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Amplification by host cell factors of a sequence contained within the herpes simplex virus 1 genome

(host origin of DNA synthesis/MCF7 cells/estrogen/host origin of DNA replication)

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ABSTRACT **We report that a cloned 1620-base-pair (bp) DNA fragment mapping in the *Bam*HI O fragment of herpes simplex virus 1 DNA is amplified after transfection into uninfected cells. The DNA fragment maps entirely within a portion of the open reading frame encoding the large subunit of the viral ribonucleotide reductase and does not contain any of the known lytic origins of viral DNA synthesis. Amplification of this sequence in transfected cells results in accumulation of full-sized *Dpn* I-resistant plasmids containing the sequence in Hirt extracts of low molecular weight DNA. Subfragments of the 1620-bp fragment were not amplified, whereas larger fragments containing the intact 1620-bp fragment were amplified. The amplification of the fragment in MCF7 cells, which express steroid receptors, was stimulated by the addition of estrogen to the medium. Addition of progesterone, dexamethasone, or testosterone was ineffective. The viral genome therefore contains at least one origin of DNA synthesis capable of supporting replication of viral DNA by cellular factors. The existence of such a host origin of DNA replication in the viral genome was predicted by the hypothesis that viral DNA is amplified by cellular enzymes in sensory neurons harboring latent virus; the link between these sequences and amplification of viral DNA during latency remains to be proven.**

The flow of events underlying the pathogenesis of herpes simplex virus 1 (HSV-1) infections *in vivo* is well known (for review, see ref. 1). After an initial, productive infection of the tissues at the portal of entry, the virus is transported through the axons of sensory neurons to the neuronal nuclei, where it establishes a latent infection. This infection can persist throughout the lifetime of the individual. Periodic stimuli such as stress, peripheral tissue damage, or hormonal changes cause the neuron to reactivate the virus to multiply and to be transported to a site at or near the portal of entry, where it may cause a lesion. In some individuals the lesions recur at frequent intervals. The latent state and associated recurrent lesions at peripheral sites enable the virus to maintain itself in the largely immune human population by allowing it to be periodically available for transmission to nonimmune individuals who come in contact with individuals exhibiting recurrent lesions.

The molecular mechanisms of latency are less well understood. The viral DNA is known to be maintained in sensory neurons in an episomal form (2). Only one region of the viral genome has been demonstrated to be transcriptionally active during latency (3). This region yields the latency-associated transcript, encoded by the repeated sequences of the viral DNA, of which the central portion accumulates in large amounts in nuclei of latently infected sensory neurons (4). To date, no viral genes or sequences, including those encoding the latency-associated transcript (5), have been demonstrated to be required for the establishment or maintenance of latency.

It is known that the copy number of viral DNA in latently infected sensory ganglia is considerably higher than one copy per neuron harboring latent virus; estimates range from 10 to >100 copies of viral DNA per latently infected neuron (1). It has been proposed (1) that the copy number of the viral DNA may play a role in the reactivation of latent virus.

There are several possible explanations for this high copy number. One hypothesis is that viral DNA may be present in only a single copy per cell, but it may be in a latent form in some neurons and in a nonfunctional form in other neurons and in nonneuronal cells. That the DNA would have to be in a nonfunctional form in a majority of the cells is based on the observation that expression of the latency-associated transcript (3) and of genes inserted into the viral genome under a host promoter (A.E.S., V. Hukkanen, J. Baines, and B.R, unpublished data) has been reported only in a small percentage of neuronal cells. A second hypothesis is that sensory neurons may be initially infected on the average with 10–100 virions per cell. The hypothesis that we favor and have explored further is that the viral genome contains cellular origins for viral DNA synthesis and that viral genomes are amplified after infection by cellular enzymes.

Three origins of viral DNA synthesis can be used to replicate HSV-1 DNA during lytic infection. These origins, designated Ori_L, Ori_{S1}, and Ori_{S2}, map in the middle of the unique sequences of the long component (6) and in the inverted repeat sequences of the short component (7, 8) (Fig. 1). In productive infection, viral DNA is synthesized by a set of viral enzymes and factors whose synthesis ultimately commits the cells to destruction. Since expression of these genes has not been detected in latently infected neurons (3), the amplification of viral DNA in neurons would have to be accomplished by cellular proteins and initiated at specific sites recognized by these proteins.

In this paper, we present evidence that a specific 1.6-kilobase-pair (kbp) fragment of the viral genome mapping in the unique sequences of the L component of HSV-1 can be amplified in transfected cells in the absence of viral proteins known to be required for viral DNA synthesis in productively infected cells (9). This fragment does not contain any previously identified open reading frame in its entirety (10) or any viral origin of lytic DNA synthesis. The fragment maps in close proximity to one of two sites previously reported to initiate the synthesis of intact viral DNA in cells released from phosphonoacetate inhibition (11).

MATERIALS AND METHODS

Cells and Viruses. Ltk$^-$, HeLa, and BHKtk$^-$ cells were obtained from the American Type Culture Collection. MCF7

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Abbreviations: HSV-1, herpes simplex virus 1; EBV, Epstein–Barr virus; Ori_S, HSV-1 S component origin of lytic replication; Ori_L, HSV-1 L component origin of lytic replication; Ori_H, host origin of DNA replication in HSV-1 genome; Ori_P, EBV latent origin of replication.

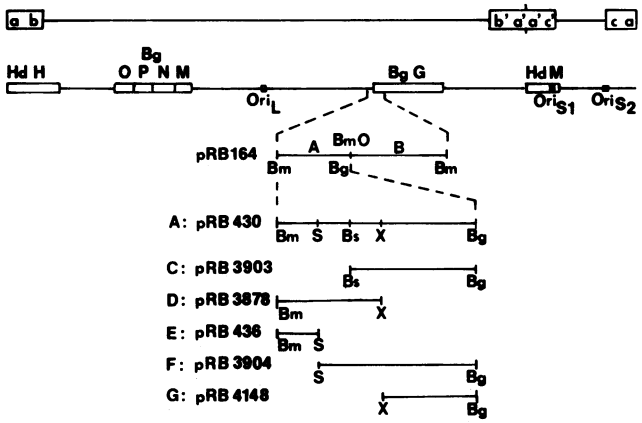

FIG. 1. Sequence arrangement of HSV-1 DNA and map location of DNA fragments tested in this study. Top line, sequence arrangement of HSV-1 DNA. Boxes indicate the sequences repeated at the termini (a b, c a) and internally in inverted orientation (b' a' a' c'). Second line, map of HSV-1 DNA. Ori_L, Ori_{S1}, and Ori_{S2} are the lytic origins of viral DNA synthesis. Open boxes indicate restriction enzyme fragments. Third line, expansion of the *Bam*HI O fragment. Fragment A, left end 1620-bp *Bam*HI/*Bgl* II fragment. Fragment B, right end *Bgl* II/*Bam*HI fragment. Fragments C–G, subclones of the A fragment; letters refer to the letters used in Fig. 2. Hd, *Hin*dIII; Bg, *Bgl* II; Bm, *Bam*HI; S, *Sal* I; Bs, *Bss*HII; X, *Xho* I.

cells were a gift from E. Fuchs (Univ. of Chicago). N18 cells were a gift from R. Miller (Univ. of Chicago). HSV-1(F) is the wild-type clinical isolate, passaged a limited number of times in cell culture, that is used as a prototype strain in this laboratory.

Plasmids. pRB201 contains the *Hin*dIII HM junction fragment of HSV-1(F) inserted into the *Hin*dIII site of pBR322. pRB168 contains the *Bam*HI Z fragment of HSV-1(F), which includes the sequences encompassing Ori_{S2}, inserted into the *Bam*HI site of pUC18. pRB164 contains the *Bam*HI O fragment of HSV-1(F) inserted into the *Bam*HI site of pUC18. pRB430 contains the left end *Bam*HI/*Bgl* II fragment (Fig. 1, fragment A) of *Bam*HI O (from pRB164) inserted into the *Bam*HI site of pUC18. pRB436 contains the left end *Bam*HI/*Sal* I fragment of *Bam*HI O (from pRB430) inserted into pUC18 digested with *Bam*HI and *Sal* I. pRB3878 was constructed by digestion of pRB430 with *Xho* I and *Eco*RI, followed by filling in of 5′ ends with T4 DNA polymerase and ligation, and contains the left end *Bam*HI/*Xho* I fragment of pRB430. pRB3903 was constructed by digestion of pRB430 with *Bss*HII and *Pst* I, followed by filling in of the *Pst* I 5′ end and digestion of the *Bss*HII 3′ end by T4 DNA polymerase to yield blunt ends, and ligation, and it contains the right end *Bss*HII/*Bgl* II fragment of pRB430. pRB3904 was constructed by ligation of a *Sal* I digest of pRB430, and it contains the right end *Sal* I/*Bgl* II fragment of pRB430. pRB4148 was constructed by digestion of pRB430 with *Bam*HI and *Xho* I, followed by T4 polymerase to fill in 5′ ends and ligation, and it contains the right end *Xho* I/*Bgl* II fragment of pRB430. pGEM3Z was purchased from Promega.

Transfections. All transfection assays were done by calcium phosphate precipitation, as modified by Chen and Okayama (12). Twenty micrograms of plasmid DNA was used per 100-mm plate of cells. Steroid hormones, where indicated, were added to the cells 24 hr after transfection, when the medium was changed.

Extraction of Low Molecular Weight DNA. Low molecular DNA was extracted by the method of Hirt (13); DNA from one-half of each plate ($\approx 10^7$ cells) was used for digestion with restriction enzymes.

Blots and Hybridization Conditions. After separation on 1% agarose gels, DNA fragments were alkaline blotted onto Zeta-Probe nylon membranes (Bio-Rad). All hybridizations were done in 30% formamide/3× SSC/1% SDS/0.5% skim milk/400 μg of sonicated salmon sperm DNA per ml at 68°C (1× SSC is 0.15 M NaCl/0.015 M sodium citrate). Blots were prehybridized in the same solution at 68°C for 1 hr before addition of probe (nick-translated ^{32}P-labeled plasmid; $\approx 10^8$ cpm/μg, $\approx 10^6$ cpm/ml). After hybridization, membrane filters were washed according to the manufacturer's instructions, with a final wash in 18% formamide/1.8× SSC/1% SDS at 68°C for 1 hr.

RESULTS

A viral DNA fragment capable of being amplified in uninfected cells was mapped to a 1620-bp *Bam*HI/*Bgl* II subfragment of the *Bam*HI O fragment of HSV-1 DNA (Fig. 1). This fragment is contained entirely within the open reading frame encoding the large subunit of ribonucleotide reductase (14) but does not encode the entire polypeptide and does not contain a known origin of DNA replication capable of functioning during productive infection. For simplicity, we shall refer to the host origin of DNA replication as Ori_H to differentiate it from the lytic origins Ori_L and Ori_S.

Replication of the Ori_H sequence in a transient replication assay is shown in Fig. 2. Plasmids consisting of pUC18 vector and either the entire 1620-bp *Bam*HI/*Bgl* II Ori_H fragment (pRB430; Fig. 1, fragment A) or subfragments of the *Bam*HI/*Bgl* II fragment (pRB4148, pRB3878, pRB436, pRB3904, and pRB3903; Fig. 1, fragments G, D, E, F, and C, respectively), as well as a plasmid containing the *Bam*HI Z fragment of HSV-1(F) (pRB168), which includes the lytic origin Ori_{S2}, were transfected into Ltk$^-$ cells. Low molecular weight DNA was isolated by Hirt extraction 4 days after transfection, cleaved with *Dpn* I and *Hin*dIII restriction

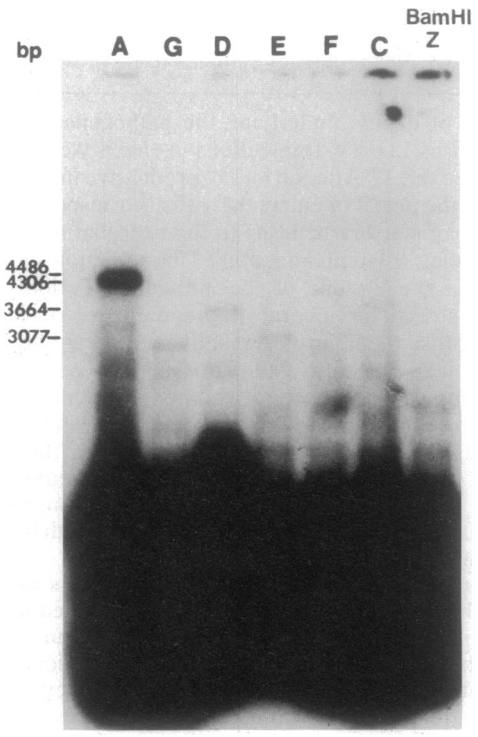

FIG. 2. Autoradiographic image of a Southern blot of electrophoretically separated *Hin*dIII/*Dpn* I digests of low molecular weight DNA extracted from Ltk$^-$ cells transfected with plasmids containing HSV-1 DNA fragments. Except for *Bam*HI Z, cloned in pRB168, each fragment is identified by the letter at the top of the lane. The map position of each fragment is shown in Fig. 1. Probe was nick-translated pUC18. Markers were *Hin*dIII digests of pRB168, pRB430, pRB436, and pRB3878 run on the same gel.

enzymes, and analyzed for the presence of full-length, *Dpn* I-resistant plasmid DNA. Only the plasmid containing the entire 1620-bp subfragment of *Bam*HI O was capable of replication in this system; none of the other plasmids showed replication to significant levels. Sequences required for replication then include the left end 391 bp and the right end 709 bp. In other assays (data not shown), we observed that the entire sequence required for replication is in this fragment; while the entire *Bam*HI O fragment is also capable of replication (Fig. 3), the right end *Bgl* II/*Bam*HI portion of *Bam*HI O (Fig. 1, fragment B) has no replication activity, nor is replication of the larger fragment enhanced over that of the smaller (1620 bp) Ori_H.

Inasmuch as the search for a host origin for viral DNA synthesis was motivated by the hypothesis that it might be used in neurons harboring latent virus, it was of interest to determine whether the amplification of the fragment containing Ori_H was affected by the fragments encoding the latency-associated RNA. A plasmid containing the entire *Bam*HI O fragment (pRB164) was cotransfected into Ltk$^-$ cells with either the pGEM3Z vector or pRB201, a plasmid containing the *Hin*dIII HM junction fragment of HSV-1(F) (Fig. 1). The *Hin*dIII HM fragment contains the sequences encoding the latency-associated transcript (4) and open reading frames U_L1, -2, -3, and -4 (10) as well as those encoding infected cell proteins 0, 4, and 34.5 (15–17). Hirt-extracted DNA was cleaved with *Dpn* I and *Bam*HI and analyzed for the presence and quantity of replicated pUC18 vector sequences (Fig. 3). No significant difference could be seen in the levels of replicated vector DNA in extracts of cells cotransfected with pRB164 and pGEM3Z as opposed to those cotransfected with pRB164 and pRB201.

Finally, to determine whether this replication is enhanced under conditions that increase cellular DNA synthesis, the plasmid pRB430 DNA was transfected into cells that were mitogenically stimulated by estrogen. MCF7 cells express the receptors for estrogen, progesterone, androgens, and glucocorticoids (18). In the presence of estrogen, the cells are stimulated to divide and cellular DNA replication is enhanced 3- to 4-fold (19). These cells were transfected with pRB430, and 24 hr after transfection duplicate plates were given medium with no hormone, 10 nM 17β-estradiol, 10 nM progesterone, 100 nM dexamethasone, or 10 nM testosterone. Four days after transfection, low molecular weight DNA was extracted by the Hirt procedure, cleaved with *Bam*HI and *Dpn* I, and assayed for the levels of full-length, linear, *Dpn* I-resistant pRB430 (Fig. 4). Replication was at least 10-fold greater in the cells receiving estradiol as compared to those cells receiving no hormone, progesterone, dexamethasone, or testosterone. In other assays (data not shown), amplification of both *Bam*HI O and the 1620-bp Ori_H has also been detected in HeLa, BHKtk$^-$, and N18 neuroblastoma cells; no significant difference was observed in the levels of amplification of these sequences in any of the various cell lines.

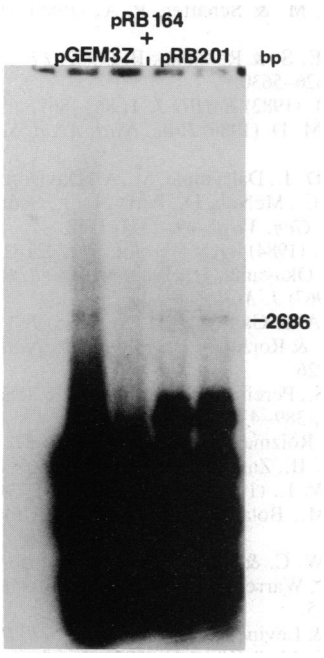

FIG. 3. Autoradiographic image of Southern blot of electrophoretically separated *Bam*HI/*Dpn* I digests of low molecular weight DNA extracted from Ltk$^-$ cells cotransfected with 10 μg of pRB430 and 10 μg of either pGEM3Z or pRB201. Map locations of all HSV-1 fragments are shown in Fig. 1. Probe was nick-translated pUC18. Marker was a *Bam*HI digest of pUC18 run on the same gel.

DISCUSSION

In this report, we demonstrate that a fragment of HSV-1 DNA is amplified by cellular factors. The minimal size of the sequence required for amplification is between 520 and 1620 bp; removal of 391 bp from the left end or of 709 bp from the right end of the 1620-bp fragment abolished its capacity to be amplified. In MCF7 cells stimulated by estrogen to divide, the fragment was overamplified with respect to amplification observed in untreated cells and in cells treated with progesterone, dexamethasone, or testosterone, in concurrence with the stimulation of cellular DNA synthesis in the estrogen-treated cells. The results suggest that the fragment contains an origin of DNA synthesis recognized by host factors, which we have designated Ori_H, and that the domains of the origin include sequences close to the termini of the 1620-bp sequence. As would be expected of an origin that is dependent on the activity of cellular DNA replication machinery, the amplification levels seen in these assays are relatively weak;

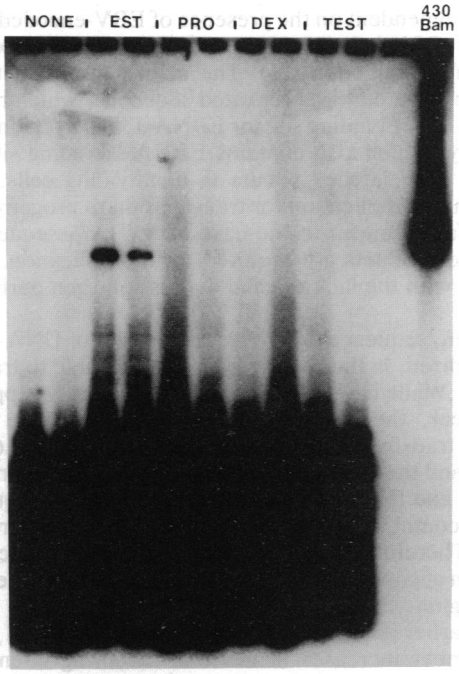

FIG. 4. Autoradiographic image of Southern blot of low molecular weight DNA extracted from MCF7 cells transfected with pRB430 and treated with no hormone (NONE), 17β-estradiol (EST), progesterone (PRO), dexamethasone (DEX), or testosterone (TEST). DNAs were digested with *Bam*HI and *Dpn* I. 430 Bam, 10 pg of pRB430 digested with *Bam*HI. Probe was nick-translated pRB430.

in comparison, transfection of the lytic origin Ori_{S2} followed by infection routinely yields levels of replicated DNA as much as 100 times greater than those observed here.

Our results indicate that a purported origin of lytic replication published in 1984 may in fact be Ori_H, to which it is juxtaposed. In those studies (11), the author infected cells in the presence of a high concentration of phosphonoacetic acid, removed the drug several hours after infection, added $^{32}P_i$, and analyzed the regions of the viral genome that first incorporated label. The results reported by Jacob (11) indicated that the label first appeared in the *Bgl* II G fragment and in a set of smaller *Bgl* II fragments at the other end of the L component (Fig. 1, *Bgl* II O, P, N, and M); these data were interpreted as an indication that those fragments contained origins of DNA replication used during lytic viral replication. Inasmuch as these fragments do not coincide with the now known lytic viral origins, and in light of the data presented in this paper, reinterpretation of those data may be based on the observation that, at high concentrations, phosphonoacetic acid inhibits cellular as well as viral DNA replication (20). Upon removal of the phosphonoacetic acid, as the intracellular concentration decreased, the first to recover would have been the less-sensitive cellular DNA synthesis, so that what was mapped in those studies may in fact have been cellular origins in the viral genome, rather than those origins used during productive infection. It remains to be determined whether the second region identified by Jacob (11) also contains sequences capable of independent replication in transfected cells.

Sequence comparisons of this origin with that used by another herpes virus during latent infection, the Ori_P sequence of Epstein–Barr virus (EBV), show no homologies between the two. We should note that EBV latency differs from that of HSV. EBV establishes latency in B lymphocytes, and at least one reason for the replication of EBV during latency is the necessity of transmission of the viral genome to the progeny cells. Ori_P is active at a relatively high level, and high levels of replication of Ori_P-containing plasmids are dependent on the presence of EBV-encoded nuclear antigen 1 (EBNA-1) (21, 22), a viral transactivator expressed during latent infection (23). The main features of the Ori_P sequence are a multiply repeated sequence, which has been shown to be the binding site for EBNA-1, and a region of dyad symmetry, which also contains EBNA-1 binding sites (24). Because HSV latency occurs in nondividing cells, the requirement for replication for transmission to progeny cells is absent; furthermore, in the case of the Ori_H sequence, no viral gene products are required for its replication, in concurrence with the lack of gene expression seen during HSV latency.

The Ori_H sequence does overlap a region of DNA that has been reported, in the case of HSV-2, to be able to transform cells (25). While HSV-1 and HSV-2 are generally supposed to be colinear, the corresponding HSV-1 sequence has no reported transformation activity; furthermore, the Ori_H sequences and the minimal transforming sequence overlap only partially, and the right end of the required Ori_H sequence is not at all contained within the minimal transforming region of HSV-2. Therefore, it appears unlikely that the replication of this origin accounts for the transforming ability of the HSV-2 mtrIII region.

In an earlier publication (1) we predicted that viral genome copy number increases in neurons harboring latent virus. Because viral genes whose products amplify viral DNA during lytic infection are not expressed in latently infected cells, amplification of viral DNA in the course of latent infection would require origins of DNA synthesis recognized by cellular factors. Furthermore, because no virus-encoded proteins are expressed, the amplification of viral DNA would have to occur in the absence of viral trans-activating proteins similar to those that have been reported in cells harboring latent EBV. Consistent with this prediction, we have demonstrated that HSV-1 DNA contains sequences that enable amplification of viral DNA by cellular proteins in the absence of viral proteins. Also, as predicted by the lack of evidence that the latency-associated transcript plays a role in the establishment or maintenance of the latent state, cotransfection of the fragment containing Ori_H with that encoding the latency-associated transcript had no effect on amplification of Ori_H.

While we have demonstrated here that HSV-1 DNA sequences can be amplified in cells in the absence of the viral factors involved in lytic DNA synthesis, proof of the amplification of latent viral DNA by the use of this origin remains to be established and will ultimately rest on testing of recombinant viruses from which the origin function has been removed. Were Ori_H to be utilized in latently infected neurons, it could be predicted to play an important role in the reactivation of virus from the latent state.

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