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19 October 1987; accepted 20 January 1988

A Gene for Dihydrofolate Reductase in a Herpesvirus

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The enzyme dihydrofolate reductase (DHFR) is found ubiquitously in both prokaryotes and eukaryotes. It is essential for de novo synthesis of purines and of deoxythymidine monophosphate for DNA synthesis. Among viruses, however, only the T-even and T5 bacteriophage have been found to encode their own DHFR. In this study a gene for DHFR was found in a specific subgroup of the gamma or lymphotropic class of herpesviruses. DNA sequences for DHFR were found in herpesvirus saimiri and herpesvirus ateles but not in Epstein-Barr virus, Marek's disease virus, herpes simplex virus, varicella-zoster virus, herpesvirus tamarinus, or human cytomegalovirus. The predicted sequence of herpesvirus saimiri DHFR is 186 amino acids in length, the same length as human, murine, and bovine DHFR. The human and herpesvirus saimiri DHFRs share 83 percent positional identity in amino acid sequence. The herpesvirus saimiri DHFR gene is devoid of intron sequences, suggesting that it was acquired by some process involving reverse transcription. This is to our knowledge the first example of a mammalian virus with a gene for DHFR.

THREE DISTINCT BUT RELATED groups of herpesviruses have generally been recognized. A human herpesvirus serves as the prototype for each of these groups (*1*). Herpes simplex virus is the prototype of the alpha group, human cytomegalovirus (CMV) is the prototype of the beta group, and Epstein-Barr virus (EBV) is the prototype of the gamma or lymphotropic herpesviruses. Herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) are T-lymphotropic viruses of New World primates closely related to each other in sequence (*2*). Although genetic relatedness between these New World primate viruses and EBV was not obvious from hybridization studies, evidence suggests HVS is more closely related to EBV than to alpha or beta herpesviruses (*3*). HVS and HVA naturally infect squirrel monkeys (*Saimiri sciureus*) and spider monkeys (*Ateles* sp.), respectively.

Herpesviruses are known to encode enzymes for nucleotide and DNA synthesis—a luxury afforded perhaps by the large size of their genomes. Herpesvirus-encoded enzymes involved in nucleotide metabolism and DNA synthesis that have been identified to date include thymidine or deoxypyrimidine kinase (*4*), ribonucleotide reductase (*5*), DNA polymerase (*6*), deoxyuridine triphosphatase (*7*), exonuclease (*8*), uracil-DNA glycosylase (*9*) and thymidylate synthase (TS; E.C. 2.1.1.45) (*10*). The TS gene has been found in HVS, HVA, and in varicella-zoster virus but not in other herpesviruses (*10–12*).

Dihydrofolate reductase (DHFR; E.C. 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate, an essential step for de novo glycine and purine synthesis and for the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate (dTMP). The central role of DHFR in DNA precursor synthesis and its sensitivity to inhibition by drugs such as methotrexate have made DHFR the target of anticancer chemotherapy. For these and other reasons, DHFR has been extensively studied and much is known about its synthesis, regulation, and escape from drug inhibition (*13*). Among viruses, however, only the T-even and T5 bacteriophage have been found to encode their own DHFR (*14*). In this report, we describe a naturally occurring DHFR gene in a mammalian virus.

The genome of HVS contains a 110-kilobase-pair (kbp) sequence of DNA called L-DNA (36% G+C). This is flanked at each end by 1444-bp repeat units of repetitive DNA called H-DNA (71% G+C) (*15*). Our nucleotide sequencing near the left H-L DNA border of HVS strain 11 revealed an open reading frame of 246 amino acids that was 187 amino acids (L-DNA nucleotides 4532–3972) in length from the first methionine (Fig. 1). The amino acid sequence predicted by this open reading frame was highly related to sequences for cellular DHFR obtained by others (*16–19*) (Fig. 2). Cellular DHFR of human, murine, and bovine origin is initially synthesized as a 187–amino acid product, with the initiating methionine then being cleaved to yield valine as the first amino acid of mature enzyme

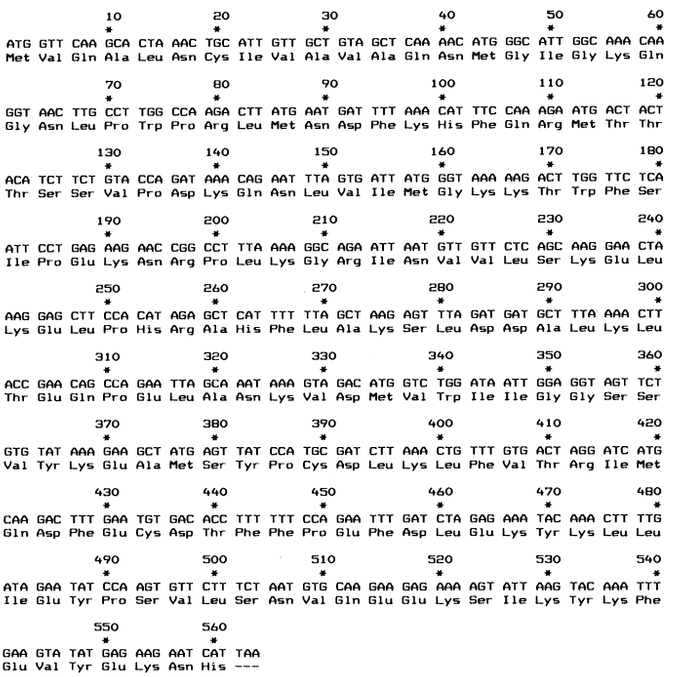

Fig. 1. Nucleotide sequence of herpesvirus saimiri strain 11 DNA encoding a predicted dihydrofolate reductase. Nucleotides 1 and 564 of the sequence shown are nucleotides 4532 and 3969, respectively, of L-DNA from the left H-L border. DNA of this region of HVS was cloned previously (*32, 34*). Several subclones were then generated into PGEM vectors (Promega). In addition, a series of nested deletion clones were obtained by using Exonuclease III and S1 nuclease (*36*). Exonuclease III was used to specifically digest DNA unidirectionally from a 5′ protruding end. Portions were removed at various time intervals, trimmed with S1 nuclease, and religated to yield clones with the desired deletions. Dideoxy sequencing reactions were resolved on buffer gradient, 6% acrylamide (1 m long) sequencing gels (American Bionetics).

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(*19*); the HVS DHFR coding sequences have thus been conserved without deletions or insertions. The human, mouse, and bovine cellular DHFRs have 83, 78, and 74% amino acid identity when compared to HVS DHFR; the predicted HVS DHFR amino acid sequence is 67% identical to chicken DHFR (Fig. 2). By comparison, the murine-bovine and murine-human DHFR amino acid identities are 84 and 89% (*16*). The sequence of squirrel monkey DFHR is not yet available. The human DHFR amino acid sequence has been compared previously with that of *Escherichia coli, Lactobacillus casei, Leishmania,* and T4 bacteriophage (*20*).

The mammalian cell DHFR gene is spread out over 26 kbp or more and is transcribed with at least five introns (*21, 22*). The genomic DHFR sequence in HVS, in contrast, is devoid of intron sequences. Since the HVS DHFR is more closely related to mammalian cell DHFR than to any other DHFR that has been analyzed to date, these results suggest that the HVS DHFR gene was acquired from the host cell by some process involving reverse transcription, as has been suggested previously for intronless human DHFR pseudogenes (*23*).

Although the HVS DHFR coding sequences appear to be well conserved, the 5′ and 3′ noncoding, flanking sequences diverge markedly from those of human origin (Fig. 3). No significant similarity was noted between sequences 5′ to the HVS DHFR initiation codon and the RNA sequences 5′ to the initiation codon of human DHFR. Flanking sequences 3′ to the HVS DHFR stop codon are similar to the equivalent sequence in human DHFR only over 28 nucleotides (68% identity) (Fig. 3). Beyond that, significant sequence relatedness was not observed. By contrast, the 5′ flanking region preceding the initiating ATG of mouse and human DHFR genes is highly conserved as far as −1200 bp and is highly G-C–rich for the first 300 nucleotides (*21, 24*). Homology between the 3′ flanking sequences of mouse and human DHFR genes extends only 100 nucleotides beyond the termination codon (*21*). The divergence of flanking sequences in HVS, particularly the loss of 5′ G-C–rich conserved sequences, may reflect its origin from reverse transcription of messenger RNA and the acquisition of viral transcriptional control signals rather than divergence from the cellular upstream sequences. The conservation of only the DHFR amino acid coding sequences in HVS suggests that it confers useful function and selective advantage to the virus.

Complete nucleotide sequences have been obtained for EBV (*25*), varicella-zoster virus (*26*), and herpes simplex virus type I (*27*). Furthermore, the sequence of more than 50% of the human CMV genome has been completed (*28*). This has allowed sequence comparison with the HVS DHFR gene. DHFR-related sequences were not identified in the genomes of EBV, varicella-zoster virus, herpes simplex virus, or in the portions of human CMV sequenced to date. We have also used a ^{32}P-labeled HVS DNA probe containing the DHFR sequence to search for related sequences in a variety of herpesvirus DNAs using Southern blot hybridization (*29*). DHFR-related sequences were detected in A, B, and nonA-nonB strains of HVS [see (*30*) for classification] and in HVA. DHFR-related sequences were not detected in Marek's disease virus, herpes simplex virus, human CMV, and herpesvirus tamarinus [a herpes simplex–related virus of New World primates (*31*)]. Thus, DHFR genes have been detected in a specific subgroup of gamma herpesviruses but not in other herpesviruses of the alpha, beta, and gamma groups.

Although we have not specifically demonstrated viral-encoded DHFR enzymatic activity, it seems likely that the HVS DHFR gene will encode an enzymatically active protein. First, an abundant 4.9-kb polyadenylated RNA, appropriate in location and direction for this open reading frame, was previously mapped to this region; the 5′ end of this 4.9-kb viral RNA in infected cells has been mapped previously to just upstream from the DHFR open reading frame (*32*). Our sequencing suggests that the ATG at L-DNA nucleotide 4532, which could encode the first methionine of the DHFR open reading frame, is likely to represent the first AUG from the 5′ end of this RNA. Second, study of cellular DHFR has suggested that certain key amino acids involved in binding nicotinamide adenine dinucleotide phosphate (NADPH) and methotrexate are con-

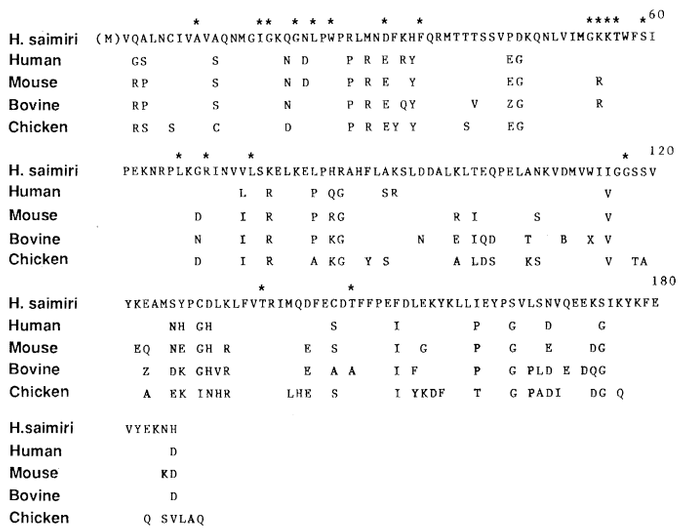

Fig. 2. Amino acid sequence comparisons of dihydrofolate reductase from various sources. The sequence of human, murine, bovine, and chicken DHFRs were described previously (*16–19*). While human, murine, and bovine DHFRs contain 186 amino acids, the chicken DHFR has 189 amino acids due to the presence of three additional amino acids at the carboxyl terminus. An asterisk (*) indicates those amino acids believed to be involved in binding to NADPH and methotrexate which are highly conserved in enzymes from several sources (*16, 17*). Where spaces are blank, the amino acid is the same as that of herpesvirus saimiri.

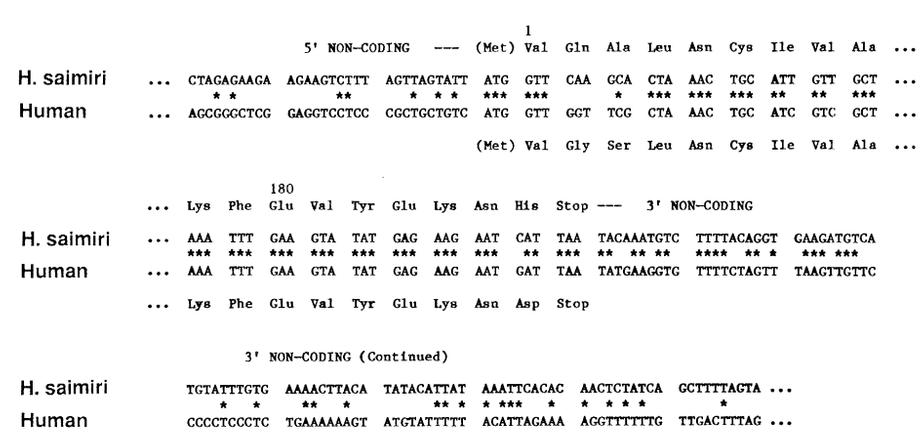

Fig. 3. Identity in the 5′ and 3′ noncoding sequences of HVS and human dihydrofolate reductase. The sequences immediately upstream and downstream from the DHFR coding sequences are compared. An asterisk (*) indicates identical nucleotides. Significant identity was not observed in sequences 5′ or 3′ beyond those shown.

served in DHFR from bacterial to mammalian enzymes (*16, 17*); these key amino acids (* in Fig. 2) are well conserved in the HVS DHFR sequence.

The central role of DHFR in DNA synthesis is demonstrated by its increase in activity in rapidly dividing cells and the sensitivity of such cells to folate analogs. Among the folate-requiring enzymes, TS is unique in its consumption of 1 mole of reduced folate for every mole of dTMP generated. This stoichiometry requires coordinated regulation of the two enzymes. This has been accomplished in T4 bacteriophage by overlapping the TS and DHFR genes (*14*) and in the protozoan parasite *Leishmania* by production of a bifunctional TS-DHFR polypeptide (*20*). The DHFR and TS genes of HVS are located near the opposite ends of the unique sequence L-DNA. Both of these regions are consistently retained in HVS-transformed T-cell lines in which large segments of the L-DNA central region are lost (*33*). The DHFR gene is apparently not required for immortalization or replication in vitro by HVS since replication-competent virus strains have been constructed that delete all or part of the DHFR coding sequences yet retain their ability to immortalize T cells and to replicate in permissive monolayer cell lines (*34, 35*). The presence of DHFR and TS genes in HVS and HVA, however, could facilitate virus reactivation or replication in resting T cells. The maintenance of TS and DHFR genes may be particularly important in providing adequate dTMP pools for viruses such as HVS and HVA, which have unusually high A+T content. A possible role for HVS DHFR in persistent infection, latency, and reactivation from latency can now be investigated by using susceptible New World primates as host.

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37. We thank B. Barrell, P. Farrell, D. McGeoch, and B. Fleckenstein for assistance in homology searches, B. Fleckenstein for gifts of cytomegalovirus and herpesvirus ateles DNA, and P. Coussens for Marek's disease virus DNA. This work was supported by Public Health Service grants 31363 from the National Cancer Institute and by grant RR00168 from the Division of Research Resources.

29 October 1987; accepted 20 January 1988

Synaptic Reorganization in the Hippocampus Induced by Abnormal Functional Activity

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Abnormal functional activity induces long-lasting physiological alterations in neural pathways that may play a role in the development of epilepsy. The cellular mechanisms of these alterations are not well understood. One hypothesis is that abnormal activity causes structural reorganization of neural pathways and promotes epileptogenesis. This report provides morphological evidence that synchronous perforant path activation and kindling of limbic pathways induce axonal growth and synaptic reorganization in the hippocampus, in the absence of overt morphological damage. The results show a previously unrecognized anatomic plasticity associated with synchronous activity and development of epileptic seizures in neural pathways.

THE HIPPOCAMPUS RECEIVES CONverging neocortical, subcortical, limbic, and brainstem inputs via the perforant pathway to dentate granule cells that in turn project to CA3 hippocampal neurons via mossy fiber axons (*1*). These pathways undergo synaptic reorganization in response to lesions (*2*) and also demonstrate long-lasting increases in synaptic efficacy in response to certain patterns of afferent activity (*3, 4*). Repeated activation of neural pathways can induce seizures and a permanent epileptic state (kindling) (*5*). Using the Timm method to identify hippocampal mossy fibers (*6*), we observed that synchronous perforant path activation and kindling of limbic pathways induce mossy fiber sprouting and synaptic reorganization in the dentate gyrus, in the absence of overt CA3/CA4 lesions.

The Timm method is a histochemical technique that stains neural elements con-

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