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Genetics

Molecular genetics of vaccinia virus: Demonstration of marker rescue

(recombination/restriction endonucleases/genomic variants/unique DNA sequences/poxviruses)

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ABSTRACT **Two genomic variants of vaccinia virus isolated from serially propagated stocks were used to demonstrate marker rescue. The smaller (S variant) virus contains a 6.3 megadalton (MDal) deletion of unique DNA sequences present in the 123-MDal larger (L variant) virus. The deletion was mapped at 6.85 MDal from the left terminus of the genome, just outside of the inverted terminal repetition. Rescue of the unique deleted DNA sequences by infectious S variant virus was obtained in CV-1 cells by using the calcium orthophosphate precipitation technique of intact or restriction endonuclease-treated L-variant DNA. Restriction fragments that overlapped the deletion allowed marker rescue, but restriction of the L-variant DNA within the unique deleted sequences gave negative results. Restriction endonuclease analysis of the DNA obtained from twice-plaque-purified recombinant virus derived from the rescue of overlap donor fragments gave a restriction pattern identical to that of L-variant virus, indicating that the donor DNA was inserted into the rescuing virus by double recombination. No amplification of the unique sequences was observed from intact L-variant DNA in the absence of infectious S-variant virus, suggesting that deproteinized vaccinia DNA is noninfectious and that the donor DNA was neither integrated into the host DNA nor present as an episomal structure. By using 1 μg of intact L-variant DNA per CV-1 monolayer in a 6-cm Petri dish, ≈1–5% of the plaques contained the L-variant genotype, and the dose–response curve was essentially linear from 0.1 to 2 μg of DNA.**

Marker rescue describes the recovery of genetic characteristics from either complete but inactive genomes or from subgenomic fragments. This technique has been used to assign specific genetic markers to discrete physical locations on the genome, facilitating the understanding of viral gene expression and regulation. Marker rescue is much more precise in gene localization than is classical recombinational analysis (which gives linear orders but not exact physical locations) and is much less laborious than the analysis of intertypic recombinants. Thus, based directly on the technique reported by Hutchison and Edgell (1) or on variations thereof, the genomes of φX174 (2), simian virus 40 (SV40) (3, 4), polyoma (5), adenovirus (6, 7), and herpesvirus (8–12), among others, have been extensively mapped by marker rescue. For viruses like φX174, SV40, and polyoma, marker rescue was facilitated by the circularity and infectious nature of the viral DNA. Thus, partial or complete heteroduplexes, formed between single-stranded mutant DNA and wild-type genomic segments, were constructed and allowed to repair and replicate in the cell. Progeny genomes contained both the mutant and wild-type genotype. For viruses like adenovirus and herpesvirus, which have linear genomes, marker rescue depends on recombination within the cell. Marker rescue in the adeno- and herpesvirus systems was facilitated by the infectious nature of the viral DNAs because recombination from coinfection of cells with both mutant and wild-type viral DNA was more efficient than when infectious virus was relied upon as the rescuing vehicle (9, 10). The sensitivity of marker rescue in the physical localization of genetic elements relies greatly on the ability to produce a series of discrete fragments whose exact physical location on the genome is known. This is achieved through the use of restriction endonucleases.

Although poxviruses are known to readily undergo genetic recombination and the phenomenon of genetic reactivation has been well characterized (13), marker rescue at the molecular level has not been demonstrated to date. The genomic complexity of poxviruses and the lack of demonstrable infectivity from naked poxviral DNA have hindered advances in this area.

We have demonstrated the presence of two major genomic variants of vaccinia in serially propagated viral stocks (14). The smaller (S variant) virus is apparently a spontaneous deletion of ≈6.3 megadaltons (MDal) from the 123-MDal larger (L variant) virus. This deletion has been mapped 6.85 MDal from the left terminus of the vaccinia genome, just outside of the inverted terminal repetition. Although RNA is transcribed from these unique deleted sequences both *in vitro* and *in vivo*, the genetic information is not essential for viral replication in either HeLa or CV-1 cells (14). We have taken advantage of these genomic variants to demonstrate the phenomenon of marker rescue in the poxvirus system. Because we looked at a deletion mutant, problems with leakiness or spontaneous reversions (as are encountered with conditional lethal mutants) did not have to be considered. The demonstration of marker rescue of the unique DNA sequences present in the L-variant genome by infectious S-variant vaccinia virus is the subject of this report.

MATERIALS AND METHODS

Viruses and Cells. The characterization of the L and S variants of the WR strain of vaccinia and their restriction maps have been described in detail (14). Virus stocks were prepared in suspension HeLa cells and purified essentially as described by Joklik (15). Titrations and marker rescue experiments were performed under agar overlay on monolayers of CV-1 cells.

Preparation of Intact and Endonuclease-Digested Vaccinia DNA. Vaccinia DNA was extracted and purified from virions as follows. Purified virions were lysed at a concentration of 50 A_{260} units/ml in 10 mM Tris·HCl, pH 7.8/50 mM 2-mercaptoethanol/100 mM NaCl/10 mM Na_3EDTA/1% Sarkosyl NL-97/26% sucrose. Proteinase K was added to 100 μg/ml, and the lysate was incubated at 37°C overnight. DNA was extracted by addition of an equal volume of phenol/chloroform, 1:1 (vol/vol). The organic phase was removed, and the aqueous phase

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Abbreviations: Me_2SO, dimethyl sulfoxide; MDal, megadaltons; SV40, simian virus 40.

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was reextracted until the interphase was clear. Two additional extractions with chloroform were carried out, and the aqueous phase was dialyzed extensively against 10 mM Tris·HCl, pH 7.4/0.1 mM Na_3EDTA at 4°C. DNA was concentrated to ≈100 μg/ml with Ficoll.

Restriction endonuclease digestions with *Sma* I, *Sst* II, *Hin*dIII, and *Ava* I were performed as described (14). Nuclease digestion with *Eco*RI and *Bst*EII was carried out under the standard conditions specified by the manufacturer, Boehringer Mannheim. DNA fragments were fractionated on agarose gels and isolated by adsorption and elution from glass powder as detailed (14).

Marker Rescue. Marker rescue was performed on CV-1 monolayers by using the calcium phosphate technique of Graham and van der Eb (16) as modified by Stow and Wilkie (17) and Wigler *et al.* (18) and reviewed by Graham *et al.* (19). Confluent monolayers (CV-1) were infected with S variant vaccinia virus to give ≈50–200 plaques per 6-cm Petri dish. After a 1-hr adsorption period, intact or restricted L variant DNA was added to the monolayers as a calcium phosphate precipitate. After 40 min, overlay media was added; 4 hr after the initial addition of DNA, the monolayer was exposed to 1 ml of buffered 25% dimethyl sulfoxide (Me_2SO) for 4 min. The Me_2SO was removed, the monolayers were washed and overlayed with nutrient agar, and, after 3 days, the cells were stained with an agar overlay containing neutral red. The next day the agar overlay was removed, and the monolayers were transferred to nitrocellulose filters.

Transfer of Monolayers to Nitrocellulose Filters and *In Situ* Hybridization. Monolayers were transferred to nitrocellulose filters and prepared for *in situ* hybridization as described by Villarreal and Berg (20). The nitrocellulose filters were interleaved with Whatman no. 1 filter paper circles in 6-cm Petri dishes and prehybridized for 6 hr at 60°C in 0.9 M NaCl/0.09 M sodium citrate/containing Denhardt's solution (0.02% bovine serum albumin/0.02% polyvinyl pyrrolidone/0.02% Ficoll) (21), 1 mM EDTA, and sonicated, denatured salmon sperm DNA at 100 μg/ml. Radioactive probe, consisting of ^{32}P-labeled nick-translated (22) L variant *Ava* I H fragment (specific activity of ≈1 × 10^8 cpm/μg) was used for hybridization at ≈1 × 10^5 cpm/ml overnight at 60°C in 0.3 M NaCl/0.03 M sodium citrate containing Denhardt's solution, 1 mM EDTA, 0.1% $NaDodSO_4$, 10% dextran sulfate (23), and sonicated, denatured salmon sperm DNA (50 μg/ml). The filters were washed repeatedly at room temperature in 0.3 M NaCl/0.03 M sodium citrate/0.1% $NaDodSO_4$, washed twice at 60°C in 0.03 M NaCl/0.003 M sodium citrate/0.1% $NaDodSO_4$, air dried, and radioautographed.

RESULTS

Rescue of Unique L Variant DNA Sequences by Infectious S Variant Virus. Three basic preparations of L variant DNA were prepared for the marker rescue studies. The first consisted of purified, intact L variant DNA. The second consisted of L variant DNA digested with appropriate restriction endonucleases generating donor DNA fragments that overlapped the unique sequences. The third preparation consisted of L variant DNA digested with restriction endonucleases that cleaved within the unique L variant DNA sequences.

The restriction endonuclease cleavage sites localized towards the left terminus and contained within the 39.1-MDal *Sst* II B fragment of the vaccinia DNA pertinent to the marker rescue experiments reported here are shown in Fig. 1. Digestion of the L variant genome with *Ava* I generated a 6.8-kilobase pair fragment, H, that resides entirely within the unique DNA sequences deleted in the S variant genome (ref. 14; Fig. 1). Thus,

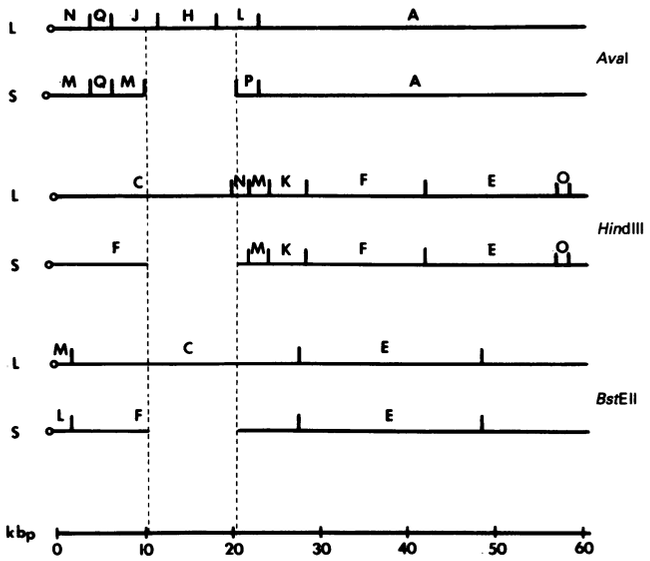

FIG. 1. Restriction endonuclease cleavage maps of the prototype L variant vaccinia genome and its S variant deletion mutant. The physical locations of the DNA fragments generated by cleavage with *Ava* I, *Hin*dIII, and *Bst*EII and localized within the left terminal 39.1-MDal *Sst* II B fragment of the prototype vaccinia genome are shown. kbp, Kilobase pair.

^{32}P-labeled nick-translated *Ava* I H fragment provided a highly specific probe for detecting the rescue of the unique L variant DNA sequences. Both *Hin*dIII (Fig. 1) and *Eco*RI (unpublished data) cleaved the L variant DNA within the unique deleted sequences. Digestion with *Bst*EII generated a 16-MDal fragment, C, that overlaps the deletion, cleaving within the left terminus. Digestion with *Sma* I cleaved the L-variant DNA once, generating a large overlap fragment of approximately 100 MDal (ref. 14; not shown).

The results of a typical marker rescue experiment are shown in Fig. 2. No hybridization of the ^{32}P-labeled nick-translated *Ava* I H-fragment probe was observed with uninfected (Fig. 2*A*) or S variant virus-infected CV-1 monolayers (Fig. 2*C*). The latter result was expected because the *Ava* I H fragment resides completely within the deleted DNA sequences (ref. 14; Fig. 1). No hybridization of the probe was observed when uninfected CV-1 cells were challenged with purified, intact L variant DNA in the absence of S variant rescuing virus (Fig. 2*B*). This result suggests that purified vaccinia DNA as prepared here is not infectious for CV-1 cells. No evidence for replication of naked vaccinia DNA was detected under a variety of experimental conditions. Under these conditions, herpesvirus DNA showed at least a 10^5-fold greater specific transfectivity (unpublished data).

Considerable hybridization of the probe was observed to filters containing imprints of CV-1 monolayers infected with L variant virus (Fig. 2*D*). When purified intact, *Sma* I-, or *Sst* II-digested L-variant DNA was introduced into CV-1 cells that had been infected with S-variant virus (Fig. 2 *E*, *F*, and *G*, respectively), rescue of the unique sequences was scored by a positive hybridization with the *Ava* I H-fragment probe, indicating that the L variant genotype had been replicated. The data presented in Fig. 2 also suggest that the rescue of unique L variant DNA sequences by S variant virus is not due to encapsidation of the intact L variant genome by S variant viral products because L variant DNA digested with either *Sma* I or *Sst* II was also capable of being rescued by S variant virus.

Table 1 summarizes the results of a number of experiments looking at the relative efficiency of marker rescue using intact

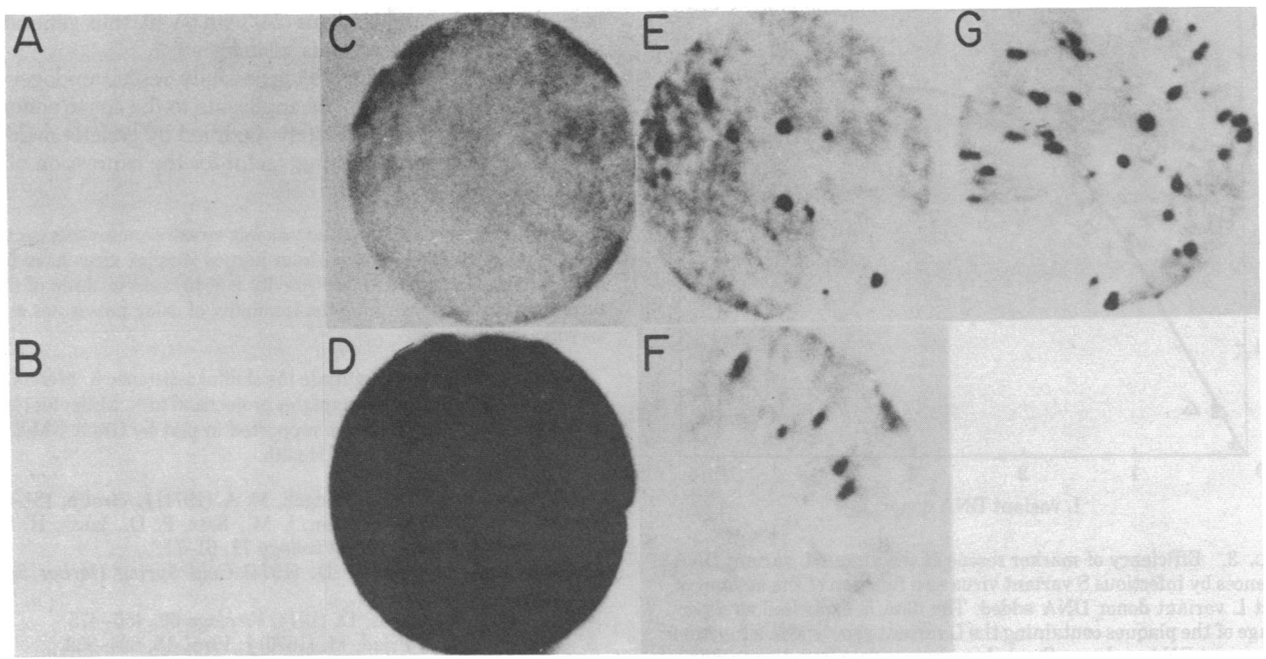

FIG. 2. Rescue of unique L variant DNA sequences by infectious S variant vaccinia virus. Monolayers of CV-1 were transferred to nitrocellulose filters and prepared for *in situ* hybridization as described by Villarreal and Berg (20). Intact or restricted L variant donor DNA and calf thymus DNA carrier in 10 mM Tris·HCl/1 mM EDTA/250 mM $CaCl_2$, pH 7.4, were added to an equal volume of 280 mM NaCl/1.5 mM sodium phosphate/50 mM Hepes, pH 7.0, slowly with constant mixing provided by bubbling N_2 through the solution. After 30 min at room temperature, 0.2 ml of the precipitate containing 1 μg of viral DNA and 2 μg of carrier DNA was added to the monolayers. ^{32}P-Labeled nick-translated *Ava* I H fragment (specific activity = $\approx 1 \times 10^8$ cpm/μg) was used for *in situ* hybridization. The dark images in the radioautograms are coincident with plaques as detected by staining the monolayer with neutral red and are indicative of the successful marker rescue and replication of the unique L variant DNA sequences. The radioautograph is of the following CV-1 monolayer imprints: uninfected CV-1 monolayers (*A*); uninfected CV-1 monolayers transfected with total purified L variant DNA (*B*); S variant (*C*) or L variant (*D*) virus-infected CV-1 monolayers; and monolayers of CV-1 cells infected with S variant virus and cotransfected with total (*E*), Sma I-digested (*F*) or Sst II-digested (*G*) L variant DNA.

or endonuclease-cleaved L variant donor DNA and infectious S variant vaccinia. It should be noted that all donor DNA fragments that overlapped the deletion allowed successful marker rescue. Significantly, restriction enzymes that cleaved the L variant DNA within the unique sequences such as *Hin*dIII, *Ava* I, and *Eco*RI did not allow rescue of the unique sequences by S variant virus. Because these three enzymes cleaved within the unique sequences, no overlap fragments were available for recombinational events. It is significant that the *Bst*EII-digested DNA could be rescued because the *Bst*EII overlap donor fragment required recombinational events on both sides of the deletion.

Table 1. Efficiency of marker rescue with intact or restricted L variant DNA

Donor L variant DNA preparation	% of plaques with L variant genotype
Intact	5
Sma I	2
Sst II	3
Sma I/*Sst* II	0.6
Sma I/*Sst* II*	0.4
*Bst*EII	0.1
Ava I	0
*Hin*dIII	0
*Eco*RI	0

All preparations of the L variant DNA were total digests at 1 μg per dish, except for the preparation of fragments isolated from agarose gels. In this latter case the exact concentration is unknown but is <1 μg per dish. A minimum of 5000 plaques were analyzed for each donor DNA preparation.

* Digest fragments purified from agarose gels.

In order to obviate the possibility that intact L variant DNA was repackaged in the presence of coinfecting S variant virus, L variant donor DNA was cleaved with a number of endonucleases to generate overlap donor fragments. *Bst*EII was particularly useful because the largest fragment generated was $\approx$26 MDal and readily separable from intact DNA. Analysis of the digested DNA on agarose gels followed by quantitative densitometry ruled out detectable levels of undigested donor DNA (data not shown). This datum, in addition to that cited in the text, strongly supports the interpretation of marker rescue by recombination rather than repackaging of intact L variant DNA.

Recovery and Restriction Analysis of Recombinant Vaccinia Virus. In order to demonstrate that the unique *Bst*EII-digested L variant DNA sequences rescued by S variant virus were present in the progeny viral genome and not amplified through integration into the host chromosome or as episomal entities, a number of plaques were isolated from a marker rescue experiment. A fraction of the isolated plaques was fixed onto a nitrocellulose filter and hybridized with the ^{32}P-labeled *Ava* I H-fragment probe. Those plaques indicating a successful marker rescue were further purified by two cycles of plaque purification, grown up in HeLa cells, and purified by sucrose gradient centrifugation (15). Analysis of the genome purified from this recombinant virus with *Ava* I resulted in a profile identical to that for the L variant genotype (data not shown), ruling out integration of the donor DNA into the host or amplification of the donor sequences through an episomal structure.

Optimal Conditions for Marker Rescue. With a constant amount of infectious S variant virus as the rescuing vehicle, an approximately linear dose–response relationship is observed with intact L variant donor DNA from 0.1 to 2 μg (Fig. 3). No dramatic increase in marker rescue was observed when L vari-

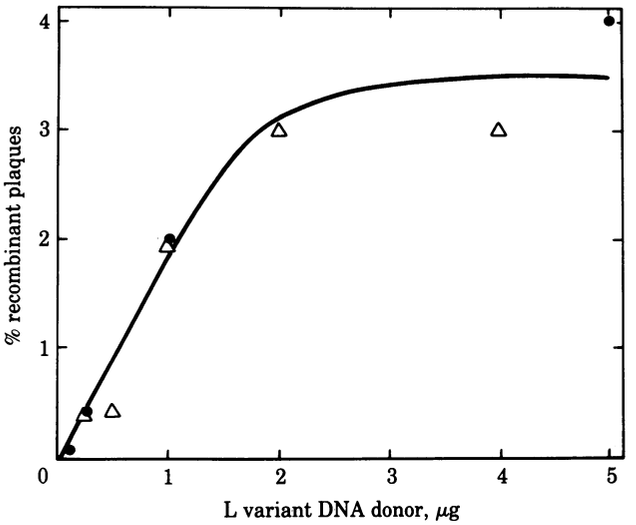

FIG. 3. Efficiency of marker rescue of the unique L variant DNA sequences by infectious S variant virus as a function of the amount of intact L variant donor DNA added. The data is expressed as a percentage of the plaques containing the L variant genotype as a function of L variant DNA as donor. Symbols represent data from two separate experiments.

ant DNA was coprecipitated in the presence of calf thymus DNA carrier from 1 to 20 μg/per ml, and, in contrast to other cell lines that respond to a Me_2SO "booster" generating an increase in the relative transfectivity of herpesvirus and adenovirus DNA (17), no increase in marker rescue was obtained with a Me_2SO booster with the CV-1 cell line used here (data not presented). Likewise the relative efficiency of marker rescue was not altered by freeze/thaw treatment of the DNA or limited digestion of the DNA with pancreatic or S1 nuclease, nor was the efficiency dependent on the metabolic state of the CV-1 cells (unpublished data).

DISCUSSION

That poxviruses are capable of genetic recombination, and genetic reactivation is well documented. We have taken advantage of the two genomic vaccinia variants isolated from serially propagated viral stocks to demonstrate the phenomenon of marker rescue. This positive finding will facilitate the precise mapping of genes of the complex vaccinia virus genome in studies similar to those used for other animal viruses such as polyoma, SV40, adenoviruses, and herpesviruses. Conditional lethal mutations and the localization of such genes as those coding for thymidine kinase and rifampicin sensitivity, for example, should be readily mapped in the genome, aiding in the understanding of the genomic architecture and regulation of gene expression in this virus system.

By manipulation of the DNA with restriction enzymes it should now be possible to construct deletion mutants, as has been done by Lai and Nathans (24) with SV40, thus generating new molecules of the vaccinia genome.

In addition, the ability to successfully rescue endogenous genomic markers also may be applicable to the construction of a vaccinia genome appropriately modified by genetic manipulation for use as a new cloning vector for the expression of exogenous DNA in eukaryotic cells.

Note Added in Proof. Six unique vaccinia virus recombinants containing the thymidine kinase gene from herpes simplex virus have been constructed. Thymidine kinase activity is expressed in three of these recombinants, demonstrating the feasibility of using poxviruses as eukaryotic viral vectors.

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