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A Subset of Herpes Simplex Virus Replication Genes Induces DNA Amplification within the Host Cell Genome

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Herpes simplex virus (HSV) induces DNA amplification of target genes within the host cell chromosome. To characterize the HSV genes that mediate the amplification effect, combinations of cloned DNA fragments covering the entire HSV genome were transiently transfected into simian virus 40 (SV40)-transformed hamster cells. This led to amplification of the integrated SV40 DNA sequences to a degree comparable to that observed after transfection of intact virion DNA. Transfection of combinations of subclones and of human cytomegalovirus immediate-early promoter-driven expression constructs for individual open reading frames led to the identification of six HSV genes which together were necessary and sufficient for the induction of DNA amplification: UL30 (DNA polymerase), UL29 (major DNA-binding protein), UL5, UL8, UL42, and UL52. All of these genes encode proteins necessary for HSV DNA replication. However, an additional gene coding for an HSV origin-binding protein (UL9) was required for origin-dependent HSV DNA replication but was dispensible for SV40 DNA amplification. Our results show that a subset of HSV replication genes is sufficient for the induction of DNA amplification. This opens the possibility that HSV expresses functions sufficient for DNA amplification but separate from those responsible for lytic viral growth. HSV infection may thereby induce DNA amplification within the host cell genome without killing the host by lytic viral growth. This may lead to persistence of a cell with a new genetic phenotype, which would have implications for the pathogenicity of the virus in vivo.

Herpesviruses are characterized by persistence in a latent state after primary infection of their hosts. They can repeatedly be reactivated from latency by a variety of stimuli. Although herpesvirus latency has been studied intensively, viral gene expression appears to be so restricted in the latent state that only little is known about it (for review, see reference 54). However, the lifelong close relationship between virus and host should offer the virus ample opportunity to induce nonlethal damage within the host cell genome as a result of restricted viral gene expression. In vitro, a variety of herpes simplex virus (HSV)-induced effects on the host cell genome have been described. HSV has been shown to induce chromosomal aberrations (8, 22, 62) and host cell DNA repair (43). HSV induces point mutations within the host cell genome, with an efficiency similar to that of chemical carcinogens (49, 50, 59). In addition, HSV induces DNA amplification of integrated simian virus 40 (SV40) DNA sequences in SV40-transformed hamster and human cell lines (38, 39, 57, 58). To explain these different genomic alterations, mechanisms that would not require the permanent presence of the virus in the host cell have been discussed. Instead, after limited viral gene expression leading to nonlethal host cell damage, the virus need no longer to be present in the damaged cell. Upon recurrent reactivations from the latent state, herpesviruses could use these mechanisms to play a role in tumor development as well (17, 33, and 70).

For investigation of pathogenetically relevant HSV effects on the host cell, HSV-induced DNA amplification is of particular interest, since DNA amplification not only plays a central role in the development of drug resistance (reviewed in references 18, 56, and 60) but also seems to be important in several stages of tumor development. Two lines of evidence support this notion. On the one hand, DNA amplification of cellular and integrated viral DNA sequences could be induced in cell lines by a variety of chemotherapeutic drugs such as methotrexate or by chemical or physical carcinogens (25, 30, 61). On the other hand, cytogenetic equivalents of DNA amplification, so-called homogeneously staining regions and double minute chromosomes, can be detected in a variety of tumor cell lines and also in many primary tumors. In these cases, amplified oncogene sequences are a frequent finding (for a review, see reference 1).

The generation of double minute chromosomes in HSV-infected cells has been demonstrated (8). In addition, it was reported recently that HSV can induce recombination between duplicated target DNA sequences unrelated to HSV DNA (64). This recombinogenic potential may play a role in the formation of double minute chromosomes, the generation of which necessarily involves extensive recombinational events. However, since HSV grows lytically on most cell lines suitable for study of HSV-induced DNA amplification, the direct demonstration of HSV-induced DNA amplification has so far been possible only in systems in which selection for the amplified phenotype is not necessary. In SV40-transformed hamster cell lines, HSV induces a high degree (up to more than 100-fold) of SV40 DNA amplification within 2 days (37, 58). This high efficiency in inducing DNA amplification within a short time period makes HSV-induced DNA amplification amenable to genetic analysis. With the use of temperature-sensitive mutants and antiviral drugs, the HSV-encoded DNA polymerase proved necessary for the induction of DNA amplification (23, 38). However, constitutive expression of the HSV DNA polymerase gene (*pol*) after transfection into hamster cells did not induce SV40 DNA amplification (R. Heilbronn, unpublished re-

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sults), suggesting that the HSV DNA polymerase alone is not sufficient.

Here we report an alternative approach to the identification of HSV genes that are necessary and sufficient for the induction of DNA amplification. This approach had proven successful for analysis of the concerted action of HSV genes in viral DNA replication; transfection of cloned HSV DNA fragments covering the entire genome provided the necessary *trans* functions for origin of replication (ori)-dependent HSV DNA replication (7). Systematic analysis demonstrated that seven early and two immediate-early (IE) genes were necessary and sufficient for *ori*-dependent DNA replication (68). On the assumption that HSV replication genes could also be amplification-inducing genes, we used a similar approach to characterize the HSV genes involved in SV40 DNA amplification. We show here that a subset of HSV replication genes not sufficient for ori-dependent HSV DNA replication is necessary and sufficient for the induction of SV40 DNA amplification upon transfection into SV40-transformed hamster cells. These results open the possibility that in vivo reactivation of herpesviruses from latency with limited viral expression could also lead to DNA amplification within the host cell genome without destroying the host by lytic viral growth, thus leading to persistence of a genetically altered cell.

MATERIALS AND METHODS

Recombinant plasmid DNAs. HSV type 1 (HSV-1; strain KOS) subclones pMC121 (*Xba*I-C), pMC124 (*Xba*I-D), pMC123 (*Xba*I-E), pMC122 (*Xba*I-F), pCW21, pMC150, pMC160, pMC160-1, pMC160-2, pNN1, pNN3, pNN4, and pNN5 were a kind gift of M. D. Challberg (7, 68). The pSG series of cloned *Eco*RI fragment of HSV-1 were a gift of M. Levine (20).

pH0, -3, and -7 are constructs of the IE genes IE110 (ICP0) and IE175 (ICP4) subcloned from pSG25 and pSG1 into Bluescript (Stratagene, San Diego, Calif.) as follows: pH0 is a *Sac*I-*Hpa*I subclone covering IE110 (ICP0); pH3 is a *Xho*I-*Sac*I subclone (created by partial digestion) for IE175 (ICP4); and pH7 covers the entire IE110-IE175 coding region (*Xho*I-*Hpa*I). All constructions relied on sequence information given previously (39, 41, 68). pH6 is a plasmid covering the DNA polymerase (*pol*) and the major DNA-binding protein (DBP) genes created by fusion of pNN1 and pNN3, using the single restriction site *Bgl*II. pH8 is a plasmid covering UL42 and UL52 created by fusion of pNN4 and pNN5. pH9 is a plasmid covering UL5 and UL8 created by fusion of pCW21 and the *Sal*I-*Kpn*I fragment of pMC160 covering the UL8 coding region.

Expression constructs for the UL5, UL8, UL9, UL29 (DBP), UL30 (*pol*), UL42, and UL52 open reading frames (ORFs) under the control of the human cytomegalovirus (HCMV) IE enhancer promoter region (3) were generated by fusion within the 5′ untranslated leader sequences as follows. A *Hin*cII-*Ava*II (positions −598 to +52) subfragment of the HCMV IE enhancer-promoter region was blunt ended with T4 DNA polymerase and cloned into the *Hin*cII site of pUC19 (pCMIEa). A vector constructed with the same HCMV fragment cloned into the *Sma*I site of pUC19 (pL82) was kindly provided by M. Pawlita. For all constructions, T4 DNA polymerase was used to create blunt ends whenever necessary. pCM-UL5 was constructed by fusion of the HCMV promoter with the UL5 ORF 30 base pairs (bp) upstream of the first ATG, using the unique *Mlu*I site. pCM-UL8 was constructed similarly, using the unique *Rsr*II site 180 bp upstream of the first ATG of the UL8 ORF. pCM-UL9 was created from pMC160-1 by partial digestion with *Sac*I, fusing 140 bp upstream of the first ATG of the UL9 ORF to the HCMV promoter. pCM-pol was created by a double partial digestion, using the *Bam*HI site 30 bp upstream of the first ATG of the first ORF of the DNA polymerase gene for fusion to the HCMV promoter and the *Kpn*I site just downstream of the 3′ end of the *pol* ORF. pCM-DBP was created by partial digestion from pNN1, using the *Bst*EII site 180 bp upstream of the first ATG of the DBP ORF for fusion to the HCMV promoter. pCM-UL42 was created by using the unique *Rsr*II site 55 bp upstream of the first ATG of the UL42 ORF, and pCM-UL52 was created by using the unique *Bss*HII site 13 bp upstream of the first ATG of the UL52 ORF for fusion to the HCMV promoter. Plasmids were prepared according to the alkali lysis procedure (34).

Cells and viruses. Elona11 is a subcloned cell line derived from Elona, an SV40-transformed Syrian hamster cell line kindly provided and described in detail by Matz (37). Cells were maintained in Dulbecco minimal essential medium, 5% heat-inactivated (30 min, 56°C) fetal calf serum, antibiotics, and 10 mM *N*-2-hydroxyethylpiperaze-*N*-2′-ethanesulfonic acid (HEPES; pH 7.2). Propagation of HSV-1 strain 17 syn^+ was performed as described previously (37). HSV-1 virion DNA was prepared from purified virions by lysis in 1% sodium dodecyl sulfate–proteinase K (100 μg/ml) at 56°C for 3 h, followed by banding in CsCl.

Transfection procedure. Elona11 cells (1.0×10^6 to 1.5×10^6) were plated onto 10-cm-diameter dishes. The next day, cells were refed and transfected 2 to 4 h later by the calcium phosphate coprecipitation method of Weber et al. (63), using 20 μg of plasmid DNA per dish. The amount of each plasmid DNA transfected is indicated in the figure legends. pUC19 DNA was added if necessary.

Plasmid DNA was suspended in 500 μl of 0.25 M $CaCl_2$–50 mM HEPES (pH 6.95). After 15 min, 500 μl of 0.280 M NaCl–0.750 mM Na_2HPO_4–0.750 mM NaH_2PO_4–50 mM HEPES (pH 6.95) was added; 15 min later, the mixture was pipetted onto the cells. At 3 to 4 h posttransfection, cells were shocked with 25% dimethyl sulfoxide in HEPES-buffered Dulbecco minimal essential medium for 4 min, washed twice with serum-free medium, and left overnight in growth medium. Cells were refed the next day and harvested 40 to 48 h posttransfection for extraction of genomic DNA. Every transfection experiment was performed at least three times with different plasmid preparations, with reproducible results.

Analysis of genomic DNA. High-molecular-weight genomic DNA was extracted from cell nuclei prepared by Nonidet P-40 lysis, digested with proteinase K (100 μg/ml) for 2 h at 56°C, and extracted with phenol, phenol-chloroform, and chloroform. DNA was dialyzed against 1× TE overnight. One-fifth of the purified DNA was digested with either *Sac*I (for amplification assays) or the combination *Eco*RI-*Hin*dIII-*Dpn*I (for replication assays) for 6 to 12 h, precipitated, and resuspended in 30 μl of 1× TE. Optical density at 260 nm was determined in 1/10 of the restricted DNA, and 5 μg per lane was run on agarose gels (0.5% for *Sac*I digestions; 0.7% for *Eco*RI-*Hin*dIII-*Dpn*I digestions). Gels were blotted with 0.5 M NaOH–1.5 M NaCl onto GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) after 15 min in 0.25 N HCl and two 15-min periods in 0.5 M NaOH–1.5 M NaCl. Blots were UV cross-linked and hybridized as described elsewhere (9). For determination of SV40 DNA amplification, a nick-translated *Taq*I-*Kpn*I fragment of SV40 DNA

was used as the hybridization probe; for determination of ori_S replication, an oligolabeled 1.4-kbp *Eco*RI-*Xho*I fragment of pSG25 covering ori_S was used.

RESULTS

Structure of the resident and amplified SV40 DNA sequences in Elona11 cells. Infection with HSV induces amplification of chromosomally integrated viral DNA sequences in a variety of rodent and human cell lines (38, 39, 57, 58). The genetic analysis of this effect in a transient cotransfection assay with a set of cloned HSV genes posed certain requirements on a recipient cell line: (i) a high degree of amplification within a short time period (days) to account for the much lower efficiency of transfection versus infection, (ii) a simple and stable pattern of the integrated SV40 DNA sequences, and (iii) good transfectability by the calcium phosphate coprecipitation technique, which is especially suitable for high-efficiency cotransfer of several different plasmids. These requirements are met by the cell line Elona11. It has one chromosomal integration site for the SV40 genome (Fig. 1). A Southern blot of Elona11 DNA digested with different noncut enzymes for SV40 DNA (*Sac*I, *Xba*I, and *Bgl*II) and hybridized to ^{32}P-labeled SV40 DNA yielded one high-molecular-weight band of varying mobility, depending on the enzymes used. *Eco*RI, a single-cut enzyme for SV40 DNA, produced a 5.2-kbp band (compare with the one-copy-per-cell SV40 DNA marker) corresponding to the full-size genome, suggesting the integration of multiple copies in tandem. The two fainter bands appeared to harbor the flanking integration sites, since at least one of the bands was mobilized upon double digestion with *Sac*I-*Eco*RI. The structure of the integrated SV40 DNA sequence was the same as that reported by Matz (37). The integration pattern remained stable over serial passage of the cell line, and no episomal molecules were detected either spontaneously or after infection or transfection (data not shown).

Upon infection of Elona11 cells with HSV a greater than 100-fold amplification of the integrated SV40 DNA sequences could be detected within 36 to 48 h postinfection. The amplified SV40 DNA sequences were arranged as tandemly reiterated oligomers of more than 40 kbp in length (37). Using *Sac*I digestion of genomic DNA, the resident SV40 DNA could be easily distinguished from the bulk of amplified sequences by its mobility in a low-percentage gel (Fig. 1). A short exposure was chosen for the blot with HSV-infected cell DNA (Fig. 1) to demonstrate the clear-cut distinction between resident and amplified SV40 DNA sequences. This distinction is the basis of the amplification assay, since upon transfection only the small percentage of successfully cotransfected cells will amplify DNA sequences.

Identification of the minimum set of HSV genes necessary and sufficient for induction of SV40 DNA amplification. HSV-induced DNA amplification is dependent on a functional viral DNA polymerase (23, 38). However, the viral DNA polymerase proved not to be sufficient for induction of DNA amplification after constitutive expression of the *pol* ORF under the control of the HCMV IE promoter (pCM-pol) in Elona11 cells (data not shown). To search for additional HSV genes whose combined action might be required for the induction of DNA amplification, an approach was chosen that had proven successful for identification of HSV genes acting in *trans* on the *cis* elements for HSV replication, ori_S and ori_L (7, 68). The assumption was that HSV replication genes could also be amplification-inducing genes.

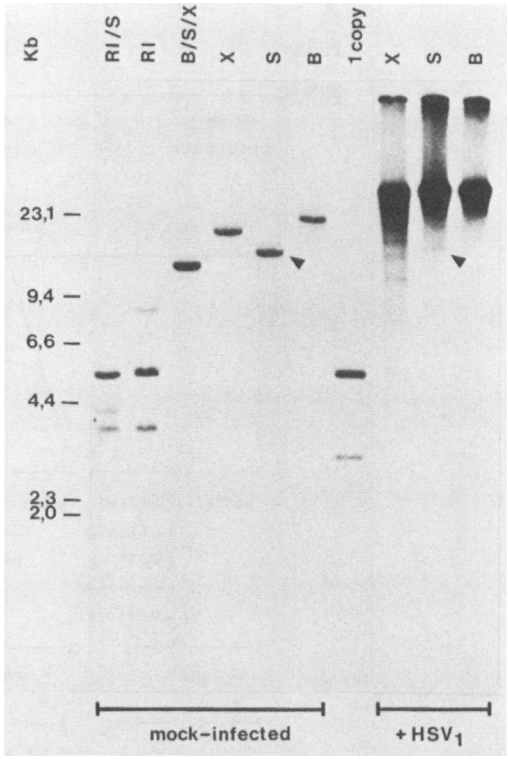
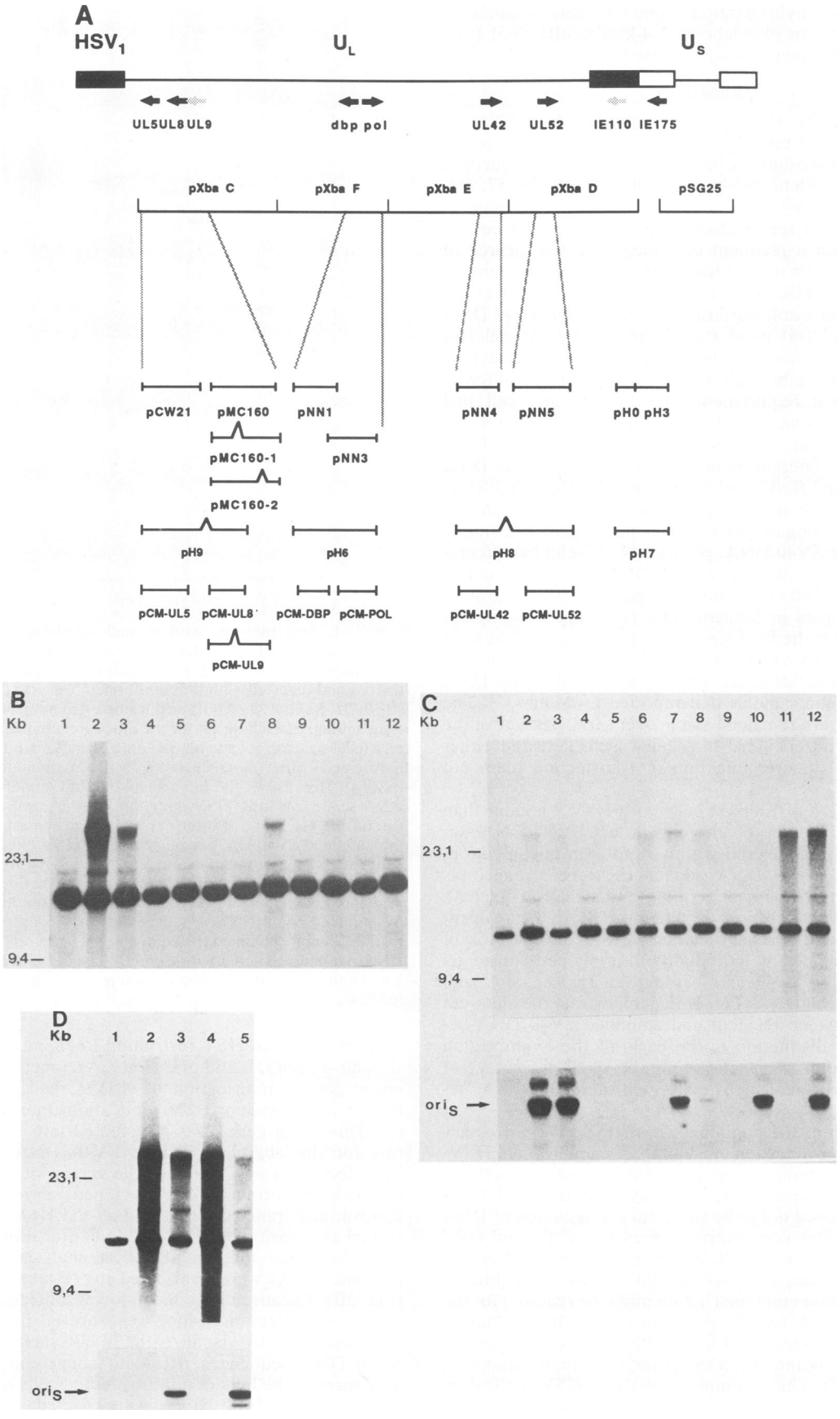


FIG. 1. Structure of resident and amplified SV40 DNA sequences in Elona11 cells. Mock-infected or HSV-1-infected Elona11 cells were harvested 40 h postinfection. Genomic DNA was extracted and digested with different noncut enzymes for SV40 DNA (B, *Bgl*II; S, *Sac*I; X, *Xba*I) or the single-cut enzyme *Eco*RI (RI). A 5-μg amount of each digest for the mock-infected cell DNA was run on a 0.7% agarose gel and blotted onto GeneScreen Plus. Blots were hybridized with ^{32}P-labeled SV40 DNA including the vector (pSP64). Because of the more than 100-fold amplification of SV40 DNA sequences after HSV infection, only 1 μg of each digest was run on the gel, and a shorter exposure was chosen to demonstrate the amplified SV40 DNA sequences after HSV infection (3-h exposure versus 24 h for the mock-infected cell DNA). Arrowheads point to the 15-kbp *Sac*I fragment harboring the resident SV40 DNA sequences, which are clearly distinguishable from the >40-kbp bulk of amplified SV40 DNA sequences after HSV infection. The lane marked 1 copy contained the equivalent of one copy of cloned SV40 DNA per cell restricted with *Bam*HI. The 5.2-kbp band represents SV40 unit length; the 3.0-kbp band represents the vector sequences (pSP64).

A set of cloned HSV restriction fragments, pXbaI-C, -D, -E, and -F, pSG25, and pH3 (Fig. 2A), together covering the entire genetic information of HSV-1, had been shown to induce ori-dependent HSV replication upon cotransfection (7). This set of genes was transfected into Elona11 cells to test for the ability to induce SV40 DNA amplification. Transfection of pUC19 DNA, the vector into which most of the HSV DNA fragments were cloned, served as a negative control, and transfection of undigested HSV-1 virion DNA served as a positive control. At 44 h after transfection, cells were harvested for Southern blot analysis. *Sac*I-digested genomic DNAs were hybridized to ^{32}P-labeled SV40 DNA (Fig. 2B). Because of the much lower efficiency of transfection versus infection, a long exposure of the Southern blot was necessary to visualize the weak signals of amplified SV40 DNA sequences. Resident single-copy SV40 DNA sequences in the bulk of nontransfected cells gave rise to the strong 15-kbp *Sac*I band. As expected, transfection of


A
HSV1
UL
US
UL5 UL8 UL9
dbp pol
UL42
UL52
IE110 IE175
pXba C
pXba F
pXba E
pXba D
pSG25
pCW21
pMC160
pNN1
pNN4
pNN5
pH0 pH3
pMC160-1
pNN3
pMC160-2
pH9
pH6
pH8
pH7
pCM-UL5
pCM-UL8
pCM-DBP
pCM-POL
pCM-UL42
pCM-UL52
pCM-UL9
B
Kb 1 2 3 4 5 6 7 8 9 10 11 12
23,1
9,4
C
Kb 1 2 3 4 5 6 7 8 9 10 11 12
23,1
9,4
oriS
D
Kb 1 2 3 4 5
23,1
9,4
oriS

pUC19 DNA did not induce SV40 amplification (Fig. 2B, lane 1), whereas HSV-1 virion DNA induced a strong amplification effect (Fig. 2B, lane 2) similar to that observed after HSV infection (Fig. 1). Transfection of the cloned HSV DNA fragments (pXbaI-C, -D, -E, and -F, pSG25, and pH3) also induced DNA amplification (Fig. 2B, lane 3). The relatively weak signal compared with the signal produced upon transfection of virion DNA can be explained by the fact that within 40 h, intact virion DNA can initiate multiple rounds of lytic viral growth with superinfection of nontransfected cells, whereas the nonoverlapping cloned HSV DNAs cannot replicate. In addition, the probability of successful transfection of one molecule of HSV virion DNA is considerably higher than the probability of successful cotransfection of six independent plasmids. To determine whether all six of these HSV plasmids code for genes necessary for the induction of DNA amplification, the set of plasmids was transfected, omitting one plasmid at a time. Omission of either pXbaI-C, -D, -E, or -F abolished the amplification effect induced by transfection of the full set of six clones (Fig. 2B, lanes 4 to 7). Omission of pSG25, which codes for genes within the unique short region, did not abolish the amplification effect of the other five clones (Fig. 2B, lane 8). Omission of pH3, coding for IE175 (ICP4), the major IE gene necessary for transactivation of early genes, abolished DNA amplification (Fig. 2B, lane 9). In summary, each of the *Xba*I DNA fragments of HSV-1 encode at least one essential gene for the induction of DNA amplification. The IE gene IE175 (ICP4) is necessary, whereas genes encoded by the unique short region are dispensable.

This situation is reminiscent of ori-dependent HSV DNA replication (7). Therefore, to further characterize the amplification-inducing HSV genes, subclones of the *Xba*I fragments that coded for genes necessary for ori-dependent HSV replication were tested. *Xba*I-F encodes two genes essential for HSV replication (7), the major DBP and the viral DNA polymerase genes, the latter already known to be a necessary function of HSV-induced DNA amplification (23, 38). Transfection of subclones covering only the coding regions for polymerase and DBP (pNN1 and pNN3) together with *pXba*I-C, -D, and -E, pSG25, and pH3 induced an amplification effect similar to that of pXbaI-F (Fig. 2B, lane 10). However, omission of either pNN1 or pNN3 completely abolished DNA amplification (Fig. 2B, lanes 11 and 12). The slightly reduced signal after transfection of pNN1 and -3 compared with results for *Xba*I-F (Fig. 2B, lane 10 versus lane 3) is most probably explained by the fact that the *Xba*I-F-carrying plasmid contains a functional ori_L and can replicate after transfection, whereas pNN1 and pNN3 are constructed from ori_L-defective subclones (7). In summary, the only two genes within *Xba*I-F necessary for induction of SV40 DNA amplification are those encoding the viral DNA polymerase and the major DBP.

So far, we have determined that the genes necessary for SV40 DNA amplification are covered by plasmids pXbaI-C, -D, and -E, pH6 (*pol* and DBP together), and pH3 (IE175 [ICP4]). To characterize the amplification-inducing genes residing on pXbaI-C, we tested subclones that code for UL5, UL8, and UL9 (Fig. 2A), the three genes within pXbaI-C necessary for ori-dependent HSV replication (68). Transfection of pXbaI-C, -D, and -E, pH6, and pH3 induced DNA amplification as well as did transfection of the same set of genes but with XbaI-C replaced by pMC160 (UL8/9) and pCW21 (UL5) (Fig. 2C, lanes 2 and 3). Omission of either pCW21 (UL5) or replacement of pMC160 by pMC160-1, thus omitting the UL8 gene, abolished the amplification effect (Fig. 2C, lanes 4 and 5). However, replacing pMC160 by pMC160-2, thus omitting the UL9 gene, in the presence of

FIG. 2. Identification of the minimal set of HSV-1 genes necessary and sufficient for HSV-induced SV40 DNA amplification. (A) Schematic representation of the 150-kbp HSV-1 genome with the unique long (U_L) and unique short (U_S) regions and flanking inverted repeats (■, long region; □, short region). Replication-amplification-inducing genes: UL5, -8, -9, -42, and -52 (terminology according to McGeoch et al. [41]); dbp (DNA-binding protein), pol (DNA polymerase), IE110 (ICP0), and IE175 (ICP4). Arrows indicate the direction of transcription; solid arrows represent genes necessary for both HSV replication and SV40 DNA amplification (UL5, UL8, *pol*, DBP, UL42, UL52, and IE175), and stippled arrows indicate genes necessary only for HSV DNA replication (UL9 and IE110). Below is shown the cloned set of *Xba*I restriction fragments of HSV-1 which, together with pH3 (encoding IE175 [ICP4]) and pSG25 (covering the unique short region), covers the entire genetic information of HSV-1. The relative positions of cloned subfragments covering individual ORFs are as follows: pH9 covers the UL5 and UL8 coding regions, pH6 covers the DBP and *pol* coding regions, and pH8 covers the UL42 and UL52 coding regions. The pCM series of expression constructs is represented at the bottom. These constructs are named according to the ORFs they express under the control of the HCMV promoter. (B) Southern blot of *Sac*I-restricted genomic DNA of Elona11 cells transfected with different combinations of cloned HSV DNAs. The blot was probed with a ^{32}P-labeled *Taq*I-*Kpn*I SV40 DNA subfragment. The bands at >40 kbp present in some lanes at various intensities represent amplified SV40 DNA sequences. Some minor bands of an intensity far below the one-copy-per-cell level always became visible in all of the lanes upon long exposure of the blots. These bands most probably represent cellular sequences cross-hybridizing with SV40 DNA. Cells were transfected with the following combinations of the DNAs described above: lane 1, pUC19, 20 μg; lane 2, HSV-1 DNA purified from virions, 20 μg, undigested; lane 3, complete set of plasmids covering the entire HSV-1 genome (pXbaI-C, -D, -E, and -F), 4 μg each, plus pH3 and pSG25, 2 μg each; lane 4, same as lane 3 but without pXbaI-C; lane 5, same as lane 3 but without pXbaI-D; lane 6, same as lane 3 but without pXbaI-E; lane 7, same as lane 3 but without pXbaI-F; lane 8, same as lane 3 but without pSG25; lane 9, same as lane 3 but without pH3; lane 10, same as lane 3 but with pNN1 and pNN3 (2 μg each) instead of pXbaI-F; lane 11, same as lane 10 but without pNN1; lane 12, same as lane 10 but without pNN3. (C) Results of a transfection experiment performed as for panel B, using the following combinations of plasmids: lane 1, pUC19, 20 μg; lane 2, the minimum set of amplification-inducing plasmids identified in panel B, namely, pXbaI-C, -D, and -E (4 μg each), pNN1, pNN3, and pH3 (2 μg each) plus pH10 (1 μg), covering ori_S for the replication assay performed in parallel; lane 3, same as lane 2 but with pCW21 and pMC160 (2 μg each) instead of pXbaI-C; lane 4, same as lane 2 but with pMC160 (2 μg) instead of pXbaI-C; lane 5, same as lane 2 but with pMC160-1 and pCW21 (2 μg each) instead of pXbaI-C; lane 6, same as lane 2 but with pMC160-2 and pCW 21 (2 μg each) instead of pXbaI-C; lane 7, same as lane 2 but with pNN5 and pH0 (2 μg each) instead of pXbaI-D; lane 8, same as lane 7 but without pH0; lane 9, same as lane 7 but without pNN5; lane 10, same as lane 2 but with pNN4 (2 μg) instead of pXbaI-E; lane 11, pH7 (IE110/175), pH6 (*pol*/DBP), pH8 (UL42/52), and pH9 (UL5/8), 4 μg each; lane 12, same as lane 11 plus pMC160-1 (coding for UL9; 2 μg). Below is a Southern blot of *Eco*RI-*Hin*dIII-*Dpn*I-restricted DNA of the same experiment probed with a ^{32}P-labeled fragment for ori_S. *Dpn*I-resistant, replicated ori_S sequences are indicated. (D) Results of a transfection experiment performed as for panel B, using the following combinations of DNAs in addition to 1 μg of pH10 (ori_S): lane 1, pUC19, 20 μg; lane 2, pH7 (IE110/175), pH6 (*pol*/DBP), pH8 (UL42/52), and pH9 (UL5/8), 2 μg each; lane 3, same as lane 2 plus pMC160-1 (UL9); lane 4, combination of constructs in which the respective ORFs are under HCMV promoter control (pCM-UL5, -UL8, -DBP, -*pol*, -UL42, and -UL52, 1 μg each); lane 5, same as lane 4 plus pCM-UL9. The replication assay for ori_S was performed as described for panel C.

the rest of the clones did elicit an amplification effect (Fig. 2, lane 6). Therefore, only the UL5 and UL8 genes, not UL9, are necessary for induction of SV40 DNA amplification. This finding differs from the situation in ori-dependent HSV replication, for which the UL9 gene is indispensable. Since ori-dependent HSV replication had been assayed in a monkey cell line (Vero) whereas DNA amplification had been assayed in hamster cells (Elona11), a *Dpn*I replication assay similar to the one described by Challberg (7) was performed in parallel with the amplification assay to exclude a bias due to the different cell systems used. pH10, an ori_S-containing plasmid that can replicate in the presence of HSV replication functions, was cotransfected together with the amplification- or replication-inducing plasmids. A Southern blot with *Eco*RI-*Hin*dIII-*Dpn*I-digested genomic DNA was hybridized to a ^{32}P-labeled ori_S subfragment of pSG25. *Dpn*I will digest the transfected plasmid DNA methylated during replication in bacteria, whereas plasmids replicated after transfection into eucaryotic cells are not methylated and therefore are *Dpn*I resistant. The data are shown below the results for the amplification assay performed in parallel (Fig. 2C). The *Dpn*I-resistant ori_S fragment revealing eucaryotic replication is indicated. It is evident that in the presence of pXbaI-D and -E, pH3, and pH6, plasmids pMC160 (UL8/9) and pCW21 (UL5) induced ori_S replication as well as did pXbaI-C (Fig. 2C, lanes 2 and 3). However, omission of the plasmid encoding UL5, UL8, or UL9 abolished ori_S replication (Fig. 2C, lanes 4 to 6), exactly as described by Wu et al. (68) for Vero cells. Since the UL9 ORF codes for a DBP specific for ori_S and ori_L (46), it is plausible that it is required only for directing the HSV replication enzymes to the HSV origins of replication.

*Xba*I-D encodes three genes playing a role in ori-dependent HSV replication: UL52, which is absolutely required for ori replication, and two IE genes, IE110 (ICP0) and IE63 (ICP27). Transfection of plasmids pNN5 (UL52) and pH0 (IE110 [ICP0]) together with plasmids pXbaI-C and -E, pH6, and pH3 induced DNA amplification and ori_S replication as well as did transfection of pXbaI-D (Fig. 2C, lane 7). Omission of pH0 (IE110 [ICP0]) did not reduce the amplification effect; however, ori_S replication was considerably lowered (Fig. 2C, lane 8). The latter result is in perfect agreement with the findings of Wu et al. (68). Omission of pNN5 (UL52), however, abolished the amplification effect as well as ori_S replication (Fig. 2C, lane 9). In summary, pXbaI-D codes for one gene (UL52) essential for both SV40 DNA amplification and ori_S replication. The IE gene IE110 (ICP0), however, is dispensible for DNA amplification but adds to the effect of IE175 (ICP4) in ori_S replication. Further addition of a plasmid for IE63 (ICP27), pMC150, did not increase the amplification effect (data not shown) but added to the effect of IE175 (ICP4) and IE110 (ICP0) in ori_S replication (68).

pXbaI-E encodes one gene absolutely required for ori-dependent HSV replication, UL42. Replacement of pXbaI-E by pNN4 (UL42) upon transfection together with pXbaI-C, and -D, pH6, and pH3 induced DNA amplification and ori_S replication (Fig. 2C, lane 10). Although in that experiment the amplification effect appeared rather weak, it was clearly established in additional experiments that UL42 is the only necessary function within XbaI-E (see below and Fig. 2C, lane 11).

The results are summarized as follows. Seven of eight HSV-1 genes necessary and sufficient for ori-dependent HSV replication are necessary and sufficient for the induction of DNA amplification of chromosomally integrated SV40 DNA sequences. These HSV-1 genes are UL5, UL8, UL42, UL52, *pol*, DBP, and IE175 (ICP4). Although the UL9 gene is required for HSV replication, it is not necessary for DNA amplification. With the approach taken, we cannot determine whether additional HSV genes might add to the amplification effect. These stimulatory genes would, however, not be absolutely required, in contrast to the set of HSV functions necessary for the induction DNA amplification that we identify here. To study DNA amplification induced by these genes without the background of other HSV genes, the identified amplification-inducing HSV genes were cloned onto three plasmids, pH6 (*pol*/DBP), pH8 (UL42/52), and pH9 (UL5/8), to reduce the number of plasmids for efficient cotransfer. The combination pH6-pH8-pH9, in addition to pH7 (IE175 [IE110]), was transfected into Elona11 cells and assayed for the induction of DNA amplification. The set of six early and two IE genes induced SV40 DNA amplification (Fig. 2C, lane 11) to a higher extent than did the original set of clones (Fig. 2C, lane 2), confirming the finding that this set of HSV genes is sufficient for the induction of DNA amplification. As expected, however, pH6, -7, -8, and -9 were not sufficient for ori-dependent HSV replication. Addition of pMC160-1, coding for UL9, fully restored ori_S replication (Fig. 2C, lane 12).

Role of the HSV-1 IE genes in DNA amplification. From these studies, an unresolved question was whether IE genes were necessary only as transactivators of the early genes (UL5, UL8, UL42, UL52, *pol*, and DBP) or whether they played an additional role in DNA amplification separate from transactivation. Since the HSV early-gene promoters are almost silent upon transfection without transactivation by IE genes (13, 45, 52), they were replaced by the strong constitutive HCMV promoter to make them independent of transactivation. These expression constructs, pCM-UL5, -UL8, -UL9, -*pol*, -DBP, -UL42, and -UL52 (Fig. 2A), were transfected into Elona11 cells in parallel with the respective genes under HSV-1 promoter control (pH6, -8, and -9) in the presence of pH7 (IE175/110) for transactivation. DNA amplification and ori_S replication were assayed as described above. It is evident that the constitutive expression of the early genes under HCMV promoter control induced SV40 DNA amplification and ori_S replication as efficiently as did expression of the same genes driven by their cognate promoters (Fig. 2D, lanes 2 and 4). Thus, efficient expression of the set of six HSV genes, UL5, UL8, *pol*, DBP, UL42, and UL52, is necessary and sufficient for induction of SV40 DNA amplification. Further addition of the constitutively expressed UL9 gene (pCM-UL9) also induced ori_S replication (Fig. 2D, lane 5), as did addition of the UL9 gene driven by its native HSV promoter in the presence of IE175/110 (Fig. 2D, lane 3). The reduced level of SV40 DNA amplification upon addition of UL9 might be explained by competition of the SV40 ori and ori_S for the HSV replication-amplification functions. In summary, IE genes seem to be necessary only as transactivators of early genes in both DNA amplification and ori-dependent HSV replication.

DISCUSSION

Amplification of chromosomally integrated SV40 DNA sequences as a model system for the amplification of authentic cellular genes. The first indication that DNA amplification can be demonstrated without selection for the amplified phenotype directly in response to chemical or physical carcinogens came from early studies by Lavi (30). She used SV40-transformed Chinese hamster cells (CO60) in which

the chromosomally integrated SV40 DNA sequences were amplified to a very high degree (up to >100-fold) within 2 to 5 days in response to a variety of carcinogens. This high degree of amplification within a short time period made SV40-transformed cell lines suitable for testing for the amplification-inducing potential of many different agents in a short-term assay system. In subsequent studies, some of these agents were also tested for the induction of dihydrofolate reductase (DHFR) gene amplification. The chemical carcinogens *N'*-acetoxy-*N*-acetylaminofluorene (NA-AAF), *N'*-methyl-*N*-nitro-*N'*-nitrosoguanidine (MNNG), ethyl methansulfonate (EMS), and arsenic, as well as UV irradiation of the cells, proved to induce increased rates of methotrexate resistance along with amplification of DHFR gene sequences (25, 32, 61). In SV40-transformed CO60 cells, parallel analysis of carcinogen-induced SV40 DNA amplification and DHFR gene amplification demonstrated that although SV40 DNA amplification occurred at a much higher rate than did DHFR gene amplification, the two events exhibited the same dose responses and time courses. In addition, DHFR gene amplification appeared to occur in the same population of cells that also exhibited SV40 DNA amplification (25, 26). The high degree of carcinogen-induced SV40 DNA amplification in a short-term assay is dependent on a functional SV40 ori in *cis* and a functional T antigen in *trans* (30, 31). The analysis of HSV-induced SV40 DNA amplification also revealed the necessity for a functional SV40 ori in *cis* and T antigen in *trans* (39). Apparently, both carcinogen- and HSV-induced SV40 DNA amplification in SV40-transformed cells occur at such a high rate because of the efficiency of T antigen to direct the cellular or viral replication enzymes toward the SV40 ori. However, apart from the difference in induction rates, SV40 DNA amplification and the amplification of authentic cellular genes appear to exhibit similar characteristics; therefore, SV40-transformed cell lines can be considered suitable model systems for the analysis of DNA amplification.

HSV replication-amplification genes have related cellular counterparts. All of the six amplification-inducing HSV genes identified in this report are necessary but not sufficient for ori-dependent HSV replication, for which an additional replication gene is required (68). The two best-studied HSV replication-amplification genes, those encoding the HSV DNA polymerase and the HSV major DBP, exhibit striking DNA sequence homology to well-studied mammalian replication enzymes. HSV *pol* shares DNA sequence homology with the gene encoding cellular DNA polymerase alpha (19, 53, 67), which reflects the very similar catalytic properties of the two enzymes. However, an important difference between these two polymerases is the presence of an associated 3'-to-5' exonuclease activity in HSV DNA polymerase, which is thought to represent a proofreading function (27). This feature of HSV DNA polymerase is shared by cellular DNA polymerase delta, which closely resembles polymerase alpha in its biochemical characteristics (5), although it is not yet known whether the two cellular polymerases are also related genetically. Polymerases delta and alpha are being discussed as leading- and lagging-strand DNA polymerases (51), whereby polymerase delta needs an auxiliary protein, the proliferating cell nuclear antigen (cyclin) for full activity (4). The proliferating cell nuclear antigen, in turn, exhibits significant DNA sequence homology to the HSV-1 major DBP (36). Purified HSV DBP has been shown to bind cooperatively to single-stranded DNA (55) and to stimulate HSV polymerase function on double-stranded DNA templates (44). This interaction of two amplification-inducing HSV functions, polymerase and DBP, could be analogous to a similar interaction between the related cellular counterparts, polymerase alpha and/or delta and proliferating cell nuclear antigen, in carcinogen-induced DNA amplification. The identification of polymerase alpha (or possibly delta) as a necessary *trans* function for carcinogen-induced SV40 DNA amplification (23) further underlines the apparent similarity of HSV- and carcinogen-induced DNA amplification.

The precise roles of the other four amplification-inducing HSV genes, UL5, UL8, UL42, and UL52, appear less clear at the moment, although it is established that all of these genes are required for viral DNA replication in cell culture (21, 35, 65, 69). UL5 appears to code for a DNA helicase (41) that has recently been purified from HSV-infected cells (10). UL42 codes for a second DBP with specificity for double-stranded DNA (designated $65K_{DBP}$) that appears to be closely associated with the viral DNA polymerase (16, 48). The roles of UL8 and UL52 are not known at present. However, in analogy to other replication systems, good candidates are an apparently HSV-encoded DNA primase that has been purified recently (24) and a topoisomerase function (2, 42). Further analysis of HSV amplification functions may lead to the identification of additional cellular homologs, which will help in the understanding of mechanisms used by cellular *trans* factors upon DNA amplification in response to carcinogen treatment.

The only single HSV gene product necessary for ori-dependent HSV replication but not for SV40 DNA amplification is the UL9 gene product. Interestingly, this gene codes for an ori-binding protein. Protein-DNA binding assays and DNase I footprint analysis with purified UL9 protein demonstrated a specific interaction of the UL9 protein with the HSV ori_L and ori_S (12, 46). The absolute requirement for this protein in viral DNA replication was demonstrated by HSV mutants for the UL9 gene that cannot synthesize viral DNA unless the defective UL9 gene is complemented in *trans* by a UL9-expressing helper cell line (6). In the study described here, UL9 was not required for induction of SV40 DNA amplification in hamster cells, although in the same experiment its presence was required for ori-dependent HSV replication, confirming the results of Wu et al. (68). In addition, the replication-deficient HSV mutants in the UL9 gene (6) induced levels of SV40 DNA amplification in Elona11 cells comparable to those induced by wild-type virus. However, as expected, the UL9 mutants could not support replication of a transfected ori_S-containing plasmid (R. Heilbronn, S. K. Weller, and H. zur Hausen, submitted for publication). These results, together with the lack of any sequence homology between the HSV ori_L and ori_S and the SV40 ori (15, 66), suggest a possible mechanistic explanation: as discussed above, the SV40 ori and T antigen are required for HSV-induced SV40 DNA amplification (39). Although the precise role of T antigen in HSV-induced SV40 DNA amplification is not yet known, it is reasonable to assume that T antigen, with its binding activity for the SV40 ori, replaces the UL9 ori_S- and ori_L-binding activity in directing the viral multiprotein complex for DNA replication to the SV40 ori. This happens even in the presence of functional UL9 protein and ori_S DNA sequences. Cooperation between SV40 T antigen and the HSV replication machinery on the SV40 ori appears to be effective enough to compete with UL9-dependent replication of the authentic HSV template. We would assume a similar interaction between the HSV replication machinery and putative cellular ori-binding proteins for the amplification of cellular genes

starting from cellular replication origins. A recent report on the interaction of the cellular and HSV replication complexes (11) further supports this assumption.

Does the amplification-inducing potential of HSV play a role in the pathogenicity of the virus in vivo? The relevance of HSV-induced DNA amplification to the pathogenicity of the virus in vivo is still unclear for several reasons. Until now it has not been known whether HSV-induced DNA amplification can take place in the absence of lytic viral growth, thus leading to survival of the infected cell. This is, of course, the prerequisite of persistence of any HSV-induced genetic alteration within the infected cell. We show in this report that this distinction is possible. A subset of HSV replication genes that are not sufficient for the induction of viral DNA replication is necessary and sufficient for the induction of DNA amplification in integrated SV40 DNA sequences. In addition, this subset of genes does not encompass the HSV genes responsible for the early phase of HSV-induced host cell shut-off, involving a general and unspecific inhibition of host cell mRNA and protein synthesis (reviewed in reference 14). This early host cell shut-off function is mediated by a virion component (28) and could be assigned to a single viral gene (UL41) (29, 40) that is unrelated to the genes identified in this report as inducing DNA amplification. The identified set of amplification-inducing HSV genes is clearly separable from lytic viral functions, e.g., early host cell shut-off, and not sufficient for viral DNA replication. In addition, IE gene effects other than transactivation of early genes do not appear to play a major role in DNA amplification. We are now in the position to directly test whether the six identified HSV genes can indeed induce DNA amplification of cellular genes. So far, the only indirect evidence of HSV-induced DNA amplification of cellular genes has been the appearance of double minute chromosomes, recognized cytogenetic hallmarks of DNA amplification (8). The direct demonstration of HSV-induced amplification of cellular genes could be of considerable importance for the pathogenicity of the virus in vivo. Since very little is known about viral gene expression during HSV latency and reactivation, we do not know whether the set of amplification-inducing genes is ever expressed separately in vivo. If this is the case, expression of these genes could eventually lead to persistence of a genetically altered cell, with possibly deleterious effects for the affected host. The analysis of gene expression of the set of HSV amplification genes during latency and reactivation will be an interesting topic for future studies.

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ADDENDUM IN PROOF

Since submission of the manuscript, a report has appeared that describes the purification of a three-subunit-enzyme complex with helicase and primase activities consisting of the genes UL5, UL8, and UL52 (J. J. Crute, T. Tsurumi, L. Zhu, S. K. Weller, P. D. Olivo, M. D. Challberg, E. S. Mocarski, and I. R. Lehman, Proc. Natl. Acad. Sci. USA **86**:2186–2189, 1989).

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