

Mapping of a Vaccinia Host Range Sequence by Insertion into the Viral Thymidine Kinase Gene

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A vaccinia virus mutant deleted of ca. 18 kilobase pairs at the left-hand end of the genome is unable to multiply on many human cell lines. To determine whether all or some of the deleted sequences were responsible for the host range property, the corresponding region from wild-type DNA was cloned in three pieces into a vaccinia transplacement vector containing the thymidine kinase gene on the *Hind*III J fragment. The next step was to transfer these pieces to the genome of the host range deletion mutant by *in vivo* homologous recombination around the thymidine kinase locus. Transfer of one 5.2-kilobase-pair *Eco*RI fragment was found to restore a wild-type phenotype on the host range mutant, thus demonstrating that only a small portion of the 18-kilobase-pair deletion contains the host range function(s). This result also illustrates that the method initially devised for inserting foreign genes into vaccinia virus DNA is useful for studies of the vaccinia genome.

Vaccinia virus can multiply in cells derived from numerous mammalian or avian species. The isolation of host range mutants from vaccinia (2, 13) or the closely related rabbit pox virus (4-6, 10, 12) suggests that the ability to cross species barriers requires virus-encoded functions. A vaccinia host range mutant (*hr*) we previously isolated is particularly interesting in that it is unable to multiply on cell lines derived from human tissues (2). The genome of this mutant is deleted of about 18 kilobase pairs (kbp) at one end of the molecule. The fact that a vaccinia variant with a smaller

developed by Mackett et al. (7) for insertion of foreign DNA into the thymidine kinase (*TK*) gene of the vaccinia genome also appeared appropriate for insertion of vaccinia wild-type DNA into the genome of a deletion mutant.

Restriction enzyme analysis of DNA from the *hr* mutant and the Copenhagen strain of the wild-type virus demonstrated that the deletion spanned an 18-kbp sequence in the left-hand end of the molecule (Fig. 1). The *Eco*RI fragments C, J, and K contained sequences overlapping the entire deletion as well as some undeleted material flanking both

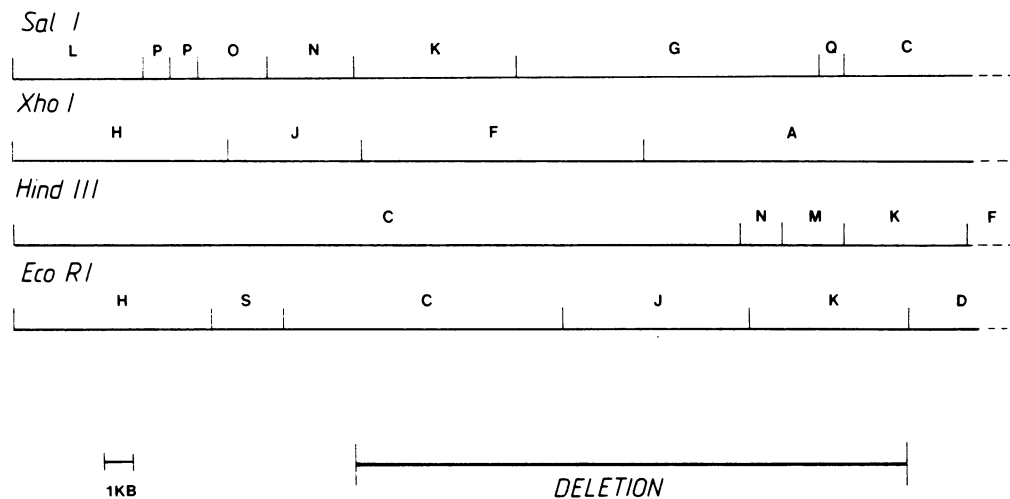


FIG. 1. Restriction enzyme map of the left-hand end of the vaccinia virus wild-type genome (Copenhagen strain). The upper limits of the deletion in the *hr* genome have been found to extend from the *Sal*I site separating *Sal*I fragments N and K to the *Eco*RI site separating *Eco*RI fragments K and D.

deletion in the same region of the genome did not display a host range phenotype on the human HeLa cell line (9, 11) suggested to us that only part of the wild-type sequences missing from the *hr* mutant is essential for multiplication on human cells. We therefore undertook to locate the region in wild-type DNA which once inserted into the *hr* deletion mutant could rescue the host range defect. The procedure

ends. We sought to clone these three *Eco*RI fragments into the vaccinia thymidine kinase gene contained in a plasmid vector pGS3. The pGS3 vector has been previously constructed by insertion of the vaccinia *Hind*III J fragment into a pBR328 vector whose *Eco*RI site had been removed (7). Copenhagen wild-type DNA was cleaved with *Eco*RI, and the fragments obtained were inserted by ligation with T4 DNA ligase into *Eco*RI-, alkaline phosphatase-treated pGS3. *Escherichia coli* HB101 was transformed with the ligation

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mix to ampicillin resistance, and the three inserts we were looking for were selected by colony hybridization to nick-translated vaccinia *SaII* fragments C, G, and K that had been previously cloned (3). Molecular cloning was carried out according to published procedures (8). Figure 2 shows the restriction profiles of the recombinant plasmids obtained as well as the maps of each construct.

Transfer of the three thymidine kinase insertion mutations to the *hr* genome required *in vivo* recombination events.

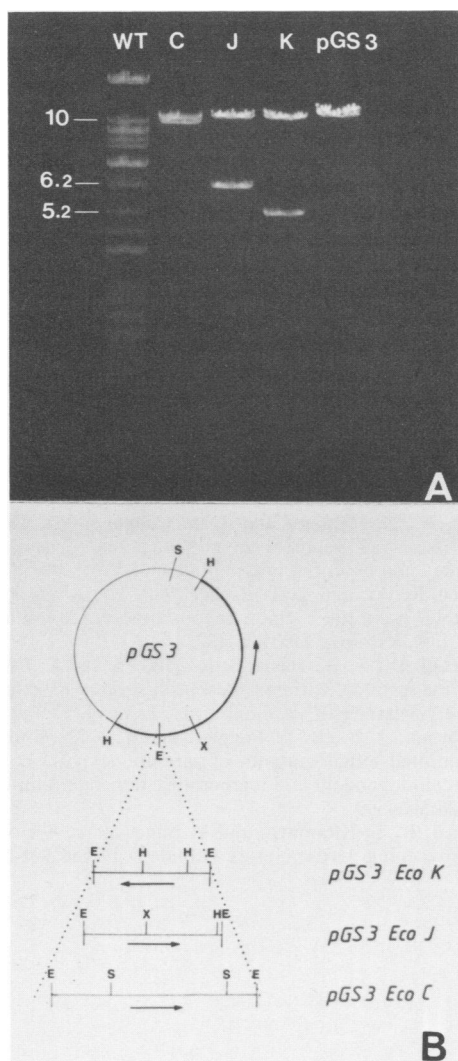


FIG. 2. Restriction digests and maps of vaccinia virus *EcoRI* fragments C, J, and K inserted into the pGS 3 plasmid vector. (A) Restriction profiles of DNA from vaccinia virus (WT), the pGS 3 plasmid vector and three plasmids derived from this vector after insertion of vaccinia fragments *EcoRI* C (10 kbp), J (6.2 kbp), and K (5.2 kbp). The viral DNA as well as the plasmid DNAs were digested to completion with *EcoRI*, and the fragments obtained were separated on an 0.6% agarose gel in the presence of ethidium bromide. (B) The vector pGS3 is represented as a circle; the thin line corresponds to pBR328 sequences, and the thick line corresponds to the vaccinia *HindIII* J fragment. Restriction maps of the three *EcoRI* fragments inserted into pGS3 are depicted beneath the plasmid. Abbreviations: E, (*EcoRI*); H, (*HindIII*); X, (*XhoI*), S, (*SaII*). The arrows indicate the polarity of the vaccinia fragments from left to right, with respect to the conventional vaccinia restriction maps.

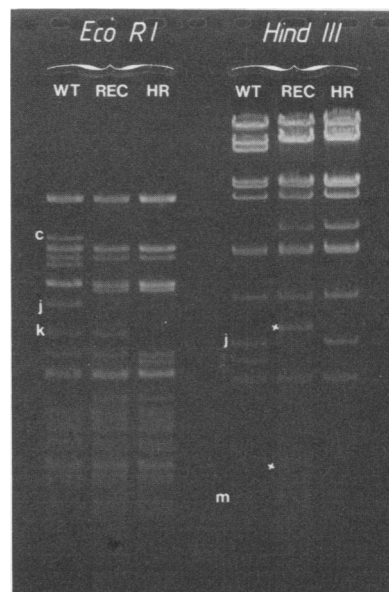


FIG. 3. Restriction enzyme profile of DNA from the wild-type virus, the *hr* mutant, and the recombinant virus. DNA purified from the wild-type virus (WT), the *hr* mutant (HR) or the recombinant virus described in the text (REC) was digested with *EcoRI* or *HindIII*, and the fragments obtained were separated by electrophoresis on an 0.6% agarose gel. The gel was stained with ethidium bromide and photographed under UV light. The *EcoRI* fragments of particular interest (C, J, K) are indicated in the margin. The *HindIII* fragments discussed in the text are indicated with letters for J or M and with a cross for the two new fragments found in the recombinant virus.

This was carried out by first infecting chicken embryo fibroblasts with the virus strain *hr ts 38* that carries both the *hr* mutation and a *ts* mutation mapping in the *HindIII* G fragment (3) and then cotransfecting cells with calcium phosphate-precipitated DNA from the *hr* mutant and one of each of the recombinant plasmids shown in Fig. 2. After incubation of the cells for 48 h at the nonpermissive temperature (39.5°C) for multiplication of the *hr ts* virus, a number of plaques appeared, presumably owing to rescue of the *ts* lesion by the addition of purified *hr* DNA. Since plaques could appear only when transfection of *hr* DNA had been successful, the procedure employing a *hr ts* virus enabled us to prevent any virus multiplication in infected cells that had not taken up exogenous DNA. Cells successfully transfected with *hr* DNA should also be competent for uptake of plasmid DNA which can participate in homologous recombination with *hr* DNA at or surrounding the *TK* locus. To reveal these recombination events cells containing plaques were frozen, thawed, and disrupted by ultrasonic treatment; the virus released was titrated on mouse LTK⁻ cells in the presence of bromodeoxyuridine and on human HepII cells. Virus capable of multiplying on both cell lines was isolated from cells that had been transfected with the plasmid containing the *EcoRI* K fragment. Virus reisolated from HepII cells could also multiply on LTK⁻ cells in the presence of bromodeoxyuridine, and vice versa. One virus isolate was further clonally purified and found to multiply with similar kinetics and to a similar yield as wild-type virus both on chicken cells and on HepII cells (not shown). These results suggest that the genetic information contained in the *EcoRI* K fragment from vaccinia DNA is capable by itself of

restoring a wild-type phenotype on the *hr* mutant. To ensure that the *EcoRI* K fragment had indeed been inserted into the vaccinia *TK* gene, DNA from the wild type, the host range mutant and the newly isolated recombinant virus were submitted to restriction enzyme analysis. It is clear that the *EcoRI* K fragment is found in the recombinant virus DNA, whereas the *EcoRI* C and J fragments are missing (Fig. 3). Moreover digestion with *HindIII* demonstrates that the *HindIII* J fragment has disappeared from recombinant virus DNA. As expected from the map of cloned *EcoRI* K fragment in Fig. 2, the recombinant virus contains the *HindIII* M fragment and two new fragments of the predicted size. The largest of the new fragments is 5.4 kbp, and it corresponds to a fusion product of the left end of the *EcoRI* K fragment and the right end of the *HindIII* J fragment. The smaller of the new fragments is 2.7 kbp, and it is a fusion product of the right end of the *EcoRI* K fragment and the left end of the *HindIII* J fragment. Therefore, the *EcoRI* K fragment was inserted into the *TK* gene of the *hr* genome in a polarity opposite the one it assumed at the left-hand end of wild-type DNA.

These results have allowed us to more precisely locate a DNA sequence that is essential for multiplication of vaccinia virus in human cells. The outcome is consistent with the isolation by others (9, 11) of a non-host range variant of vaccinia WR that is deleted in a region corresponding approximately to our *EcoRI* fragments C and J. The function of this very large region containing more than 10 kbp is still unknown. Whether the entire 5.2-kbp *EcoRI* K fragment or only part of it is needed to rescue the *hr* mutant phenotype is now being investigated. As it has been shown that this region encodes a number of early viral proteins (1), one or several of them may be needed for multiplication in human cells. Our investigation demonstrates the usefulness of the procedure devised for inserting foreign DNA into the vaccinia genome to also solve problems in vaccinia genetics. Considerable shuffling of vaccinia sequences appears to be possible without any loss of virus infectivity.

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