Regeneration of Herpesviruses from Molecularly Cloned Subgenomic Fragments

MADDY VAN ZIJL,[1] WIM QUINT,[1]† JAN BRIAIRE,[1] TONY DE ROVER,[1] ARNO GIELKENS,[1] AND ANTON BERNS[2]*

Division of Molecular Genetics, The Netherlands Cancer Institute, and Department of Biochemistry, University of Amsterdam, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam,[2] and Department of Virology, Central Veterinary Institute (CDI), 8221 RA Lelystad,[1] The Netherlands

The ability to manipulate the genomes of herpesviruses is of eminent importance for obtaining insight into gene function and regulation of gene expression of these complex viruses. Here we report the use of in vivo overlap recombination to generate pseudorabies virus mutants. Cotransfection of up to five overlapping cloned subgenomic fragments, which together constitute the entire genomic information of pseudorabies virus, results in the efficient reconstitution of virus. This allows the efficient introduction of multiple well-defined mutations in herpesvirus genomes in a single step, without any selection or screening for a particular phenotype.

Pseudorabies virus (PRV), a member of the alpha-herpesviruses, is the causative agent of Aujeszky's disease in domestic and wild animals (2) and leads to severe economic losses in the swine industry. As part of a study designed to generate an effective and well-characterized bioengineered live PRV vaccine, we have shown that it is possible to generate infectious virus from subgenomic fragments by cotransfection of two adjacent but nonoverlapping fragments of 120 (HindIII-A) and 25 kilobase pairs (kbp) (HindIII-B), respectively (Fig. 1a) (7, 13). Reconstitution of infectious virus from subgenomic fragments has also been reported for smaller viruses like spleen necrosis virus (12) and adenovirus (5, 6). Regeneration of these viruses was performed by cotransfection of overlapping cloned fragments. We have used a similar methodology to regenerate herpesviruses with a genome of 150 kbp.

For this purpose, a DNA cosmid library of the genome of PRV Northern Ireland Aujeszky-3 (NIA-3) (2) was produced. NIA-3 virus was isolated from the medium of infected BHK cells by precipitation with polyethylene glycol and was purified by discontinuous sucrose gradient centrifugation. Isolation of DNA from the virus pellet was as described previously (9). For the construction of a DNA cosmid library, fragments were prepared in one of two ways. Either viral DNA was sheared by forcing the DNA solution (20 µg of DNA in 500 µl of TE [pH 8.0]) twice through a 25-gauge needle or DNA was treated with 2.5 µg of DNase per ml for 10 min at 15°C in 50 mM Tris–(pH 7.4)–1 mM MnCl$_2$–100 µg of bovine serum albumin per ml. The resulting fragments were separated on a 15 to 40% glycerol gradient in 50 mM Tris (pH 8.0)–300 and mM NaCl–1 mM EDTA at 40,000 rpm in an SW40 rotor for 5.5 h at 20°C. The tubes were then punctured, and 0.5-ml fractions were collected. Fractions containing fragments with sizes between 35 and 45 kbp were pooled, and the DNA was pelleted after twofold dilution with TE by centrifugation in an SW50 rotor at 50,000 rpm for 5 h. The fragments were treated with bacteriophage T4 polymerase, and EcoRI linkers were ligated to the termini (there is no EcoRI site in the NIA-3 genome). The fragments

were cloned in the EcoRI site of the cosmid vector pJBF, a derivative of pJB8. A physical map of the clones was obtained by Southern blot analysis with restriction endonucleases BamHI, KpnI, and BamHI+KpnI. Cosmids which showed an altered physical map (compared with the corresponding region of the parental virus) were discarded. This was particularly relevant for cosmid clones containing the inverted repeat region of PRV; this region was not only underrepresented in the cosmid library, but a significant fraction of the clones showed rearrangements as well.

Sets of four or five overlapping apparently nonrearranged clones, together constituting the complete viral sequence, were tested for the capacity to generate intact virus after transfection into pig kidney cells (PK-15 cells) by the calcium phosphate precipitation method (10). The genomic regions covered by a set of four clones are shown in Fig. 1b, and a set of five clones is depicted in Fig. 1c. The overlaps of these clones range from 1.5 to 16 kbp. The fragments used in the transfection assays were prepared as follows. Plasmid and cosmid DNA was isolated by the alkaline lysis method and purified by a CsCl density gradient (11). To enhance the efficiency of homologous recombination, linear DNA was used in the transfection experiments. The clones were digested with the appropriate restriction endonuclease to release the inserts from the cosmid vector. Cosmid DNA was digested with EcoRI and plasmid DNA was digested with HindIII in order to release the inserts. In cases in which progeny virus was used for further experiments, the inserts were purified by glycerol gradient centrifugation to prevent incorporation of procaryotic vector sequences in the viral genome. Almost all (95%) of the vector sequences were removed by centrifugation in a 15 to 40% glycerol gradient (50 mM Tris [pH 8.0]–300 mM NaCl–1 mM EDTA) at 40,000 rpm in an SW40 rotor at 20°C for 6 to 8 h. Fractions containing highly enriched DNA insert, as monitored on a 0.7% agarose gel, were pooled and recovered by centrifugation. The DNA was extracted once with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]), precipitated with ethanol, and dissolved in TE. Mixtures of viral fragments were cotransfected into PK-15 cells in a T25 flask at 25 to 30% confluency. Positive transfections were scored by the occurrence of cytopathic effect 3 days after transfection.

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* Corresponding author.
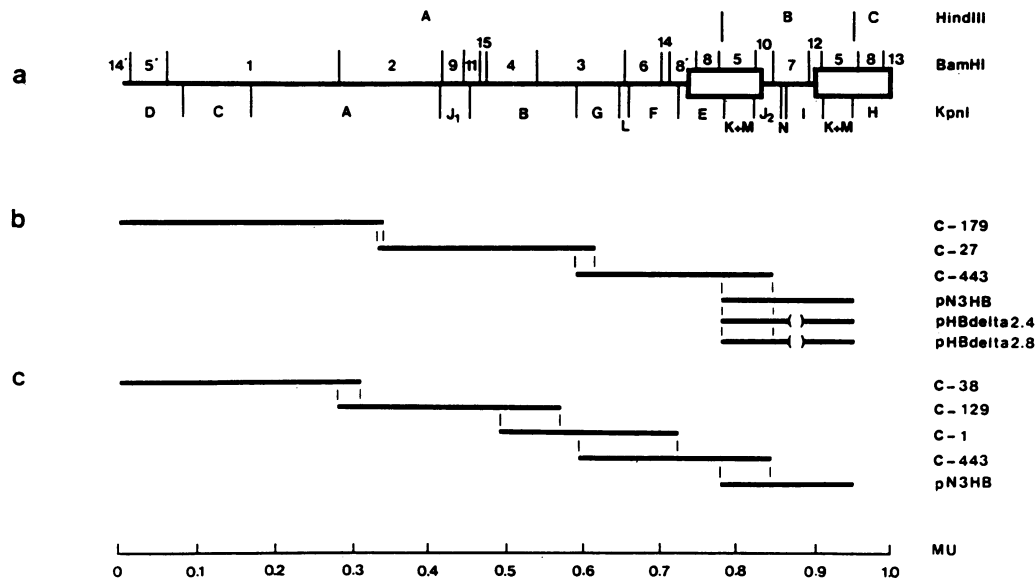† Present address: Stichting Samenwerkende Delftse Ziekenhuizen, 2600 GA Delft, The Netherlands.

FIG. 1. Positions of the transfected clones on the physical map of PRV strain NIA-3. (a) Physical map of NIA-3 (13). The genome consists of a linear double-stranded DNA molecule (17) which can be divided into a unique short region bracketed by inverted repeats (indicated by open rectangles) and a unique long region (4). The sites of *Bam*HI, *Hin*dIII, and *Kpn*I are depicted. The fragments are given in alphabetical or numerical order according to size. (b) Set of four overlapping clones. Cosmid clones are designated c-179, c-27, and c-443. pHBdelta2.4 and pHBdelta2.8 are derived from pN3HB by BAL 31 exonuclease digestion and carry deletions in *Bam*HI-7 of approximately 2.4 and 2.8 kbp, respectively (13). (c) Set of five overlapping clones. Cosmid clone and plasmid designations are shown to the right. MU, Map units.
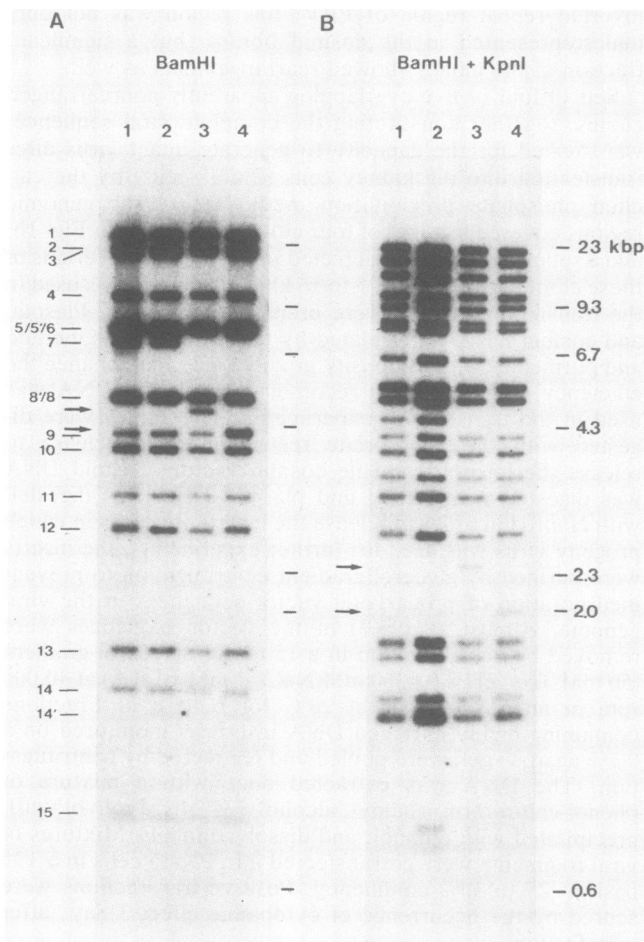
Transfections with an appropriate mixture (see below) of four or five fragments resulted in virus production. To establish the genomic structure of the reconstituted viruses, progeny viral DNA which had been isolated from plaque-purified viruses was characterized by Southern blot analysis. Filters were probed with nick-translated NIA-3 or vector DNA. As can be seen from the *Bam*HI and the *Bam*HI + *Kpn*I patterns in Fig. 2A and B, no differences in the hybridization patterns were detectable between the parental and reconstituted viral DNA (Fig. 2A and B, lanes 1 and 2). Hybridization of *Bam*HI digestion products with the vector pJBF revealed no hybridizing fragments (data not shown), indicating that vector sequences are not incorporated in the genome of progeny virus. More detailed Southern blot analysis also revealed no differences between the parental and the reconstituted virus. DNA was digested with various endonucleases (as described in the legend to Fig. 3) and probed with a *Bam*HI-5 and a *Bam*HI-10 clone (Fig. 3A and 3B, respectively), which together make up the overlap region of the clones c-443 and pN3HB. All restriction fragments of the reconstituted virus showed the same mobility as the corresponding fragments of the parental virus. Even when DNA from total lysates of the primary transfec-

FIG. 2. Southern blot analysis of the reconstituted viruses. *Bam*HI- (A) and *Bam*HI + *Kpn*I- (B) digested fragments of the parental NIA-3 virus (lanes 1) and three reconstituted viruses (lanes 2 to 4) obtained by hybridization of viral DNA with nick-translated NIA-3 genomic DNA. Lanes 2, 3, and 4 represent virus reconstituted from four overlapping fragments (Fig. 1b) including pN3HB (lanes 2), pHBdelta2.4 (lanes 3), or pHBdelta2.8 (lanes 4). Arrows mark the positions of the altered *Bam*HI 7 (A) and 4.5-kbp *Bam*HI-*Kpn*I (B) fragments in the deletion mutants. NIA-3 fragments are numbered at the left. Agarose gel electrophoresis, transfer of DNA to nitrocellulose filters, and hybridization with total nick-translated NIA-3 DNA were performed as described previously (14, 19).
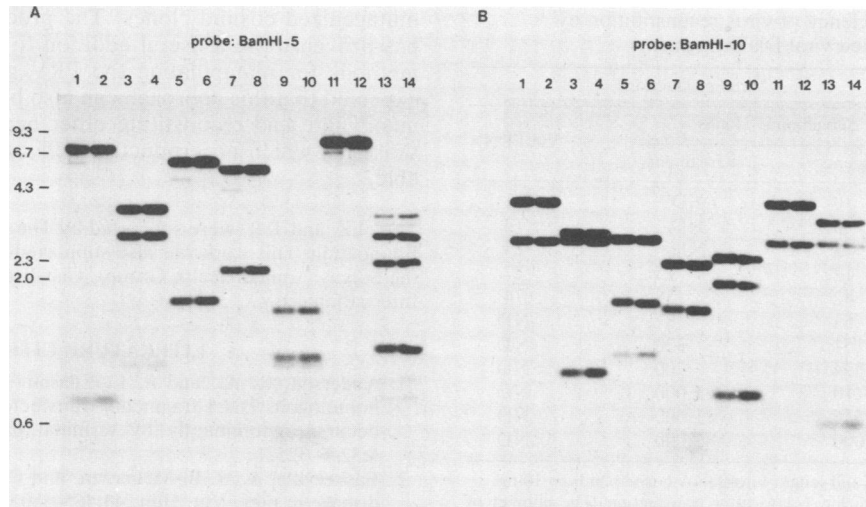
FIG. 3. Southern blot analysis of the overlap region of reconstituted virus. Viral DNA from the parental virus (odd-numbered lanes) and the DNA from virus reconstituted from four wild-type cosmid clones (even-numbered lanes) was digested with BamHI + BalI (lanes 1 and 2), BamHI + PstII (lanes 3 and 4), BamHI + SacI (lanes 5 and 6), BamHI + SalI (lanes 7 and 8), BamHI + SmaI (lanes 9 and 10), BamHI + StuI (lanes 11 and 12), and BamHI + XhoI (lanes 13 and 14) and hybridized to a nick-translated BamHI-5 (A) and a BamHI-10 (B) NIA-3 clone. The hybridizing fragments in the odd-numbered lanes (parental virus) which have no counterpart in the even-numbered lanes (reconstituted virus) are due to an impurity in the DNA preparation of the parental virus.

tants was used, the restriction endonuclease pattern was indistinguishable from that of wild-type PRV. This indicated that the vast majority of viable viruses reconstituted via overlap recombination are unaltered. Minor differences detectable among plaque-purified, reconstituted viruses reflected the characteristic variations also observed between independent isolates of the parental virus, that is, minor differences in the repeat fragments.

To show that defined mutants can be generated by this method, the wild-type pN3HB plasmid was replaced by pHBdelta2.4 or pHBdelta2.8. Each of the latter two plasmids carries a different well-characterized deletion. These plasmids have been described previously (13). Transfections of clones c-179, c-27, and c-443 together with one of these plasmids yielded replication-competent viruses. A Southern blot analysis of the DNA of these viruses is shown in Fig. 2. BamHI and BamHI + KpnI restriction endonuclease digestions show the expected pattern. Only the 6.8-kbp BamHI 7 and the 4.5-kbp BamHI-KpnI fragments, in which the BAL 31-introduced deletions reside (13), are altered in the mutant (cf. Fig. 2A, lanes 1, 3, and 4, and Fig. 2B, lanes 1, 3, and 4). The wild-type BamHI 7 fragment is absent from both of these mutants and replaced by fragments of 4.6 and 4.2 kbp, respectively. A similar reduction in size is observed in the 4.5-kbp BamHI-KpnI fragment.

To provide further evidence for the appropriate reconstitution of the virus, the biological properties of a reconstituted wild-type virus were compared with those of the parental virus. Piglets were infected with either one of these viruses and were monitored during the disease period. No significant differences between the two groups were observed. In the course of the disease, we monitored the occurrence of fever (duration and temperature), loss of weight as a function of days postinfection, titer of neutralizing antibodies, and mortality (data not shown). To our knowledge this is the most sensitive assay to detect alterations in the biological properties of these viruses. Therefore, we conclude that reconstitution of PRV by overlap recombination gives rise to intact and unaltered virus.

The efficiency of virus reconstitution was studied first by comparing the infectivity of mixtures of four or five subgenomic clones (in equimolar amounts) with that of full-length viral DNA. The subgenomic clones were transfected in linear form into PK-15 cells at 25 to 30% confluency in T25 flasks. Equimolar amounts of the three cosmid clones c-179, c-27, and c-443 were mixed and digested with EcoRI, and the plasmid pN3HB was digested with HindIII. The DNA was extracted twice with a mixture of phenol:choloroform: isoamyl alcohol (25:24:1 [vol/vol/vol]), precipitated with ethanol, and dissolved in TE. An equimolar amount of linearized pN3HB was added to the cosmid clones, and this mixture was diluted to the appropriate concentrations. Full-length viral DNA was isolated as described above. Calf thymus DNA was used to adjust the amount of DNA to 10 μg per transfection. DNA was transfected into PK-15 cells by the calcium phosphate precipitation technique (10). At 4 h after the addition of the precipitate, the cells were subjected to a glycerol shock (15% glycerol added to the tissue culture medium for 45 sec).

The results of these transfections are presented in Table 1. The minimal amount of full-length viral DNA required for virus production was 0.1 fmol, whereas the minimal amount of cosmid DNA was approximately 0.4 fmol per clone. Four or five cosmids were equally efficient in reconstituting the virus. Titration studies indicated that the use of equimolar amounts of fragments was not the most efficient in virus reconstitution. Higher efficiencies were recorded when the fragment containing the repeat and the unique short region was present in submolar concentration (data not shown). The high efficiency of virus reconstitution from the cosmid-based fragments was unexpected. One could argue that total viral DNA is also partly degraded during the transfection procedure and that therefore production of progeny virus under these conditions also requires overlap recombination. However, this is not compatible with the observation that five cosmid clones are as efficient as four cosmid clones in virus reconstitution. Furthermore, the linear relationship which was observed between the amount of fragments used

TABLE 1. Efficiency of virus reconstitution by
transfected viral DNA fragments

Amt of DNA (fmol)[a]	Virus production[b]		
	Subgenomic clones		Viral DNA
	4 fragments	5 fragments	
0.1	0	ND[c]	1
0.2	0	ND	3
0.4	1	ND	23
0.8	12	15	27
1.6	42	38	74
3.2	53	87	ND
6.4	151	320	ND
13.0	394	700	ND
25.0	410	>1,000	ND
50.0	545	>1,000	ND
100.0	>1,000	>1,000	ND

[a] A quantity of 0.1 fmol of full-length viral DNA corresponds to 10 ng.

[b] Number of plaques appearing 3 days after transfection. The number of plaques is the average of two independent transfections performed at the same time. Infectivity between duplicates and independent experiments (not shown) varied on average by no more than a factor of 2.

[c] ND, Not done.

and the number of plaques scored indicates that a cell takes up all the cotransfected fragments in a wide concentration range and that the subsequent recombination among the transfected fragments is not the rate-limiting step in this process.

We have not determined the minimal requirements for overlap recombination. The overlap regions in the cosmid clones varied from 1.5 to 16 kbp, which is far more than what is assumed to be required for efficient recombination (8, 18). Linearization of the DNA before transfection increased the recombination efficiency significantly. In order to regenerate virus from four circular clones, 25 to 50-fold more DNA was required. This is in agreement with the results of others, who have shown that linear molecules are preferred substrates for homologous recombination (1, 21). Furthermore, viruses could never be produced from transfections of five circular clones (with up to 3 μg of DNA per clone). The majority of the viruses obtained from circular DNA contained vector sequences. However, vector-free, genuinely reconstituted virus could be obtained from these transfections after plaque screening (data not shown).

Part of the terminal repeat, i.e., 1.5 kbp of the BamHI 5 fragment and the BamHI 8 and BamHI 13 fragments, is not included in the transfection mixture (Fig. 1b and c). The reconstituted viruses, however, do contain two identical and complete repeats, as was demonstrated by the presence of the BamHI 13 fragment (Fig. 2A). This can be explained by homologous recombination (3) or by a copying mechanism which regenerates the terminal repeat from the internal repeat (16). The reconstitution of PRV from subgenomic fragments illustrates that homologous recombination of foreign DNA in mammalian cells is extremely efficient, as has also been shown by others (12, 21).

The use of cloned fragments will obviate many of the problems associated with manipulating the 150-kbp-long PRV genome. In the search for genes involved in the virulence of PRV, viral fragments can now be easily manipulated in vitro and subsequently transferred to viruses without requiring selection or screening procedures (15, 20). Even mutant viruses with poor replicating capacity and carrying multiple genetic alterations can now be obtained in pure form in a single transfection step by using in vitro

mutagenized cosmid clones. The procedure described here has proven to be a useful addition to the already available methods for manipulating the PRV genome. Finally, it is expected that this approach can also be used successfully to manipulate and reconstitute other herpesviruses, including those for which no productive cell culture system is available.

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