Identification, Transfer, and Characterization of Cloned Herpes Simplex Virus Invasiveness Regions

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Following peripheral inoculation of experimental animals, herpes simplex virus type 2 (HSV-2) strains are more virulent than HSV-1 strains, and clinical studies suggest that they possess enhanced virulence in humans. One dramatic type-specific difference in virulence is observed following inoculation of the chorioallantoic membrane (CAM) of the chicken embryo: HSV-2, but not HSV-1, makes large pocks on the CAM, invades the mesoderm, generalizes in the embryo, and kills the chicken. These properties have been believed to be specific for HSV-2, and their molecular basis is unknown. We now report that an HSV-1 strain, ANG, behaves in this system like HSV-2 strains, making large, invasive pocks, generalizing, and ultimately killing even more efficiently than HSV-2. In addition, we have transferred restriction fragments of ANG DNA to another HSV-1 strain, 17 syn$^+$, conferring the CAM virulence phenotype on the normally CAM-avirulent 17 syn$^+$. Like ANG, these recombinant viruses are 10^6-fold more virulent (PFU/50% lethal dose [LD$_{50}$] ratio, $\leq 10^2$) than the parental 17 syn$^+$ strain (PFU/LD$_{50}$ ratio, $\geq 10^8$). A molecularly cloned library of ANG DNA was used to identify two distinct regions containing the virulence functions. Transfer of sequences contained in either cloned ANG *Eco*RI fragment A (0.49 to 0.64 map units) or F (0.32 to 0.42 map units) DNA to 17 syn$^+$ confers CAM virulence, whereas other cloned regions of the ANG genome do not. Using cloned DNA, we derived and plaque purified several virulent recombinant viruses with inserts from either the ANG *Eco*RI fragment A (INV-I) or F (INV-II) areas. In each instance, the transfer of the cloned INV-I or INV-II sequences enhanced virulence for the chicken embryo 10^6-fold (PFU/LD$_{50}$ ratio, $\leq 10^2$). In addition, the transfer of the cloned ANG *Eco*RI-F INV-II sequences resulted in a 10^3-fold enhancement of neuroinvasiveness and virulence for mice. Following footpad inoculation, these recombinants kill mice with a PFU/LD$_{50}$ ratio of approximately 10^3 (similar to HSV-2 strains) compared with 10^6 for 17 syn$^+$. Thus, we have identified, cloned, and transferred two DNA regions from HSV-1 ANG which contain virulence genes (INV-I and INV-II) important in mesodermal invasiveness on the CAM and, in the case of INV-II, neuroinvasiveness in the mouse. In each instance, the recombinant HSV-1 viruses have attained enhanced virulence beyond that described for HSV-1 strains and similar to that seen with HSV-2. Possible explanations for the existence of these two distinct virulence functions in ANG include selection for a unique mesodermal growth-enhancing function or selection for a mutation conferring CAM virulence by another mechanism. In either case, the sequences responsible could be either unique for HSV-1 or related to those conferring the similar phenotype upon HSV-2. These could arise either through mutation of HSV-1 or through in vivo recombination with HSV-2.

Identification of genes specifically involved in herpes simplex virus (HSV) pathogenesis has been difficult, since the large HSV genome encodes 70 gene products, most with unknown functions. In addition, the natural history of infection is complex. Although host resistance is important in determining disease outcome, HSV strains themselves can vary with respect to their pathogenic properties. Thus, laboratory-passaged (and often intentionally genetically altered) as well as clinical isolates exist with altered virulence in the animal host (8). Pathogenically defective HSV strains have recently been used to identify a number of genomic areas encoding gene products required for full virulence (2, 5, 33, 34).

Researchers in our laboratories have been interested in identifying genomic areas involved in HSV invasiveness and subsequent generalization in the host. In particular, we have been interested in the enhanced invasiveness of HSV-2

compared with HSV-1. In general, HSV-2 strains are 100- to 1,000-fold more virulent after peripheral inoculation of mice or rabbits (21, 23). In addition, they appear to cause viremia (7) and severe disseminated disease in human neonates (6) and adults more frequently (J. Goodman, manuscript in preparation) and are more efficient at reactivating and causing symptomatic disease in the human genital tract (15).

We have observed a dramatic difference in type-specific viral invasiveness following inoculation of HSV on the chorioallantoic membrane (CAM) of fertile chicken embryos. Over 50 years ago, Goodpasture et al. first demonstrated that HSV could produce pocks on the CAM (11). Once it was appreciated that there were two antigenically distinct HSV strains, Nahmias et al. noted that HSV-2, but not HSV-1, makes large, necrotic pocks which invade the CAM mesoderm and provoke a marked inflammatory response (22). Rodgers (27) and subsequently Goodman and Stevens (10) found that HSV-2, but not HSV-1, can cause generalized infection after CAM inoculation and kill the

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embryo, and we found a 10^6-fold superiority in virulence for HSV-2 as measured by PFU/50% lethal dose (LD_{50}) ratios (10).

Through marker rescue techniques we have attempted to transfer HSV-2 DNA into HSV-1 to identify the HSV-2 sequences(s) responsible. To date, we have been unsuccessful. However, we now report that an HSV-1 strain, ANG, makes large pocks on the CAM, invades the mesoderm, generalizes in the chicken embryo, and kills even more efficiently than does HSV-2. Through intratypic recombination of viral DNAs by cotransfection, we have been able to transfer fragments of the ANG genome and confer the full virulence phenotype on the normally CAM-avirulent HSV-1 strain 17 syn$^+$. Furthermore, we have used molecularly cloned ANG DNA to show that two different ANG DNA fragments, EcoRI fragments A (0.49 to 0.64 map units m.u.) and F (0.32 to 0.42 m.u.), contain unique transferable virulence properties. Each virulence region enhances killing of the chicken embryo 10^6-fold, and the EcoRI F fragment additionally enhances neuroinvasiveness in the mouse to a level similar to that of HSV-2 strains. Thus, transferable HSV DNA sequences have been discovered which enhance a virulence property beyond that usually observed with HSV-1 strains.

MATERIALS AND METHODS

Viruses. Strains used included reference HSV-1 and HSV-2 strains 17 syn$^+$ and HG52, respectively (10; kindly provided by J. Subak-Sharpe, Institute of Virology, Glasgow, Scotland); ANG (14, 20; kindly provided by H. C. Kaerner, Institute for Virus Research, Heidelberg, Federal Republic of Germany); MP (12); ts$^+$ syn, a syncytial mutant derived from 17 syn$^+$; 17 hep syn, a syncytial mutant derived from 17 syn$^+$ by passage in heparin and mapping to the glycoprotein B (gB) syn 3 region (the 0.4-kilobase [kb] KpnI-BamHI fragment spanning 0.348 to 0.351 m.u.; J. Engel and J. Goodman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, S38, p. 321, and manuscript in preparation); and freshly isolated genital tract HSV-1 strains obtained from the Clinical Virology Laboratory, University of Minnesota. Syn$^+$ denotes that a virus is nonsyncytial, whereas strains called syn are syncytial.

Cells. All virus stocks were grown in rabbit skin (RS) cells maintained in minimal essential medium with 5% calf serum (10). Primary chicken embryo fibroblasts (CEFs) were prepared by repeated trypsinization of 10-day-old chicken embryos and were maintained in MEM–10% fetal calf serum and 10% tryptose phosphate broth. Secondary cells were prepared by trypsinization and used for studies of viral replication kinetics when just confluent.

Virus titers were determined as described previously (10), and plaque purifications were performed by limiting dilution in RS cells.

Experimental animals. Marek-disease-free, specific-pathogen-free eggs were obtained from SPAFAS, Inc., Roanoke, Ill., and maintained in a humidified environment at 39°C for 10 days prior to inoculation. A 0.1-ml portion of virus was inoculated onto the CAM by using the false-air-sac technique (11), and the eggs were maintained at 37°C and candled daily to determine viability (10). Outbred Swiss Webster mice were obtained from Simonsen, Gilroy, Calif.

Molecular methods. Viral DNA was isolated from fully infected RS cells by using ultracentrifugation in sodium iodide gradients (35) followed by extensive dialysis against 1

mM Tris (pH 7.4)–0.1 mM EDTA. The DNA was quantitated and checked for purity both by UV absorbance and on agarose minigels.

Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs Inc., Beverly, Mass.; or Boehringer Mannheim Biochemicals, Indianapolis, Ind.; they were used as specified by the manufacturers. Digested DNA was examined electrophoretically by separations in 0.6 to 0.8% agarose in TPE buffer and stained with ethidium bromide (17). Nomenclature of the fragments was as described earlier for the prototype conformation of viral DNA (28).

For transfections, unit-length 17 syn$^+$ DNA was mixed with a two- to fivefold molar excess of either restriction endonuclease-cleaved fragments of genomic ANG DNA or cloned ANG DNA fragments, cotransfected in 60-mm petri dishes with calcium phosphate in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, and subjected to osmotic shock (34). When complete viral cytopathic effect was evident, progeny virus were harvested.

Stringent Southern blotting was performed as described previously (17) with endonuclease-cleaved DNA transferred to either nitrocellulose or Nytran (Schleicher & Schuell, Inc., Keene, N.H.) membranes and hybridized to nick-translated, cloned, excised, electroeluted, and [α-^{32}P]dCTP (3,000 Ci/mmol; Du Pont, NEN Research Products, North Billerica, Mass.)-labeled probes. The HSV fragments used both for probes and for localizing the virulence genes were cloned into pUC vectors (18) and carried in bacterial hosts Escherichia coli JM83, DH5, or HB101. Plasmids were propagated, the DNA was isolated, and inserts identified by standard methods (17). The specific cloned ANG probes used in these studies were EcoRI-A (0.49 to 0.64 m.u.), EcoRI-F (0.32 to 0.42 m.u.), BamHI-R (0.40 to 0.42 m.u.), and BamHI-O (0.57 to 0.60 m.u.). In addition, EcoRI and BamHI libraries of cloned ANG DNA fragments were made for the virulence studies.

RESULTS

Establishment of relevant biologic properties of HSV-1 ANG. Our initial observation that HSV-1 ANG could kill chicken embryos after CAM inoculation led us to systematically study the virus-host interaction in vivo. We found that when examined 3 days after infection, HSV-1 ANG, unlike other HSV-1 strains, made large pocks on the CAM similar to those seen with HSV-2. These pocks were visible with the naked eye and comparable in size to HSV-2 pocks while being considerably larger than those seen with HSV-1 17 syn$^+$. When the pocks were examined microscopically, the histopathology caused by ANG (Fig. 1A) also resembled that seen with HSV-2 (Fig. 1B). Pathologic changes including inflammation, hemorrhage, and necrosis extended through the CAM and involved the mesoderm, whereas those with HSV-1 17 syn$^+$ did not (Fig. 1C).

We next established PFU/LD_{50} ratios (26) for ANG by inoculation of serial 10-fold viral dilutions on the CAM. Its PFU/LD_{50} ratio is $\leq 10^{1.7}$, and this reflects a consistently higher virulence than that of HSV-2 HG52 ($10^{2.3}$) or 333 (10^2; data not shown) and 10^6-fold-higher virulence than that of prototype HSV-1 17 syn$^+$ ($>10^8$) (Table 1). We also found that after inoculation of the CAM, HSV-1 ANG rapidly infects the embryo and can be recovered from viscera. Of 26 livers removed and examined after embryos died 24 (92%) yielded HSV-1 ANG. In contrast, among the rare embryos
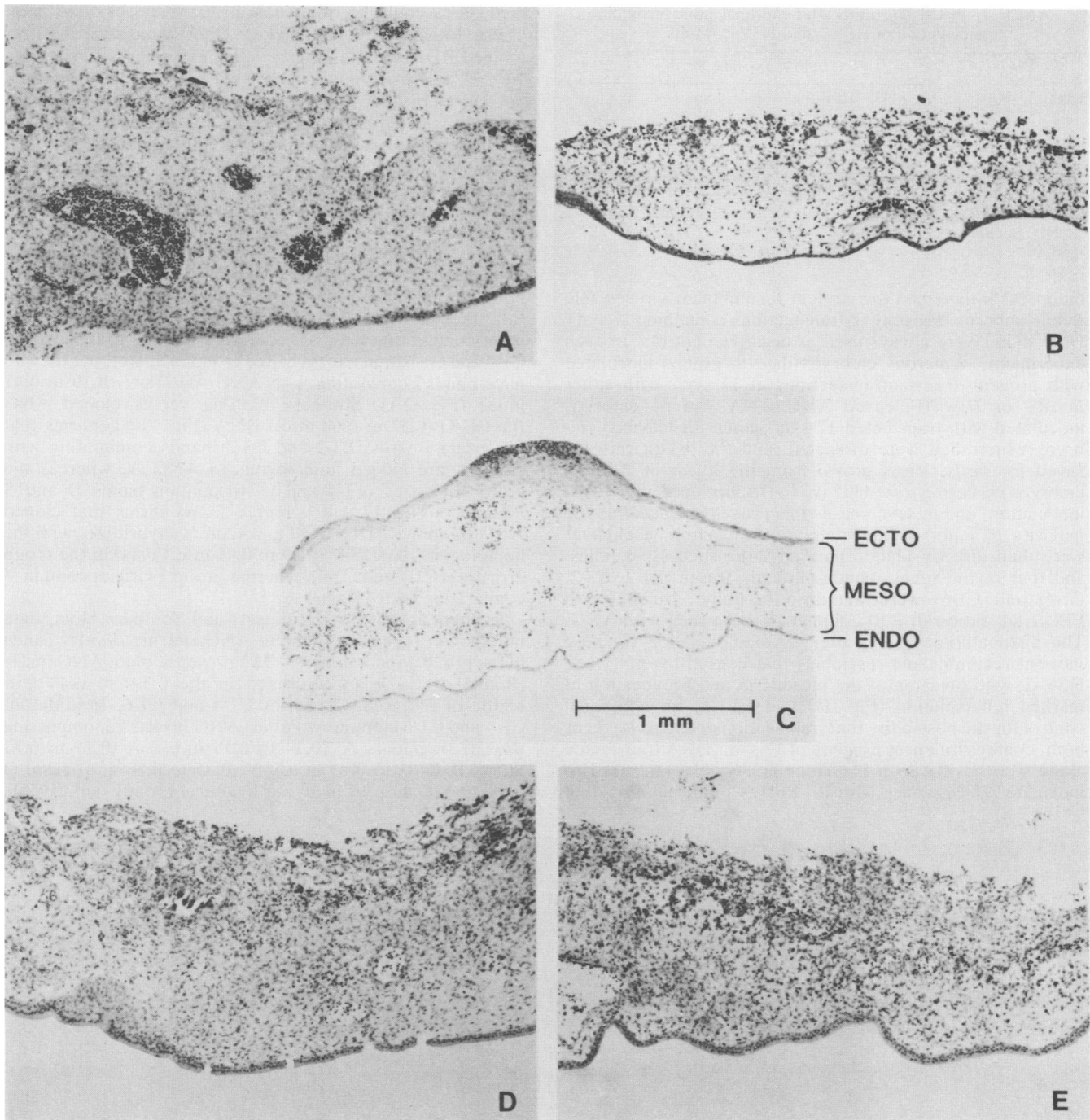
FIG. 1. Photomicrographs of pocks produced on the CAM 3 days following inoculation with (A) HSV-1 ANG, (B) HSV-2 HG52, (C) HSV-1 17 syn$^+$ or the recombinant viruses, (D) B1-1, and (E) C1-6 (see text). Note that like HSV-2, HSV-1 ANG and the virulent recombinants cause extensive necrosis and inflammation which extend through the mesoderm (MESO) to the endoderm (ENDO), whereas HSV-1 17 syn$^+$ primarily involves the ectoderm (ECTO).

dead after 17 syn$^+$ inoculation, there have been only 2 recoveries of virus from the liver in 30 attempts (6.7%) ($X^2 <$ 0.001).

Transfer of uncloned virulence fragment(s) from HSV-1 ANG to HSV-1 17 syn$^+$. Unit-length (CAM-avirulent) 17 syn$^+$ DNA competent to transfect RS cells was isolated from sodium iodide gradients. Similarly isolated (CAM-virulent) ANG genomic DNA was completely cleaved with either

HindIII or EcoRI. Such cleaved DNA was always found to be incapable of producing infectious virus when transfected alone into RS cells. Endonuclease-generated ANG DNA fragments were then cotransfected with unit-length 17 syn$^+$ DNA. The progeny of these transfections consists of a mixture of wild-type 17 syn$^+$ and various ANG × 17 syn$^+$ recombinants. The mixtures from individual transfection plates (usual titer, 10^6 PFU/0.1 ml) were inoculated directly

TABLE 1. PFU/LD$_{50}$ ratios after chorioallantoic membrane inoculation of HSV-1 and HSV-2 strains

Expt	PFU/LD$_{50}$ ratio for:		
	HSV-1		HSV-2 HG52
	17 syn$^+$	ANG	
1	>10^8	10$^{1.5}$	10$^{2.3}$
2	>10^8	10$^{1.7}$	ND^a
3	>10^7	10$^{1.0}$	10$^{2.3}$

^{a} ND, Not done.

onto CAMs to screen for virulent recombinant viruses able to kill embryos. Progeny of transfections containing 17 syn$^+$ DNA alone were always used as negative controls. In each experiment, numerous embryos died in groups inoculated with progeny from cotransfections of 17 syn$^+$ with either EcoRI- or HindIII-cleaved ANG DNA, but no embryos inoculated with transfected 17 syn$^+$ alone died. Next, embryos which died were dissected, and the livers were assayed for virus. Virus grown from the livers of 17 such embryos was again tested in vivo for its virulence after CAM inoculation, and in seven cases this progeny virus killed the majority of embryos inoculated. Isolates from each liver were then directly plaque purified three successive times, and four of the seven plaque-purified viruses (B1-2, C3-2, C1-4, and C1-6) were found to be fully virulent, with PFU/LD$_{50}$ ratios all ≤10^2, similar to those seen with ANG. The histopathology seen after CAM inoculation of these virulent recombinants resembles that caused by ANG and HSV-2, with invasion of the mesoderm and provocation of marked inflammation (Fig. 1D and E). As an additional control for the possibility that transfection or egg passage, or both, confer virulence, progeny of 17 syn$^+$ DNA transfected alone were grown to a high titer in RS cells and used to inoculate 220 eggs each with 10^7 PFU. Of nine embryos that

died, virus could be isolated from the livers of only three. These transfected and chicken embryo-passaged viruses (named RD1, RD2, and RE1) were grown in RS cells, and 10^7 PFU was used to inoculate eggs. RD1, RD2, and RE1 did not kill embryos. As a final control, 10^7 PFU of the viral progeny isolated after three serial passages of 17 syn$^+$ in vivo on CAMs was also found to be avirulent after CAM inoculation.

Identification and mapping of ANG DNA inserts in the virulent recombinant viruses. Viral DNA was isolated from each of the four plaque-purified ANG × 17 syn$^+$ transfection progeny viruses which killed embryos and cleaved with Asp718. Each viral genome contains bands comigrating with both 17 syn$^+$ and ANG (Fig. 2A). Recombinants B1-2 and C3-2 (henceforth called group 1 viruses) each have a fragment comigrating with ANG Asp718-A (0.57 to 0.67 m.u.). C1-4 and C1-6 (henceforth called group 2) do not, but they do have bands comigrating with ANG Asp718-V (0.39 to 0.41 m.u.) (Fig. 2A). Southern blotting versus cloned ANG BamHI-O (0.57 to 0.60 m.u.) DNA (Fig. 2B) confirms that the group 1 virus (B1-2 and C3-2) bands comigrating with ANG A are indeed homologous to ANG A, whereas the group 2 viruses (C1-4 and C1-6) contain bands D and S homologous to 17 syn$^+$. Figure 2C confirms that cloned ANG BamHI-R DNA (0.40 to 0.42 m.u.) hybridizes with the homologous Asp718-V (0.39 to 0.41 m.u.) band in the group 2 viruses (C1-4 and C1-6), whereas group 1 viruses contain V comigrating with 17 syn$^+$.

Examination of BamHI digests and Southern blots (data not shown) reveals no definite ANG-specific BamHI bands in the group 1 recombinants. The presence of an ANG insert (BamHI-U) is again confirmed in the 0.39- to 0.40-m.u. region of the group 2 viruses C1-4 and C1-6. In addition, C1-4 and C1-6 contain two other ANG inserts encompassing BamHI fragments A' (0.14 to 0.15 m.u.), A (0.15 to 0.22 m.u.), K (0.83 to 0.85 m.u.), Y (0.85 to 0.86 m.u.), and Q (0.98 to 1.00 m.u.). Finally, as a control for any conceivable
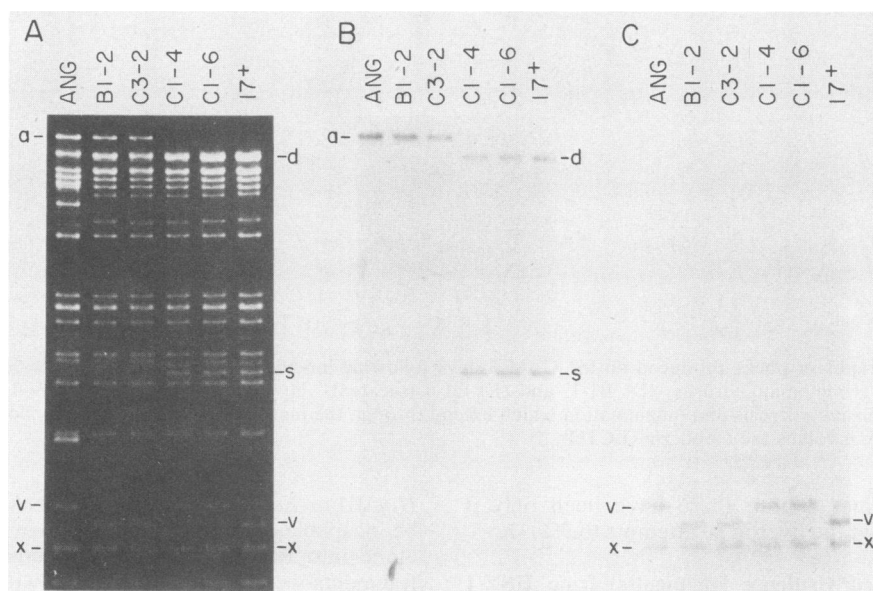

FIG. 2. (A) Electrophoretic pattern of Asp718-cleaved viral DNA from HSV-1 ANG and 17 syn$^+$ and the virulent recombinants B1-2, C3-2, C1-4, and C1-6. (B and C) Southern transfers of the gel in panel A hybridized to either cloned, ^{32}P-labeled ANG BamHI-O (0.58 to 0.60 m.u.) (B) or ANG BamHI-R (0.41 to 0.43 m.u.) DNA (C). Note that the recombinant viruses B1-2 and C3-2 each contain ANG Asp718-A (a) and that recombinants C1-4 and C1-6 contain ANG Asp718-V (v) (see text).
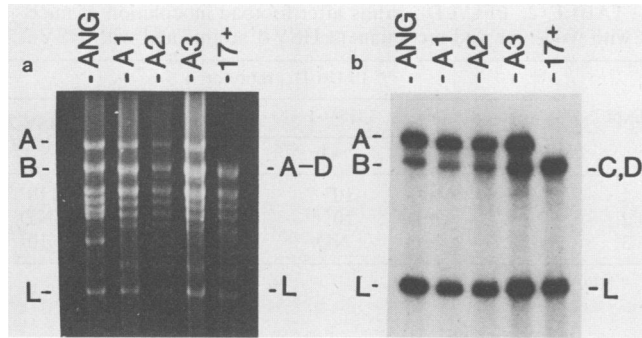
FIG. 3. (a) Electrophoretic pattern of Asp718-cleaved viral DNA from HSV-1 ANG and 17 syn⁺ and the virulent recombinants A1, A2, and A3. (b) Southern transfer of the gel in panel a hybridized to ³²P-labeled cloned ANG EcoRI-A (0.49 to 0.64 m.u.) DNA. Note that each recombinant has incorporated the Asp718-A site from ANG.

changes in restriction sites resulting from transfection and egg passage, DNA from the previously mentioned egg-passaged RD1 and RE1 viruses, as well as the CEF-passaged CEFP10, was also examined and showed no alterations in restriction enzyme profiles when compared with ANG (data not shown).

The data indicate that all four viruses are recombinants between 17 syn⁺ and ANG. Both group 2 viruses were derived from the same transfection plate and contain three ANG inserts, which appear to be identical. The group 1 viruses, B1-2 and C3-2, were derived from different transfections with, respectively, EcoRI and HindIII ANG fragments, but both have still nonetheless incorporated the ANG Asp718-A restriction site into a genomic background that appears to be predominantly 17 syn⁺ DNA.

Transfer and identification of cloned ANG virulence regions. The studies described above recombined uncloned ANG DNA with unit-length 17 syn⁺ DNA and demonstrated that the virulence property could be transferred from ANG to recombinant viruses. The fact that two genetic groups of recombinants were identified without a definite common ANG insert suggested either that hidden inserts were present or that the virulence function is polygenic. Therefore, to determine precisely which ANG sequence(s) confers virulence, we molecularly cloned a library of ANG DNA in pUC vectors. Cloned ANG DNA fragments were then individually cotransfected with unit-length 17 syn⁺ DNA. In this case, progeny virus consists only of unit-length wild-type 17 syn⁺ DNA and recombinants containing part or all of the specific cloned ANG DNA fragment. As in the studies with uncloned ANG DNA, the progeny of these cotransfections were inoculated directly onto CAMs to screen in vivo for virulent recombinants. Progeny virus from transfections containing either cloned ANG EcoRI fragment A (0.49 to 0.64 m.u.) or F (0.32 to 0.42 m.u.) DNA killed embryos. In contrast, embryos were not killed by 17 syn⁺ alone or by the progeny of cotransfections also containing cloned ANG EcoRI fragment B (0.72 to 0.85 m.u.), C (0.00 to 0.08 plus 0.82 to 0.85 m.u.), D (0.08 to 0.19 m.u.), G (0.19 to 0.29 m.u.), H (0.85 to 0.95 m.u.), or I (0.64 to 0.72 m.u.) or cloned ANG BamHI fragment A (0.16 to 0.22 m.u.) or B (0.74 to 0.80 m.u.) DNAs. We then plaque purified viruses isolated from the livers of embryos killed by the progeny from cotransfections with cloned ANG EcoRI fragment A (isolates A1 to A3) and F (isolates F1 to F2) DNA. These viruses each killed embryos with PFU/LD$_{50}$ ratios of $\leq 10^2$, and in all
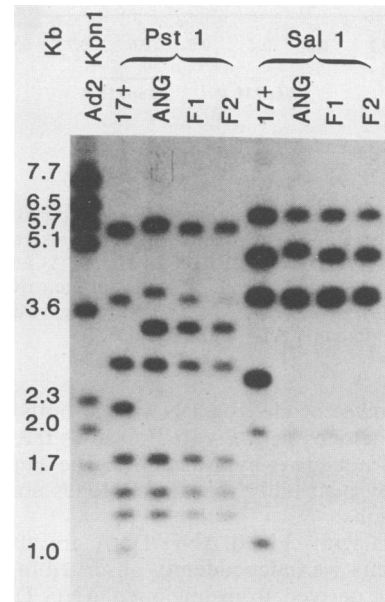

FIG. 4. Southern transfer of electrophoresed viral DNA from HSV-1 17 syn⁺ and ANG and from the recombinant viruses F1 and F2. The DNA was cleaved with PstI or SalI as noted, transferred after electrophoresis, and hybridized to ³²P-labeled cloned ANG EcoRI-F (0.32 to 0.42 m.u.) DNA. Note that F1 and F2 have both incorporated a unique 3.3-kb ANG PstI fragment and are missing the corresponding 2.2- and 1.1-kb 17 syn⁺ bands. In addition, the unique 2.6- and 1.1-kb syn⁺ SalI bands have been replaced by a 3.7-kb ANG fragment.

instances livers removed from dead embryos were positive for virus.

We next sought to prove that each of these virulent viruses indeed contains inserts of the transfected cloned ANG DNA. As shown by the Asp-718 digests of the virulent viruses A1 to A3 (Fig. 3a), each virus is truly a recombinant and has incorporated a band comigrating with ANG Asp718-A. The origin of this band as ANG Asp718-A is confirmed by Southern blotting and hybridization against cloned ANG EcoRI-A DNA (Fig. 3b).

No definite ANG inserts were seen in the BamHI or Asp718 digests of F1 or F2 DNA derived from the virulent viruses obtained after cotransfection with ANG EcoRI-F. We therefore examined other polymorphisms between ANG and 17 syn⁺ in the EcoRI-F region by using frequently cutting enzymes. The PstI and SalI digests of the F1 and F2 recombinants, when probed with cloned ANG EcoRI-F, both revealed a definite ANG insert. This is illustrated in Fig. 4 by the presence of the unique 3.3-kb ANG PstI fragment and the absence of the corresponding 1.1- and 2.2-kb 17 syn⁺ PstI bands in the recombinants. In addition, the corresponding unique 17 syn⁺ 2.6- and 1.1-kb SalI bands have been replaced by a 3.7-kb ANG fragment. In contrast, digests with several other enzymes revealing ANG versus 17 syn⁺ polymorphisms in the EcoRI-F area (BglI, MluI, BspMI, and HincII) showed no other areas of ANG inserts.

Thus, cloned ANG EcoRI-A and EcoRI-F DNA each contain sequences which we have been able to successfully transfer to recombinant viral clones, conferring a 10⁶-fold increase in virulence. To be certain that, either because of repeated sequences within ANG or through a mishap in the cloning process, related sequences are not present in the two virulence-conferring clones, we performed a Southern blot
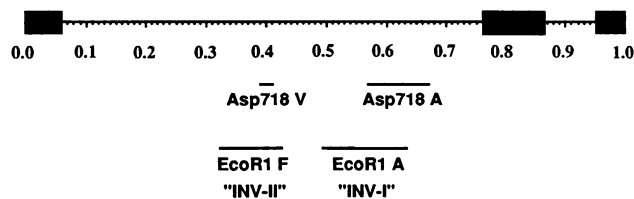
FIG. 5. Schematic drawing illustrating the approximate physical locations on the HSV-1 genome of the ANG inserts found in the group 1 recombinants B1-2 and C3-2 (*Asp*718-A) and the group 2 recombinants C1-4 and C1-6 (*Asp*718-V) and their overlap with the ANG *Eco*RI-A and *Eco*RI-F clones conferring virulence, respectively, to recombinants A1-3 and F1 and F2.

(data not shown) of cleaved DNA from both the *Eco*RI-A and *Eco*RI-F clones with *Eco*RI-F used as the probe. ANG *Eco*RI-F did not cross-hybridize with the *Eco*RI-A DNA, ruling out any possibility that these clones are transferring identical regions.

Our results with cloned ANG DNA are also consistent with the results we independently observed in mapping the recombinants derived from uncloned ANG DNA (Fig. 5). Thus, the ANG *Asp*718-A (0.57 to 0.67 m.u.) site present in the group 1 recombinants B1-2 and C3-2 (Fig. 2) is also seen in recombinants A1 through A3 made with cloned ANG *Eco*RI-A DNA (0.49 to 0.63 m.u.), and the ANG *Asp*718-V (0.39 to 0.41 m.u.) insert observed in the group 2 viruses C1-4 and C1-6 (Fig. 2), although not itself present in the recombinants, maps nearby and within the ANG *Eco*RI-F (0.32 to 0.42 m.u.) clone which transferred virulence to the F1 and F2 viruses.

Action of the cloned ANG invasiveness functions upon neuroinvasiveness in the mouse. Because virulence for the mouse and for the chicken embryo are not always directly linked (10), an important question is whether the ANG virulence regions which we have identified have general biologic activity. With the production of CAM-virulent recombinants by using cloned ANG DNA, we were able to directly address this issue. For these studies, we inoculated 5-week-old male Swiss Webster mice on the left rear footpad with serial 10-fold dilutions of the parental HSV-1 17 syn+,

TABLE 2. PFU/LD$_{50}$ ratios after footpad inoculation of mice with wild-type and recombinant HSV-1 strains and with HSV-2

Expt	PFU/LD$_{50}$ ratio for:					
	HSV-1					HSV-2
	17 syn+	A1	A3	F1	F2	HG52
1	10^6	$10^{5.7}$	10^6	$10^{3.1}$	$10^{2.7}$	$<10^{3.8}$
2	10^7	ND[a]	$10^{7.2}$	ND	$<10^{4.0}$	ND
3	$10^{5.8}$	ND	ND	$10^{2.8}$	$10^{3.0}$	$10^{2.8}$

[a] ND, Not done.

HSV-2 HG52, and our recombinant viruses A1 and A3 and F1 and F2. Recombinants A1 and A3, although attaining full virulence for the chicken embryo, were no more virulent for the mouse (Table 2). In contrast, the F1 and F2 recombinants, which incorporated ANG sequences from the cloned *Eco*RI-F region, have each attained a marked enhancement of their neuroinvasiveness (Table 2). Their PFU/LD$_{50}$ ratios of approximately 10^3 are 10^3-fold lower than that of their parental HSV-1 17 syn+ and are similar to those seen with HG52, a typical HSV-2 strain.

Studies of viral replication in CEFs. We previously derived, by repetitive passage in CEFs, a virus, CEFP10, which grows more rapidly and to higher titers in CEFs than does its parental strain 17 syn+ and which has attained some virulence for chicken embryos (10). Although the replication kinetics and yields of ANG and 17 syn+ do not differ from each other in RS cells or mouse embryo fibroblasts (K. Izumi and J. Stevens, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, S36, p. 321), we wished to determine whether ANG and/or the virulent recombinant viruses have any growth advantage in chicken cells which might be pathogenically significant. We performed studies of replication kinetics at a low (0.01 PFU per cell) multiplicity of infection, similar to the in vivo multiplicity of infection after CAM inoculation. Soon after infection ANG had a slight but consistent growth advantage over 17 syn+, with peak titers occurring earlier and at a fivefold-higher level (Fig. 6A). However, despite being far more virulent, ANG replicated similarly to CEFP10 and, in fact, achieved lower peak titers. The CAM-virulent
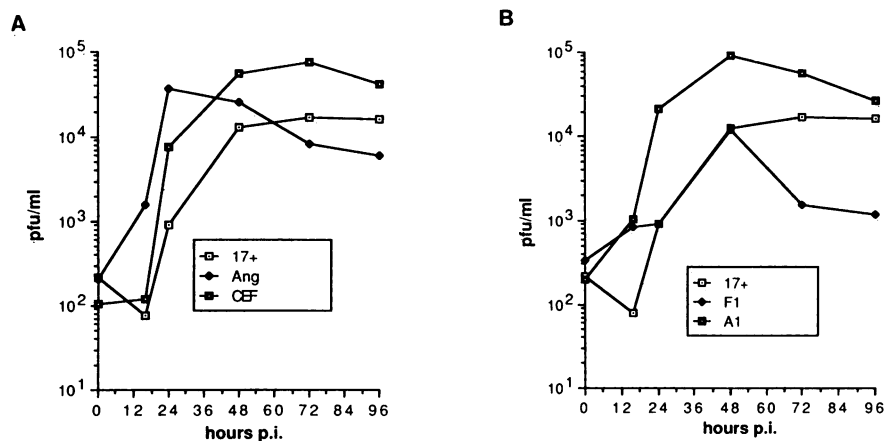

FIG. 6. (A) Replication kinetics of HSV-1 17 syn+, ANG, and the chicken embryo-passaged virus, CEFP10, in CEFs. (B) Replication kinetics of the virulent recombinant viruses A1 and F1 compared with 17 syn+. For these studies, just confluent CEFs were infected at a multiplicity of infection of 0.01 PFU per cell in 24-well tissue culture plates. At the indicated times, the contents of triplicate wells were harvested and combined, and viral PFU was assayed in RS cells. The results from one of two experiments performed are shown. p.i., Postinfection.

recombinants containing inserts from cloned ANG *Eco*RI-A DNA also, like ANG, replicated to higher titers than 17 syn$^+$ did. However, the recombinants which contain inserts from cloned ANG *Eco*RI-F had no apparent replicative advantage, despite their enhanced virulence for both the chicken embryo and the mouse (Fig. 6B).

DISCUSSION

We have found that an HSV-1 strain, ANG, is able to make large pocks, invade the CAM, and kill chicken embryos after CAM inoculation, properties heretofore recognized only in HSV-2 strains and thus previously believed to be type-specific properties of HSV-2. Furthermore, we have shown that ANG DNA contains at least two distinct virulence regions (tentatively designated INV-I and INV-II) containing functions which can be transferred by DNA cotransfection to confer mesodermal invasiveness and virulence on a previously CAM-avirulent HSV-1 strain, 17 syn$^+$. We have plaque purified pathogenically stable virulent viruses from the livers of chicken embryos infected with the progeny of such cotransfections with both cloned and uncloned ANG DNA and have used genetic mapping to demonstrate that the virulent clones are all recombinant viruses, which contain ANG DNA sequences. We have used cloned ANG DNA both to specifically transfer these virulence functions and to localize them to the *Eco*RI fragment A (INV-I) and F (INV-II) regions of HSV-1 ANG. We have demonstrated that both fragments confer a 10^6-fold increase in virulence for chicken embryos and that the INV-II, but not INV-I, region also enhances neuroinvasiveness and virulence 10^3-fold in a mammalian host. In contrast, control virus made with 17 syn$^+$ DNA transfected alone failed to kill embryos even after being subjected to passage through both the CAM and the embryo liver. Finally, cloned DNA from regions of the ANG genome other than *Eco*RI-A and *Eco*RI-F did not confer virulence. From these controlled studies, we believe that genes in ANG *Eco*RI-A and *Eco*RI-F, when inserted into the 17 syn$^+$ genome, confer virulence. Although the differing biologic properties of the *Eco*RI-A and *Eco*RI-F recombinants make it unlikely, we cannot, without sequencing, absolutely exclude the possibility that these two regions are transferring a small closely related virulence sequence or share a similar mutagenic property.

Which HSV genes are responsible, and how might they confer virulence? Characterized genes in the *Eco*RI-A INV-I CAM virulence region (0.49 to 0.64 m.u.) include the virion function associated with the shutoff of host cell protein synthesis (19), a property that is type divergent, being far more rapid with HSV-2 (24). The 5' end of the gC gene also maps within this region, and gC is also highly type divergent in terms of both sequence and antigenicity (32). In addition, the genes encoding ribonucleotide reductase and infected-cell polypeptide 10 (ICP10) as well as five transcripts with unknown gene products reside totally or partially within this area (31). It is also interesting that a genetic abnormality in strain KOS was roughly mapped to this general region and is at least in part responsible for the inability of that virus to invade the mouse central nervous system after peripheral inoculation (33).

The *Eco*RI-F INV-II CAM and mouse virulence region (0.32 to 0.42 m.u.), although smaller, is also complex. gB (0.348 to 0.368 m.u.) (4) and its associated syn 3 (syncytial) mutation locus (29) map in this area. This is of potential pathogenic importance, especially when considering mesodermal invasiveness, since gB is important in viral penetra-

tion and fusion of cells (30) and is highly immunogenic (3, 9). Of note, both F1 and F2, like ANG (but not 17 syn$^+$), produce syncytia, and we have always found syncytial variants in cotransfections containing the ANG *Eco*RI F fragment. Thus, we have also mapped the ANG syn locus to this area. Therefore, we did ask whether the syncytial phenotype alone might allow HSV-1 to become CAM virulent. However, three unrelated laboratory-derived HSV-1 syn mutants, strains MP, ts$^+$ syn, and 17 hep syn, were found not to be CAM virulent (data not shown), and syn mutants previously evaluated elsewhere (8) are avirulent after footpad inoculation of mice. These findings suggest either that virulence is specific for this particular syn genotype or that these properties, although genetically linked, may not be directly related. Two virion proteins important in encapsidation map at the 5' end of this region (1, 25); a temperature-sensitive mutant of one of these proteins, mapping between 0.318 and 0.324 m.u., is also deficient in penetrating cells (1). In addition, the far 5' end of the DNA polymerase gene (0.41 to 0.43 m.u.) maps within the *Eco*RI-F clone. Although previous studies have shown that mutants deficient in DNA polymerase are not fully neurovirulent (16), the virulent F1 and F2 recombinants do not demonstrate the ANG *Asp*718-V polymorphism (0.39 to 0.41 m.u.), making it unlikely that an ANG insert is present in this region. Also mapping within the *Eco*RI-F area are the major DNA-binding protein and several transcripts with unknown products (31).

Why is ANG different from other HSV-1 isolates with respect to CAM mesodermal invasiveness and virulence? The first explanation to consider is that ANG has somehow acquired a replicative advantage specific for chicken cells analagous to that which we previously described for the CEF-passaged virus CEFP10. Although ANG was apparently first isolated on the CAM, there is no history of multiple in vivo or in vitro passages in chicken cells. The results of our studies concerning CEFP10, RD1, and RE1 show no increase in CAM virulence after one to three such passages. Although ANG and the recombinants made with cloned ANG *Eco*RI-A do have a growth advantage over 17 syn$^+$ in vitro in CEFs, the similar replicative advantage of CEFP10 does not confer the degree of virulence seen with ANG. Unlike CEFP10, ANG makes large pocks on the CAM and is fully CAM virulent, exceeding even HSV-2 strains in its killing capacity. Perhaps most significant, transfer of the ANG *Eco*RI-F INV-II invasiveness function to F1 and F2 enhances invasiveness in both the chicken embryo and the mouse, while these virulent recombinants do not grow better in CEFs. Thus, although a growth advantage of ANG in chicken mesodermal cells may be in part responsible for the virulence of the *Eco*RI-A recombinants for chicken embryos, we doubt that such a replicative advantage is responsible for the pathogenic actions of the ANG invasiveness sequences in the *Eco*RI-F INV-II region. It is, however, possible that the CAM virulence of ANG is due to the presence of a unique HSV-1 mutation arising de novo and conferring virulence by a different mechanism.

ANG has another unusual characteristic, its reportedly genital origin, which might explain the presence of these invasiveness genes. We could not, however, find evidence for CAM virulence in seven other genital HSV-1 isolates which we studied (data not shown). Thus, the ability of an HSV-1 isolate to cause genital disease is not itself sufficient to confer CAM virulence. Nonetheless, the possible genital origin of ANG remains of great interest in the context of the known CAM virulence of HSV-2 isolates. It could suggest

that CAM virulence can be selected for, albeit rarely, within the genital tract. Perhaps more interesting, although clearly not a common clinical event, is the recent demonstration that intratypic recombination between strains of HSV can occur in vivo in an experimental setting (13). The unique biology of ANG raises the possibility that it acquired HSV-2 virulence sequences through in vivo intertypic recombination with an HSV-2 strain.

The ANG invasiveness regions have been molecularly subcloned in our laboratory, and these subclones are being recombined with 17 syn$^+$ to precisely map both virulence functions. Once localized, molecular aspects of the areas of interest can be further defined, and the involved genes can be identified. Furthermore, the recombinants constructed with cloned ANG DNA can now be used in both the CAM model and in the mouse to allow studies defining the pathogenic action of both virulence genes and their contributions to the important but heretofore largely unexplained properties of viral invasion and generalization in the living host.

LITERATURE CITED

1. **Addison, C., F. J. Rixon, J. W. Palfreyman, M. O'Hara, and V. G. Preston.** 1984. Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology **138:** 246–259.

2. **Becker, Y., J. Hadar, E. Tabor, T. Ben-Hur, I. Raibstein, A. Rosen, and G. Darai.** 1986. A sequence in Hpa1-P fragment of herpes simplex virus-1 DNA determines intraperitoneal virulence in mice. Virology **149:**255–259.

3. **Blacklaws, B. A., A. A. Nash, and G. Darby.** 1987. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constitutively expressed on L cell lines. J. Gen. Virol. **68:**1103–1114.

4. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133:**301–314.

5. **Centifanto-Fitzgerald, Y. M., T. Yamaguchi, H. E. Kaufman, M. Tognon, and B. Roizman.** 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. J. Exp. Med. **155:**475–489.

6. **Corey, L., E. F. Stone, R. J. Whitley, and K. Mohan.** 1988. Difference between herpes simplex virus type 1 and type 2 neonatal encephalitis in neurological outcome. Lancet **i:**1–4.

7. **Craig, C. P., and A. J. Nahmias.** 1973. Different patterns of neurologic involvement with herpes simplex virus types 1 and 2: isolation of herpes simplex virus type 2 from the buffy coat of two adults with meningitis. J. Infect. Dis. **127:**365–372.

8. **Dix, R. D., R. R. McKendall, and J. R. Baringer.** 1983. Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of BALB/c mice. Infect. Immun. **40:**103–112.

9. **Eberle, R., and R. J. Courtney.** 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. **35:**902–917.

10. **Goodman, J. L., and J. G. Stevens.** 1986. Passage of herpes simplex virus type 1 on chick embryo fibroblasts confers virulence for chick embryos. Virus Res. **5:**191–200.

11. **Goodpasture, E. W., A. M. Woodruff, and G. J. Buddingh.** 1931. The cultivation of vaccine and other viruses in the chorioallantoic membrane of chick embryos. Science **74:**371–372.

12. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70:**208–219.

13. **Javier, R. T., F. Sederati, and J. G. Stevens.** 1986. Two avirulent herpes simplex viruses generate lethal recombinants in vivo. Science **234:**746–748.

14. **Kaerner, H. C., C. H. Schroder, A. Ott-Hartmann, G. Kumel, and H. Kirchner.** 1983. Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a nonpathogenic herpes simplex virus type 1 virus strain in mouse brain. J. Virol. **46:**83–93.

15. **Lafferty, W. E., R. W. Coombs, J. Benedetti, C. Critchlow, and L. Corey.** 1987. Recurrences after oral and genital herpes simplex virus infection. N. Engl. J. Med. **316:**1444–1449.

16. **Larder, B. A., J. J. Lisle, and G. Darby.** 1986. Restoration of wild-type pathogenicity to an attenuated DNA polymerase mutant of herpes simplex virus type 1. J. Gen. Virol. **67:** 2501–2506.

17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

18. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.

19. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26:**389–410.

20. **Munk, K., and G. Ludwig.** 1972. Properties of plaque variants of herpes virus hominis strains of genital origin. Arch. Gesamte Virusforsch. **37:**308–315.

21. **Nahmias, A. J., W. R. Dowdle, J. H. Kramer, C. F. Luce, and S. C. Mansour.** 1969. Antibodies to herpesvirus hominis types 1 and 2 in the rabbit. J. Immunol. **102:**956–962.

22. **Nahmias, A. J., W. R. Dowdle, Z. M. Naib, A. Highsmith, R. W. Harwell, and W. E. Josey.** 1968. Relation of pock size on the chorioallantoic membrane to antigenic type of herpesvirus hominis. Proc. Soc. Exp. Biol. Med. **127:**1022–1028.

23. **Plummer, G., J. L. Waner, and C. P. Bowling.** 1968. Comparative studies of type 1 and 2 'herpes simplex' viruses. Br. J. Exp. Pathol. **49:**202–208.

24. **Powell, K. L., and R. J. Courtney.** 1975. Polypeptides synthesized in herpes simplex virus type 2 infected HEp-2 cells. Virology **66:**217–228.

25. **Preston, V. G., J. A. V. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45:**1056–1064.

26. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.

27. **Rodgers, F. G.** 1973. Infection, haemorrhage and death of chick embryos after inoculation of herpes simplex virus type 2 on the chorioallantoic membrane. J. Gen. Virol. **21:**187–191.

28. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16:**481–494.

29. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29:**677–697.

30. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. **29:**1149–1158.

31. **Schaffer, P. A., E. K. Wagner, G. B. Devi-Rao, and V. G. Preston.** 1987. Herpes simplex virus, p. 93–98. In S. J. O'Brien (ed.), Genetic maps 1987, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

32. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. In B. Roizman (ed.), The herpesviruses,

vol. 3. Plenum Publishing Corp., New York.

33. **Thompson, R. L., M. L. Cook, G. B. Devi-Rao, E. K. Wagner, and J. G. Stevens.** 1986. Functional and molecular analysis of the avirulent wild-type herpes simplex virus-strain KOS. J. Virol. **58:**203–211.

34. **Thompson, R. L., E. K. Wagner, and J. G. Stevens.** 1983.

Physical location of a herpes simplex virus type-1 gene function(s) specifically associated with a 10 million-fold increase in HSV neurovirulence. Virology **131:**180–192.

35. **Walboomers, J. M. M., and J. T. Schegget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology **74:**256–258.