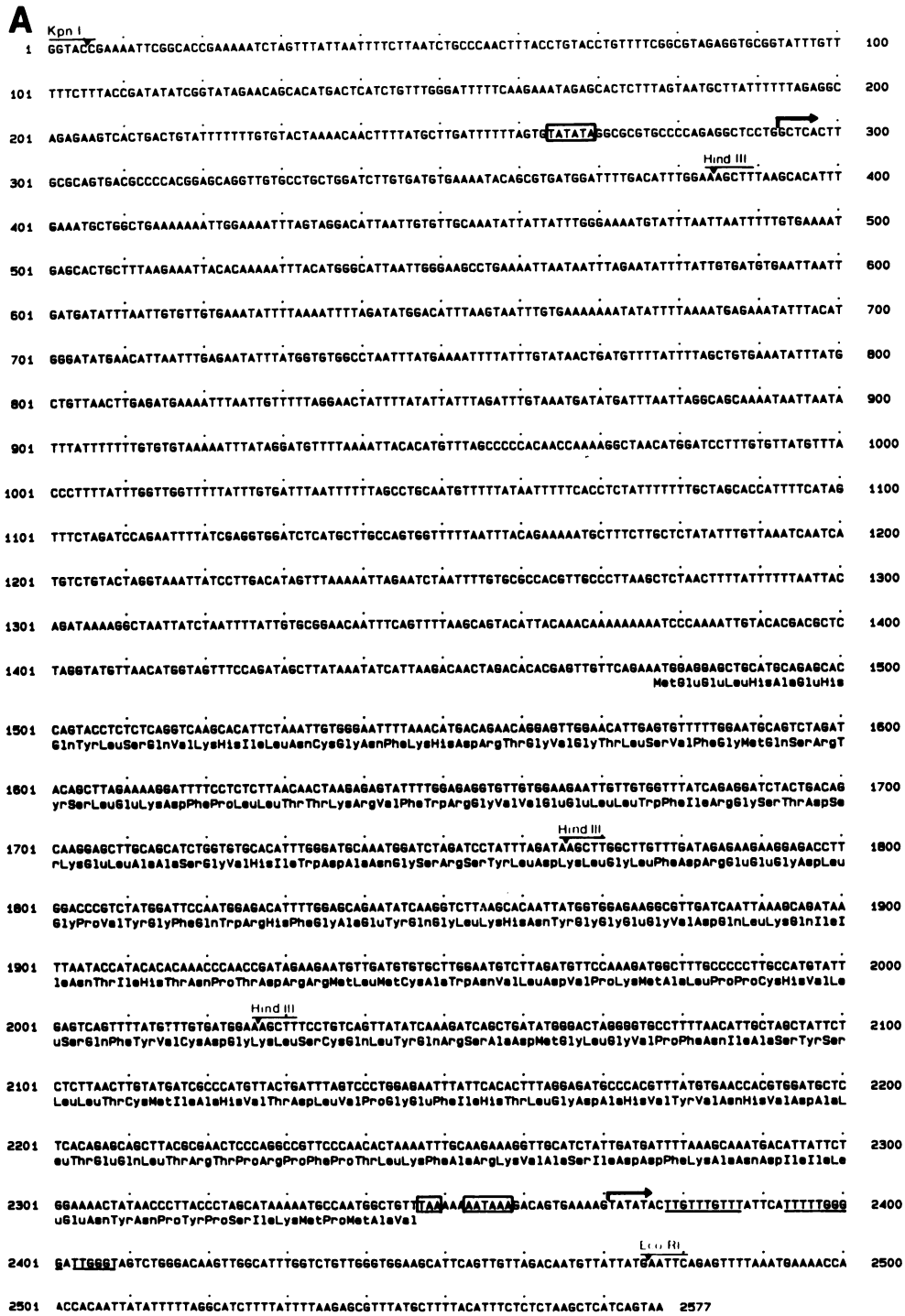

FIG. 2. (A) Primary structure of the TS gene of herpesvirus ateles (strain 810). Putative signals for initiation and termination of transcription are boxed. The cap site and polyadenylation addition site, as derived from sequence similarities with the herpesvirus saimiri TS gene, are indicated by arrows. The amino acid sequence corresponds to the single long ORF in the direction of transcription. The reading frame analyses of the TS genes from herpesvirus ateles (B) and herpesvirus saimiri (C) are shown. The open translational reading frames (for 32.9- and 33.5-kilodalton polypeptides, respectively) are symbolized by hatched boxes. Bars above the lines indicate ATG sequences; bars crossing the lines are stop codons.

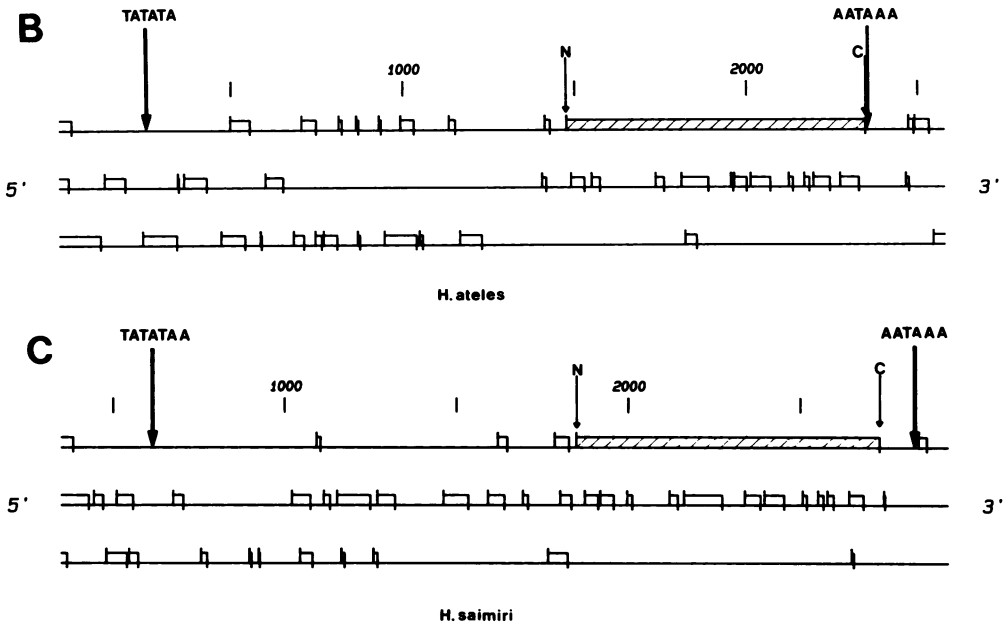

FIG. 2—*Continued.*

the platelet-derived growth factor product (37). The highest number of upstream ATGs was found in the *bcl-2* (B-cell leukemia, lymphoma 2) gene, from which, among others, a 5.5-kb mRNA is transcribed with six upstream AUG codons (41). The upstream ATG codons of eucaryotic genes sometimes meet the sequence requirements for an efficient translational start (26); they are recognized as translational initiation sites in yeast genes, such as the *CPA1* gene encoding the glutaminase subunit of the arginine pathway carbamoylphosphate synthetase (44) and the *GCN4* gene for transcriptional activation of amino acid biosynthesis (35); short peptides encoded thereof are assumed to be *cis*-acting inhibitors or stimulators of protein synthesis. It is reminiscent of mRNAs for EBNA proteins of Epstein-Barr virus that encode a leader polypeptide of 176 amino acids with, up to now, unknown function (6, 38, 43). In contrast, the upstream minicistrons in primate herpesvirus TS genes are far shorter and are not conserved with regard to nucleotide sequence and colinearity; only a few of the 29 and 22 upstream ATG codons of herpesvirus ateles and herpesvirus saimiri TS genes, respectively, are juxtaposed to nucleotides sequences favoring translation. This, in summary, suggests that the minicistron-loaded TS mRNA leaders of herpesvirus ateles and herpesvirus saimiri are functionally relevant sequences, perhaps down-regulating translation without being expressed as peptides.

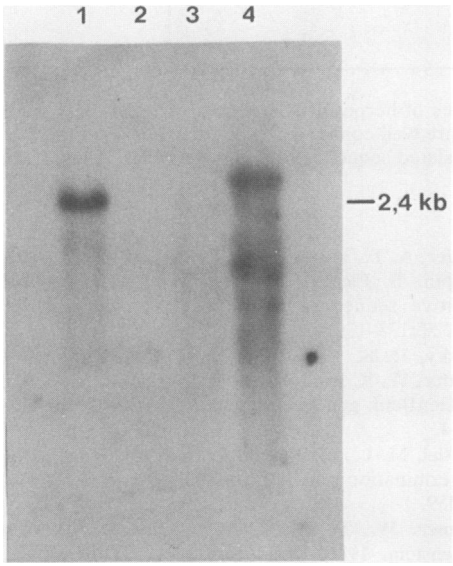

FIG. 3. Northern blot analysis of late TS transcripts from lytically infected cells. A *Hin*dIII-*Eco*RI fragment of 452 nucleotides (Fig. 1) of herpesvirus ateles L-DNA was used as the ^{32}P-labeled probe. Lanes: 1, RNA from herpesvirus ateles-infected OMK cells; 2, *Escherichia coli* RNA; 3, RNA from mock-infected OMK cells; 4, RNA from herpesvirus saimiri-infected cells. The herpesvirus ateles transcripts are shorter by about 100 nucleotides than herpesvirus saimiri TS mRNA. The smaller, heterogeneous, TS-specific RNA seen in lane 4 was described before for herpesvirus saimiri-infected cells (28).

Unlike the VZV and herpesvirus saimiri TS genes, the herpesvirus ateles TS gene does not have a sizable 3′ untranslated sequence; the TAA stop codon is immediately followed by the polyadenylation cleavage signal AATAAA. The nucleotide sequences further downstream are colinear between herpesvirus ateles and herpesvirus saimiri L-DNAs (Fig. 4). It appears that a 3′ untranslated sequence of herpesvirus ateles is missing because a sequence has been deleted rather than because a new polyadenylation signal exists in a more upstream position. The TS mRNA of herpesvirus ateles resembles the structure of the mouse cell mRNA, in which, in contrast to other eucaryotic mRNA, a 3′ untranslated sequence is entirely missing (24).

The TS polypeptides of herpesvirus ateles and herpesvirus saimiri are 83.8% identical in amino acids. Although this may appear highly divergent for a gene that is extremely conserved from bacteria to mammalian cells, it shows that the constraints for sequence conservation are far higher in the TS-coding reading frames than in untranslated mRNA parts. The T-lymphotropic herpesviruses and VZV represent two

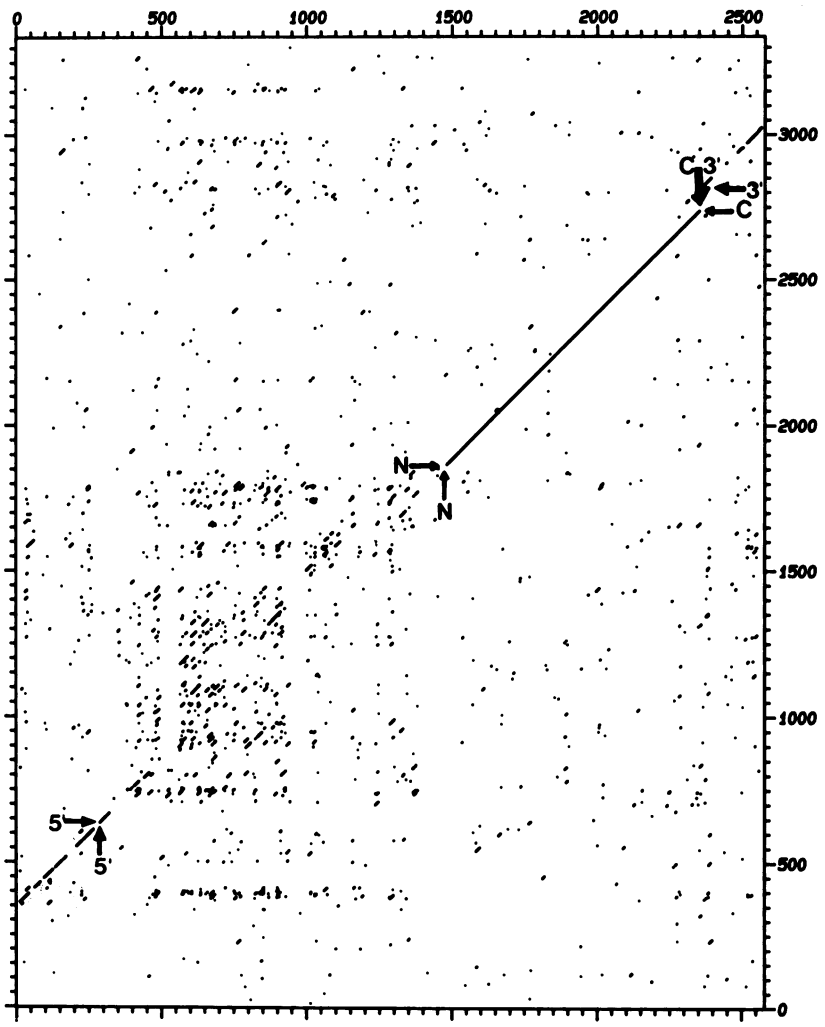

FIG. 4. Dot matrix comparison of the nucleotide sequences from the TS genes of herpesvirus ateles (horizontal axis) and herpesvirus saimiri (vertical axis). The stringency is 14 of 21 nucleotides. The ORFs (N to C) are well conserved, while most parts of the 5′ untranslated sequences (5′ to N) do not reveal any sequence homology. The missing 3′ untranslated sequence of the herpesvirus ateles TS gene appears as a deletion in otherwise colinear genomes.

distinct subgroups, γ and α herpesviruses, respectively. TS genes are missing, however, in the genomes of other members of the same herpesvirus subgroups (21, 40). Thus, the presence or absence of a TS gene correlates with the average AT content of a herpesvirus genome not with taxonomic subgrouping. Since the TS DNAs of VZV and T-lymphotropic herpesviruses are not localized within conserved gene blocks of colinear organization (18), it is not possible to decide whether the TS genes of present day herpesviruses are derived from one ancestral viral DNA sequence or whether they are independently acquired from the cellular genome.

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