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A Subset of Herpes Simplex Virus Replication Genes Provides Helper Functions for Productive Adeno-Associated Virus Replication

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Herpesviruses are helper viruses for productive adeno-associated virus (AAV) replication. To analyze the herpes simplex virus type 1 (HSV-1) functions mediating helper activity, we coinfected HeLa cells with AAV type 2 (AAV-2) and different HSV-1 mutants defective in individual HSV replication genes. AAV replication was fully accomplished in the absence of HSV DNA replication and thus did not require expression of late HSV genes. In addition, HSV mutants lacking either the origin-binding protein or the functional DNA polymerase fully maintained the capacity to replicate AAV. Cotransfection of the cloned, replication-competent AAV-2 genome together with the seven HSV replication genes (UL5, UL8, UL9, UL29, UL30, UL42, and UL52) led to productive AAV replication. Cotransfections with different combinations of these genes demonstrated that a subset of four of them, coding for the HSV helicase-primase complex (UL5, UL8, UL52) and the major DNA-binding protein (UL29), was already sufficient to mediate the helper effect. Thus, the HSV helper activity for productive AAV replication seems to consist of DNA replication functions. This appears to be different from the helper effect provided by adenovirus, which predominantly modulates AAV gene regulation.

Adeno-associated viruses (AAVs) belong to the parvovirus family, a group of small single-stranded DNA viruses (4, 5). Three viral functions have been mapped on the 4.7-kb AAV type 2 (AAV-2) genome. The right-hand open reading frame codes for the three capsid proteins, and the left-hand open reading frame codes for Rep, a family of multifunctional nonstructural AAV proteins required for AAV gene expression and DNA replication. The 145-bp terminal repeat sequences serve as origins of replication.

Parvoviruses exhibit unique replication properties. Autonomous parvoviruses replicate independently in proliferating cells, whereas AAVs either integrate into the host cell genome or make use of helper viruses for productive replication (4). Since low-level AAV replication has also been observed after treatment of AAV-infected cells with chemical or physical carcinogens (22, 51, 59, 60), the replication defectiveness of AAV is not considered to be absolute. Helper viruses for efficient AAV replication include several adenovirus strains (5) and viruses of the herpesvirus group, such as herpes simplex virus (HSV) (6) and human cytomegalovirus (HCMV) (35). Coinfection with either varicella-zoster virus (17) or vaccinia virus (50) has been shown to induce AAV antigen synthesis without direct demonstration of de novo-synthesized infectious AAV. Likewise, different avian herpesviruses provide helper activity for avian AAV (1, 2). So far, only the helper functions provided by adenovirus have been characterized in some detail. The adenovirus early genes E1A, E1B, E2A, E4 and the virus-associated (VA) RNA I exhibit helper activity at different stages of AAV gene expression (reviewed in reference 4).

Although it is not clear at present how the human AAVs are replicated in vivo, adenoviruses as well as herpesviruses are assumed to play a role in the primary infection of AAV (17, 42). Although both adenoviruses (20, 38) and herpesviruses (45) can establish latency after primary infection, regular in vivo reactivations from the latent state are inherent to herpesvirus infections only. During these episodes, herpesviruses may be able to reactivate AAV from the latent state. We therefore decided to analyze herpesvirus functions with helper activity for AAV replication. Except for one report defining a small HSV type 1 (HSV-1) DNA fragment that appeared to support AAV antigen synthesis (53) and the finding that an HSV temperature-sensitive mutant for the polymerase gene supported AAV synthesis like wild-type AAV (13), the herpesvirus genes providing the full helper effect for AAV have not been characterized in detail.

Two different approaches have been used to identify the HSV genes involved in HSV replication. First, temperature-sensitive mutants and null mutants, generated by targeted insertional mutagenesis of specific HSV genes, were tested for HSV DNA synthesis (reviewed in reference 56). In an alternative approach, the transfection of cloned HSV DNAs demonstrated that seven HSV early genes provided the necessary *trans* functions for the replication of the cotransfected HSV origins of replication, ori_S and ori_L (9, 58). The HSV replication genes code for enzymes and factors directly involved in viral DNA replication (33), namely, the HSV DNA polymerase (encoded by the UL30 gene), the single-stranded (major) DNA-binding protein (UL29), a double-stranded DNA-binding protein (UL42) (15, 16, 19, 43), an origin-binding protein (UL9) (14, 40), and a complex of three tightly associated proteins (UL5, UL8, UL52) with helicase and primase activity (11). In previous studies, we have shown that a subset of six of seven HSV replication genes induced DNA amplification within the host cell genome (24) and that this was completely suppressed by the AAV *rep* gene (21). Furthermore, *rep* concomitantly reduced HSV DNA replication (21). Since these studies indicated an interaction between the HSV replication genes and the AAV replication function *rep* (25, 54), we decided to directly test these HSV genes for helper activity. By the use of HSV

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mutants defective in individual replication genes and by cotransfection of expression plasmids for these genes, we show here that a subset of the HSV replication genes provides helper activity for productive AAV replication.

MATERIALS AND METHODS

Recombinant plasmid DNAs. The AAV-2 wild-type genome cloned into pBR322 (pAV2) was kindly provided by C. Laughlin (29). The series of plasmids carrying the open reading frames of the HSV-1 replication genes (UL5, UL8, UL9, UL29, UL30, UL42, and UL52) under the control of the HCMV immediate-early enhancer-promoter (pCM-UL5, -UL8, -UL9, -DBP, -POL, -UL42, and -UL52) have been described before (24). Plasmids were purified by the alkaline lysis procedure (30) with two subsequent gradients in CsCl.

Cells and viruses. HeLa and Vero cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10 or 5% heat-inactivated (30 min, 56°C) fetal calf serum, respectively, antibiotics, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.2).

AAV-2 was propagated in HeLa cells with adenovirus type 2 as the helper, as described previously (60). The HSV-1 wild-type strains KOS and 17 syn^+ were propagated and titrated by plaque assay in Vero cells as described previously (32). HSV mutant *ts*H (temperature-sensitive mutation in the DNA polymerase gene) is derived from HSV-1 strain 17 syn^+ and was propagated in Vero cells at the permissive temperature (33°C), as described previously (10, 31). The HSV null mutants *hr*80 (disrupted UL8 gene) (8), *hr*94 (disrupted UL9 gene) (23, 56), *d*21 (disrupted UL29 gene) (41), and *hr*114 (disrupted UL52 gene) (18) are derived from HSV-1 strain KOS and were a kind gift of S. K. Weller and P. A. Schaffer. They were propagated on helper cell lines which provide the defective functions in *trans*. The virus stocks of the null mutants were tested for the presence of contaminating wild-type virus by performing a plaque assay with Vero cells which are not permissive for the mutants (Table 1).

Infections. HeLa cells were infected in suspension with AAV-2 (multiplicity of infection [MOI] = 2) for 1 h at 37°C, medium was added, and 2×10^6 cells were seeded into 6-cm-diameter dishes. Twenty to 28 h later, cells were infected with HSV-1 wild type or mutants (MOI = 2) for 1 h and subsequently supplied with 2 ml of growth medium. After another 40 to 48 h, newly synthesized AAV was harvested by three cycles of freeze-thawing.

Transfections. Transfections were performed by the calcium phosphate transfection protocol as described before (24). In brief, 2×10^6 HeLa cells were plated onto 10-cm-diameter dishes. The next day, fresh medium was supplied, and 2 to 4 h later, cells were transfected with 20 μg of plasmid DNA per dish. The amounts of the individual plasmid DNAs transfected were as indicated in the figure legends and, if necessary, supplemented with vector DNA to a total of 20 μg. The cells were treated for 4 min with 25% dimethyl sulfoxide 3 to 4 h later. The next day, cells were refed with 3 ml of growth medium and were analyzed 40 to 48 h after transfection for AAV DNA replication and de novo AAV synthesis.

AAV replication assay. Infected or transfected cells were lysed by three freeze-thaw cycles. The disrupted cells were pelleted and analyzed for AAV DNA replication. Genomic DNA was extracted by digestion with proteinase K, repeated extractions with phenol and chloroform, digestion with RNase A, and dialysis against Tris-EDTA buffer. For titration of infectious AAV, freeze-thaw supernatants were heat treated (56°C for 30 min) to inactivate helper virus (HSV) and then inoculated in 1:10 dilution steps on fresh HeLa cells grown on 96-well plates. Cells were infected with HSV-1 as a helper (MOI = 10) 20 to 24 h later. Finally, 40 to 48 h after HSV infection, cells were lysed by three rounds of freeze-thawing, sucked onto GeneScreen membranes (NEN, Boston, Mass.), denatured, and hybridized with ^{32}P-labeled AAV-2 DNA.

RESULTS

Helper activity of HSV-1 mutants for AAV-2 propagation. HSV-1 and HSV-2 have been shown to act as helper viruses for AAV replication with an efficiency similar to that of adenoviruses (6). Under the assumption that the HSV replication genes could represent helper functions for AAV, HeLa cells were infected with AAV-2 (MOI = 2) and 20 to 24 h later with different HSV mutants (MOI = 2) carrying defects in individual replication genes (Fig. 1). Forty to 48 h later, infected cells were processed for the determination of newly synthesized AAV. Supernatants of heat-inactivated freeze-thaw lysates were titrated in duplicate on fresh HeLa cells with HSV-1 as a helper virus. The AAV titer was analyzed by hybridization of membrane-bound denatured cells with ^{32}P-labeled AAV DNA.

The HSV wild-type strains KOS and 17 syn^+ yielded an AAV titer of 6.7×10^7 infectious particles per ml of supernatant (Fig. 2, KOS wt, 17 syn^+ wt), in accordance with published data (6). HSV mutants *hr*94 (Fig. 2, ΔUL9) and *ts*H (Fig. 2, ts pol) induced an AAV titer similar to that induced by HSV wild type. This is in agreement with data previously published by Drake et al. (13) using another temperature-sensitive mutant for the HSV polymerase gene. With the null mutants *hr*80 (Fig. 2, ΔUL8), *d*21 (Fig. 2, Δdbp), and *hr*114 (Fig. 2, ΔUL52), the AAV titer dropped to 6.7×10^3, 6.7×10^5, and 3.7×10^6 infectious AAV particles per ml of lysate, respectively, suggesting a role for these genes in providing helper functions for AAV replication. All mutant virus stocks had been tested for possible wild-type contamination (Table 1), which was found to be low enough that it could not explain the observed helper effect for AAV. In addition, to exclude the possibility that the temperature-sensitive HSV mutant *ts*H was leaky at the nonpermissive temperature (39.5°C), *ts*H was plaque assayed on Vero cells at both 33 and 39.5°C in parallel with the coinfection experiment. At 33°C, the titer was 3×10^5 PFU, whereas no plaques could be detected at 39.5°C (Table 1). This difference in plaquing efficiency at the permissive versus nonpermissive temperature suggests that AAV can be efficiently replicated in the absence of an enzymatically functional HSV DNA polymerase.

From the above findings, three conclusions can be drawn. First, HSV DNA replication and subsequent expression of late genes does not appear to be necessary for AAV replication, since the replication-defective HSV-1 mutants for the origin-binding protein (*hr*94) as well as for the DNA polymerase (*ts*H) exhibited a helper effect for productive AAV replication similar to that of wild-type HSV. In addition, neither UL9 nor the HSV DNA polymerase themselves appear to play an essential role as helper genes. The reduced levels of AAV production with HSV mutants defective in the replication gene UL8, UL29, or UL52 suggest that these genes are directly required for AAV replication.

Identification of individual HSV-1 genes with helper activity

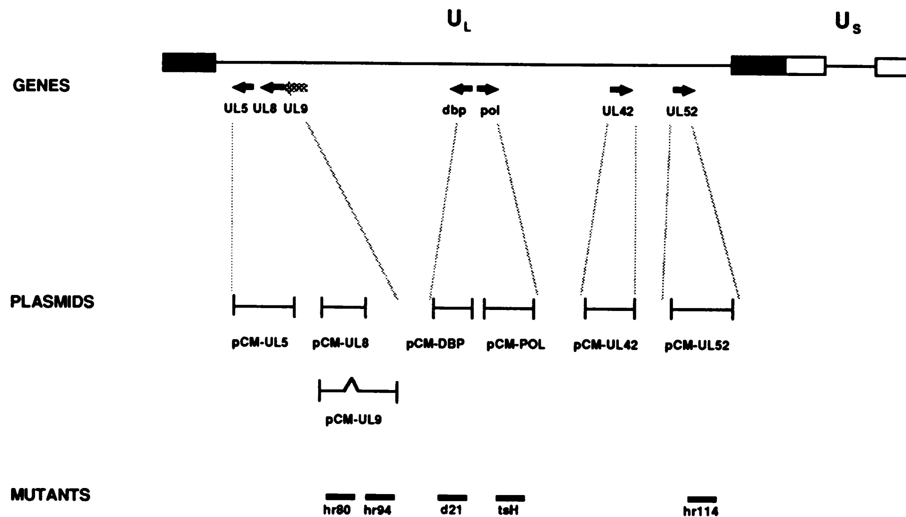

FIG. 1. Schematic representation of the 153-kb HSV-1 genome. The unique long (U_L) and unique short (U_S) regions are flanked by inverted repeats (filled boxes for the long region and open boxes for the short region). The relative positions of the HSV replication genes are indicated below: UL5, UL8, UL9, *dbp* (major DNA-binding protein [UL29]), *pol* (DNA polymerase [UL30]), UL42, and UL52. The arrows indicate the direction of transcription. Black arrows represent genes which are necessary for both HSV DNA replication and host cell DNA amplification (UL5, UL8, *dbp*, *pol*, UL42, and UL52). The grey arrow represents UL9, which is necessary only for HSV DNA replication. The plasmid series representing the HSV replication genes under the control of the heterologous HCMV immediate-early enhancer-promoter (pCM series of expression constructs) is given below. The bottom line indicates the HSV-1 mutants tested for AAV helper activity. The mutants *hr*80, *hr*94, *d*21, and *hr*114 are null mutants in the HSV genes UL8, UL9, *dbp* (UL29), and UL52, respectively, whereas *ts*H carries a temperature-sensitive mutation in the HSV DNA polymerase gene.

for AAV-2 replication. To test the role of the HSV replication genes for AAV replication separately, we transfected subsets of expression constructs for these genes driven by the heterologous HCMV immediate-early promoter into HeLa cells together with cloned replication-competent AAV-2 DNA (pAV2). Cells were harvested 48 h after transfection for the determination of AAV DNA replication by Southern blot analysis (Fig. 3A) and, in parallel, production of infectious AAV particles (Fig. 3B). Blots of genomic DNAs doubly digested with *Bgl*II and *Dpn*I were hybridized to ^{32}P-labeled AAV-2 DNA. *Dpn*I digests the input DNAs, whereas AAV molecules replicated in eukaryotic cells are resistant to *Dpn*I and appear as the typical replicative intermediates of AAV.

Transfection of the seven HSV replication genes (pCM-UL5, -UL8, -UL9, -DBP, -POL, -UL42, -UL52) and pAV2 led to AAV DNA replication (Fig. 3A, lanes all) and to de novo synthesis of infectious AAV (Fig. 3B, lane all). On a short exposure of the Southern blot, the typical replicative intermediates are visible (data not shown). The 4.65-kb band seen upon transfection of cloned AAV-2 DNA alone (Fig. 3A, AAV-DNA) represents double-stranded monomer AAV-2 (RF1), possibly generated by second-strand synthesis on single-strand AAV templates, thus rendering the double strand *Dpn*I resistant. However, neither higher oligomeric replicative intermediates nor infectious progeny were seen (Fig. 3B, AAV-DNA).

To test the role of individual HSV replication genes, we transfected pAV2 together with the seven HSV replication genes, omitting one of them at a time. Omission of pCM-UL9, coding for the HSV origin-binding protein, did not hamper AAV DNA replication or particle synthesis (Fig. 3A and B, no UL9). This is in line with the result of the coinfection of AAV and the UL9-defective HSV mutant, which had already suggested that UL9 was not required for AAV replication. Omission of plasmids coding for either the DNA polymerase (pCM-POL) or the polymerase accessory protein (pCM-UL42) allowed AAV DNA replication and particle synthesis, albeit to a slightly reduced level (Fig. 3A and B, no pol, no UL42). This reduction was not observed after coinfections of AAV and the temperature-sensitive HSV mutant for the DNA polymerase (*ts*H) (Fig. 2, ts pol). This may be due to the fact that *ts*H synthesizes an enzyme whose polymerase activity is impaired at the nonpermissive temperature but in which other functions, such as a potential protein-protein interaction in a multienzyme complex, may still remain intact. Since the HSV DNA polymerase is known to interact not only with UL42 (15, 19) but also with the major DNA-binding protein (39), it is conceivable that omission of pCM-POL or pCM-UL42 from the set of cotransfected genes would interfere with the function of other replication factors acting in a complex with the HSV DNA polymerase and its accessory protein (UL42).

In contrast to the HSV polymerase, the major DNA-binding protein appeared to be essential for AAV replication, since omission of pCM-DBP from the cotransfected DNAs allowed neither AAV particle synthesis nor AAV DNA replication (Fig. 3A and B, no dbp). Omission of either pCM-UL5 or pCM-UL8 from the set of cotransfected replication genes strongly reduced AAV DNA replication and particle synthesis (Fig. 3A and B, no UL5, no UL8). Omission of pCM-UL52 resulted in an intermediate phenotype leading to slightly reduced AAV DNA replication and particle synthesis (Fig. 3A and B, no UL52). This points to an essential role of the HSV helicase-primase complex composed of UL5, UL8, and UL52 in AAV replication and is in line with the reduced AAV titers obtained with the UL8- or the UL52-defective HSV mutant (Fig. 2).

Since the HSV replication genes UL5, UL8, UL52, and the gene coding for the major DNA-binding protein appeared to play an essential role as helper functions for AAV replication, they were tested together. AAV DNA replica-

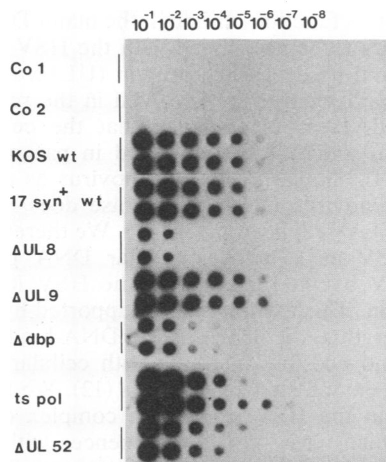

FIG. 2. AAV-2 helper activity of HSV-1 mutants defective in individual replication genes. HeLa cells were infected with AAV-2 (MOI = 2) and 24 h later with HSV-1 wild type or HSV-1 mutants as indicated (MOI = 2 PFU). Cells were harvested 48 h later by three freeze-thaw cycles. Heat-inactivated cell-free supernatants (15 μl) were titrated in duplicate in 1:10 dilution steps on HeLa cells grown in 96-well microtiter plates. Multiplication of the mean value of the indicated dilution steps by 67 leads to the actual AAV titer per milliliter of lysate. Cells were infected with HSV-1 20 h later. After another 48 h, cells were sucked onto a Gene Screen filter and hybridized to ^{32}P-labeled AAV-2 DNA. Supernatants from noninfected cells (Co 1) or from cells only infected with AAV-2 (Co 2) served as negative controls. KOS wt, HSV-1 wild-type strain KOS; 17 syn$^+$ wt, HSV-1 wild-type strain 17 syn$^+$ (incubated at 39.5°C); ΔUL8, HSV-1 null mutant *hr*80 deficient in the UL8 gene; ΔUL9, HSV-1 null mutant *hr*94 deficient in the UL9 gene; Δdbp, HSV-1 null mutant *d*21 deficient in the gene for the major DNA-binding protein (UL29); ts pol, HSV-1 mutant *ts*H with a temperature-sensitive mutation in the DNA polymerase gene (UL30), grown at the nonpermissive temperature (39.5°C); ΔUL52, HSV-1 null mutant *hr*114 deficient in the UL52 gene.

tion and the synthesis of infectious particles could be initiated by cotransfecting these four genes together with pAV2 (Fig. 3A and B, no UL9/pol/UL42, and Fig. 4A and B, UL5 + UL8 + UL52 + dbp). Further analysis demonstrated that omission of any one of these genes abolished AAV replication entirely (Fig. 4A and B). Similarly, no AAV replication could be detected when the genes UL5, UL8, UL29, and UL52 were transfected individually (data not shown). These experiments were repeated with AAV-infected cells, in which the AAV template DNA has the authentic configuration. Upon subsequent transfection of the HSV replication genes, similar results were obtained (data not shown). Thus, the requirement for a given HSV helper gene is not due to the fact that the cloned AAV genome enters the cell as a double-stranded genome without the proper origin conformation.

Taken together, these experiments demonstrate (i) that the HSV replication complex is sufficient to induce de novo AAV particle synthesis; (ii) that a subset of four HSV replication genes, namely, UL5, UL8, UL29, and UL52, is sufficient to allow the production of infectious AAV progeny almost to the level obtained with all seven genes; and (iii) that there is a strong correlation between AAV DNA replication and the synthesis of infectious particles.

TABLE 1. Determination of wild-type contamination of HSV mutants

Virus	Mutant gene	Wild type/mutant[a]
*hr*80	UL8	$<1/10^6$
*hr*94	UL9	$21/10^6$
*d*21	UL29 (*dbp*)	$7/10^6$
*hr*114	UL52	$37/10^6$
*ts*H[b]	UL30 (*pol*)	$<1/10^5$

[a] Plaquing frequencies were measured by determining the titers of mutant virus stocks on the respective helper cell lines providing the defective functions in *trans* (see Materials and Methods) and, in parallel, on nonpermissive Vero cells on which only the wild type would grow.

[b] Plaquing efficiency of the temperature-sensitive mutant was determined on Vero cells at the permissive (33°C) and non permissive (39.5°C) temperatures.

DISCUSSION

In this report, we describe the identification of HSV helper functions for the de novo synthesis of infectious AAV. Although we cannot exclude the possibility that additional HSV genes play a role in the full helper effect for AAV replication, we clearly show here that a subset of HSV replication genes is sufficient to induce productive AAV replication in the absence of HSV DNA replication. The HSV helper genes code for the major DNA-binding protein and a multienzyme complex composed of the genes UL5, UL8, and UL52. Neither the HSV DNA polymerase together with its accessory protein (UL42) nor the HSV origin-binding protein (UL9) are required for AAV replication.

UL9, however, is absolutely required for HSV DNA replication (7, 23, 56, 58). It appears to initiate HSV DNA replication by specifically binding to the HSV origins of replication, ori_S and ori_L, thus directing the HSV replication complex to the correct initiation sites (14, 40). Since there is no significant sequence homology between the HSV origins ori_S and ori_L, on one hand, and the AAV origin, on the other hand, it appears plausible that an HSV mutant for UL9 provided a full helper effect for AAV replication similar to wild-type HSV. Omission of UL9 from the set of transfected replication genes even caused an increase in AAV production (Fig. 3), which may be explained by competition between HSV and AAV DNA for the HSV replication complex in the presence of UL9. For AAV DNA replication, the AAV *rep* gene is also required in the presence of HSV as the helper (21), and it was recently shown that Rep68 directly binds to an AAV origin promoting the first steps in AAV replication (26, 52). The AAV origin of replication comprises a hairpin structure which is assumed to prime AAV DNA replication (47, 48). Rep68 not only binds to this hairpin but is also directly involved in several of the early steps in AAV DNA replication. Rep68 is an ATP-dependent site-specific endonuclease and unwinds the self-primed AAV origin by its helicase activity (26). In the light of these findings, it is difficult to understand why AAV needs the HSV helicase-primase complex for its replication. However, it is entirely possible that there exist as yet undefined additional functions associated with this three-subunit enzyme complex which may turn out to be more relevant for AAV replication. The role of the HSV major DNA-binding protein for AAV DNA replication appears more obvious. The major DNA-binding protein binds single-stranded DNA and is assumed to hold the replication fork in an extended configuration (46). The replication of the single-stranded AAV genome may well be

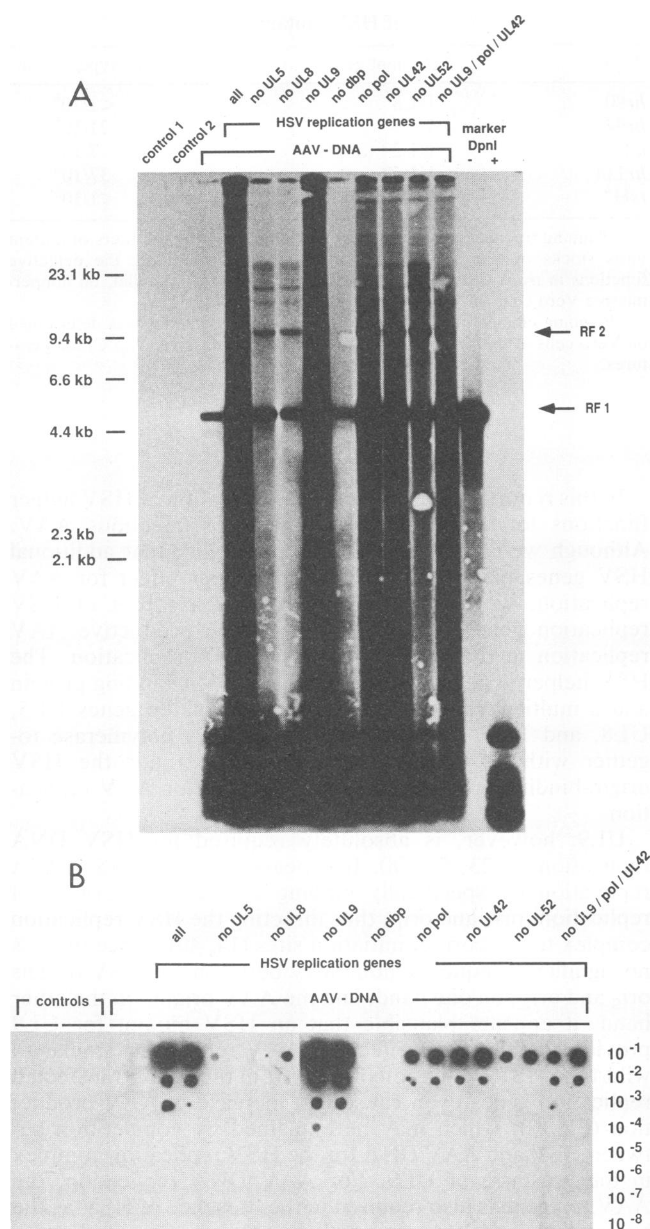

FIG. 3. Identification of individual HSV-1 replication genes with helper activity for AAV-2 synthesis. HeLa cells were transfected with replication-competent cloned AAV-2 DNA (pAV2) and different combinations of cloned HSV replication genes as indicated below. Cells were harvested 48 h posttransfection by three freeze-thaw cycles, and the disrupted cells were pelleted. Genomic DNA was extracted from the pellet for a Southern blot analysis (A). Cell-free supernatants were used for titration of infectious AAV-2 particles (B). (A) Southern blot of *Bgl*II and *Dpn*I doubly digested genomic DNA (5 μg per lane) hybridized to ^{32}P-labeled AAV-2 DNA. The replicative intermediates are indicated: RF1 (4.65 kb) represents the double-stranded monomer, and RF2 (9.3 kb) is the double-stranded dimer. In addition, higher oligomeric forms are visible. The bands in the low-molecular-weight range represent *Dpn*I-digested input DNAs which indicate comparable transfection efficiencies in all samples. The following combinations of DNAs were transfected: Co 1, 20 μg of Bluescribe; Co 2, pCM-UL5, -UL8, -UL9, -DBP, -POL, -UL42, -UL52, 2.5 μg of each; AAV-DNA, 2 μg of pAV2; HSV replication genes, pCM-UL5, -UL8, -UL9, -DBP, -POL, -UL42, -UL52, 2.5 μg of each. Sensitivity markers are 125 pg of *Bgl*II-digested or 250 pg of *Bgl*II and *Dpn*I doubly digested pAV2, respectively. (B) Titration of de novo-synthesized infectious AAV-2 particles. Supernatants (15 μl) were titrated in duplicate in 1:10 dilution steps on HeLa cells grown in 96-well plates. After 24 h, the cells were infected with HSV-1 (MOI = 10), and 40 h later, they were sucked onto a Gene Screen filter and hybridized to ^{32}P-labeled AAV-2 DNA.

facilitated by this function, although the major DNA-binding protein appears to be associated with the HSV DNA polymerase (39) and its accessory protein (UL42) (15, 16, 19), neither of which seem to be involved in the replication of AAV. Instead, there is evidence that the cellular DNA polymerases α and/or δ are involved in parvovirus DNA replication (44). Similarly, with adenovirus as a helper for AAV, the adenovirus DNA polymerase does not seem to play a role in AAV replication (34, 37). We therefore assume that with HSV as a helper, cellular DNA polymerases replicate AAV by interacting with the HSV major DNA-binding protein. This assumption is supported by the recent demonstration that the HSV major DNA-binding protein colocalizes and possibly interacts with cellular replication complexes in the infected cell nucleus (12). We have shown previously that the HSV replication complex can amplify integrated simian virus 40 DNA sequences within the host cell chromosome, where the simian virus 40 T antigen is assumed to direct the HSV replication machinery toward the simian virus 40 origin (24). In the light of those findings and those described above, it appears that the components of the HSV replication machinery are capable of interacting with other viral or cellular replication factors on several different templates. These may be viral replication origins, as in simian virus 40 or AAV, but possibly also cellular replication origins from which DNA amplification within the host cell genome is assumed to start.

A recent report by Mishra and Rose (36) described a similar experimental approach which identified a different subset of the HSV replication genes (UL5, UL8, UL9, UL29, and UL30) as helpers for AAV DNA replication. Since these investigators never tested the set of seven HSV replication genes separate from the rest of the HSV genome, did not measure AAV virus production, and did not control their results by the use of HSV mutants, the two studies cannot be compared directly. It is difficult to understand how individual components of the HSV helicase-primase complex (UL5, UL8, and UL52) (11) on the one side and the complex of DNA polymerase (UL30) and its accessory protein (UL42) (15, 16, 19) on the other side could function separately. In addition, our study clearly shows that UL9 is not required. Whatever experimental difference led to the conclusions drawn, we have no evidence from our experiments (24, 55a) which would suggest that the use of different cell types (Vero versus HeLa) or promoters for the transfected HSV genes (authentic HSV promoters versus heterologous strong HMCV promoters) plays a crucial role.

Although the roles of the HSV helper genes may appear to be well defined, so far it is still difficult to fully understand the HSV helper effect for productive AAV replication. All HSV helper functions identified in this study are replication enzymes or factors (56). However, these proteins seem to be capable of doing more than just replicate the AAV template. In our study, the extent of AAV DNA replication always correlated with the amount of infectious progeny produced. From our earlier studies employing carcinogen treatment as

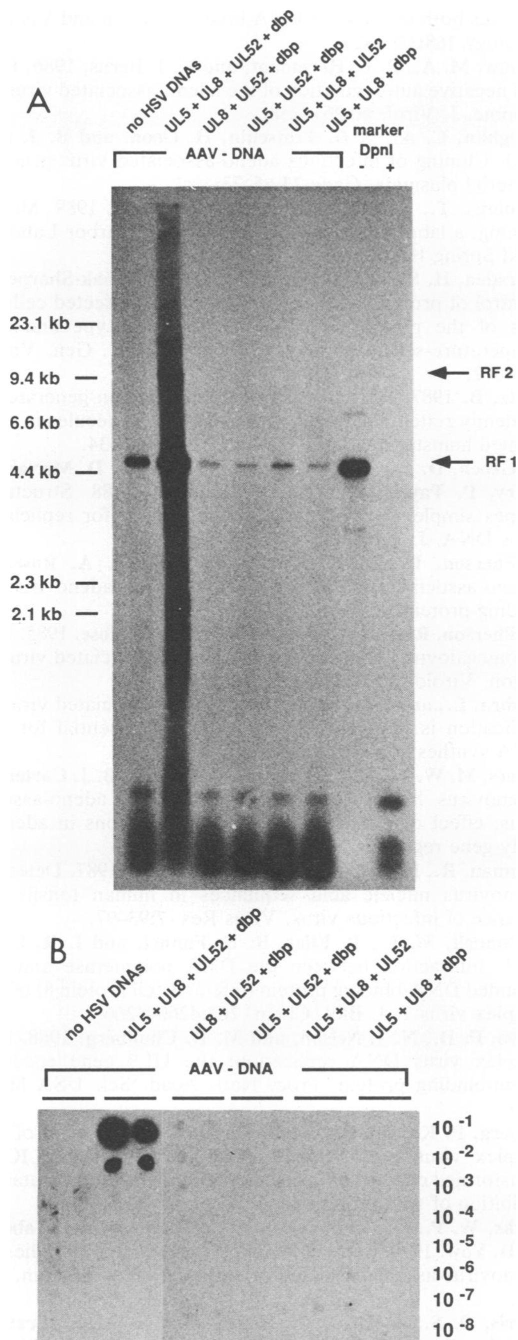

FIG. 4. The HSV helicase-primase complex and the major DNA-binding protein are sufficient for AAV synthesis. The experiment was performed as described in the legend to Fig. 3. (A) Southern blot analysis was performed for the detection of AAV replicative intermediates. (B) Cell extracts were titrated in duplicates in 1:10 dilution steps to detect de novo synthesis of infectious AAV-2 particles.

the inducer of AAV replication, we know that, although AAV DNA replication could be induced to a high level in a variety of cell lines, it proved difficult to demonstrate infectious viral progeny, which was, in addition, restricted to some specific cell lines (59, 60). These data suggest that the mere induction of AAV DNA replication does not necessarily mean that the AAV infectious cycle can progress without additional helper functions. From studies with adenovirus as a helper, it is clear that this virus group provides helper activity at several steps in AAV gene expression. In contrast to HSV, the adenovirus proteins that are directly involved in adenovirus DNA replication, the adenovirus DNA polymerase, the DNA-binding protein, and the terminal protein, do not appear to be essential for AAV DNA replication (4). Instead, adenovirus early genes are involved in the coordinated transactivation or repression of AAV promoters (3, 28, 55). Posttranscriptional regulation mechanisms have also been proposed (27, 49, 57). Whether HSV provides similar, as yet unidentified functions remains an unresolved question at present. Since the adenovirus single-stranded DNA-binding protein not only appears to play a role in DNA replication but also affects gene expression, it would be interesting to look for a similar dual function of the HSV single-stranded DNA-binding protein (UL29). To our knowledge, however, there is no indication so far that the HSV helper genes which we have identified may have functions apart from those involved in DNA replication. Since the replication functions of these genes are well characterized (reviewed in reference 56), it is reasonable to assume that these genes provide helper activity directly for AAV DNA replication. Further biochemical studies will add to the full understanding of the underlying molecular mechanisms. It appears that efficient AAV replication can be achieved by different molecular mechanisms which are induced by herpesviruses, adenoviruses, or different drugs. The elucidation of these mechanisms may also provide insights into the mechanisms used to reactivate AAV from latency. Since AAV can be replicated under specific conditions in the absence of helper viruses (59, 60), the study of viral helper functions may also lead to the identification of related cellular activation pathways which may be relevant for the propagation of AAV in vivo.

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