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Physical Mapping of the Herpes Simplex Virus Type 2 *nuc*$^-$ Lesion Affecting Alkaline Exonuclease Activity by Using Herpes Simplex Virus Type 1 Deletion Clones

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The *nuc*$^-$ lesion affecting alkaline exonuclease activity in the herpes simplex virus type 2 (HSV-2) mutant *ts*1348 had previously been mapped to the *Eco*RI-D restriction enzyme fragment of HSV-1. Eight clones with deletions representing most of HSV-1 *Eco*RI fragment D were selected with lambda gtWES hybrids. These clones were tested for their ability to rescue the alkaline exonuclease activity of HSV-2 *nuc*$^-$ *ts*1348 virus. The sequences colinear with the HSV-2 *nuc*$^-$ lesion were found to map between 0.169 and 0.174 map units on the HSV-1 Patton genome, representing an 0.8-kilobase-pair region that is 12.9 to 13.7 kilobase pairs from the left end of HSV-1 *Eco*RI fragment D.

Infection of cells with herpes simplex virus type 1 (HSV-1) or HSV-2 results in the induction of many enzymes associated with DNA synthesis, one of which is an alkaline exonuclease (5, 6, 10, 17). This enzyme is virus encoded since it can be synthesized in *Xenopus laevis* oocytes after microinjection of specific HSV-1 DNA fragments (14). The thermolability of the alkaline exonuclease in cells infected with nuclease-negative (*nuc*$^-$), temperature-sensitive mutants, such as the HSV-2 mutants *ts*13 and *ts*1348, also suggest a viral origin (3, 12). Using the *ts*1348 mutant, we determined the *nuc*$^-$ mutation to be between 0.12 and 0.21 map units on the HSV-2 genome (12). Fine mapping of a viral gene by marker rescue requires uncontaminated and distinct genome fragments. Generation of such fragments by subcloning larger restriction enzyme fragments can be time consuming and requires the fortuitous location of appropriate restriction enzyme sites in the region of interest. Umene and Enquist (18, 19) have demonstrated that many distinct deletions can be produced in HSV fragments inserted into a lambda phage vector by selection in the presence of 0.4 mM EDTA. EDTA chelates magnesium ions, which are required for the stable packaging of oversized phage genomes. We used this technique to generate deletions spanning *Eco*RI fragment D of HSV-1 (0.08 to 0.19 map units) and further demonstrated that these deletion phage hybrids may be used in marker rescue experiments to fine-map a viral gene (alkaline exonuclease).

BHK-21 C13 cells were grown in Eagle medium supplemented with 10% newborn calf serum and 10% tryptose phosphate broth. The virus used was the HSV-2 *nuc*$^-$ mutant *ts*13 revertant 4-8 (1348) (12). This mutant did not synthesize levels of alkaline exonuclease at the nonpermissive temperature of 38.5°C. *Eco*RI fragment D was mapped by secondary digestion of the purified fragment by restriction enzymes *Bgl*II, *Bam*HI, and *Sal*I (Bethesda Research Laboratories Inc., Gaithersburg, Md.). Restriction enzyme fragments were isolated from low-melting agarose gels (SeaPlaque; FMC Corp., Rockland, Maine) by the method of Langridge et al. (7). The purified restriction enzyme fragments were redigested with *Bgl*II, *Bam*HI, *Sal*I, or *Pst*I and analyzed by electrophoresis in agarose gels as previously described (1). The λgtWES hybrid containing the *Eco*RI fragment D of HSV-1 Patton was provided by L. Enquist (2). Single-plaque lysates of phages were grown in *Escherichia coli* LE392 as previously described (18). Lysate preparation and determination of titers were carried out as described by Sternberg et al. (15). Each lysate was used to isolate a single deletion phage. Phage containing deletions in the HSV-1 insert were selected by plating with *E. coli* LE392 in tryptone broth plates and top agar containing 0.4 mM EDTA (13). One plaque capable of growth in the presence of 0.4 mM EDTA was picked from each agar plate. Phage lysates from the single-plaque isolates were prepared as described above. DNA from plaque-purified phage isolates were prepared as described above. DNA from plaque-purified phage hybrids suspected of containing deletions in HSV-1 *Eco*RI fragment D was purified as previously described (18). The location of each deletion was mapped by the loss of restriction enzyme sites. Baby hamster kidney cells infected with HSV-2 *ts*1348 at a multiplicity of infection of 0.01 PFU per cell were washed twice with phosphate-buffered saline and harvested when the cells exhibited maximum cytopathic effect. DNA was isolated from these cells by the procedure of Walboomers and Schegget (20). The marker rescue technique used was essentially that of Stow et al. (16). Briefly, baby hamster kidney cells were cotransfected with DNA from intact *ts*1348 virus and individual deletion phage hybrids by the calcium phosphate infectivity technique of Graham and van der Eb (4) and then treated with 15% dimethyl sulfoxide in HEPES-buffered saline for 4 min. After plaques had appeared (approximately 3 days postinfection), the infected cells were dispersed throughout the monolayer by trypsinization. The cells were harvested 2 days later and disrupted by sonic treatment. Released virus was used to infect baby hamster kidney cells at 38.5 and 32°C. At 18 h postinfection, the infected cells were washed twice in phosphate-buffered saline and harvested for use in the exonuclease assay. Extracts were prepared by resuspending cells from two petri dishes (60 mm in diameter) in 200 μl of a buffer containing 2 mM $MgCl_2$, 2 mM dithiothreitol, and 20

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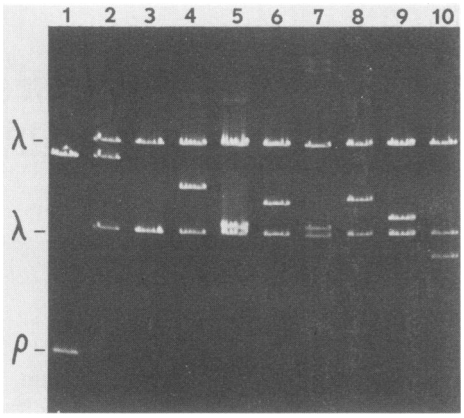

FIG. 1. Deletions in *Eco*RI fragment D of HSV-1 Patton cloned into lambda. Phage hybrids containing deletions in the HSV-1 *Eco*RI-D insert were selected by their ability to grow in the presence of 0.4 mM EDTA. DNA from eight plaque-purified isolates were digested with *Eco*RI and fractionated by electrophoresis in a 0.5% agarose gel. The isolates were D1-2 (lane 3), D1-3 (lane 4), D1-4 (lane 5), D1-5 (lane 6), D1-7 (lane 7), D1-8 (lane 8), D1-9 (lane 9), and D1-10 (lane 10). Undeleted *Eco*RI fragment D cloned into both plasmid (lane 1) and lambda (lane 2) vectors were digested with *Eco*RI and run in the gel as reference. The fragments due to lambda (λ) or plasmid (p) vectors are indicated.

mM Tris-hydrochloride (pH 7.5). The cells were disrupted by sonic treatment, and debris was pelleted at 12,000 × *g* for 2 min in a microcentrifuge. The assay mixture (100 μl) contained 7 μg of [^{3}H]thymidine-labeled baby hamster kidney cell DNA (7,000 cpm/μg), 50 mM Tris-hydrochloride (pH 9.0), 10 mM dithiothreitol, 5 mM $MgCl_2$, and 2 μl (32°C extract) or 25 μl (38.5°C extract) of the extract prepared as described above to keep all of the reactions in the linear phase. After incubation at 37°C for 30 min, the mixture was cooled on ice and acid-soluble counts were measured essentially by the procedure of Morrison and Keir (10).

Deletions in *Eco*RI fragment D of HSV-1 Patton were generated by a selection of lambda HSV-1 phage hybrids in the presence of 0.4 mM EDTA as described above. Spontaneous deletions which allowed packaging in the presence of 0.4 mM EDTA occurred at a frequency of 2×10^{-4} to 5×10^{-5} in the various phage lysate preparations. DNA from eight selected deletion clones was digested with *Eco*RI and analyzed by electrophoresis in an agarose gel (Fig. 1). All of the deletions were contained within the HSV-1 sequences, since the migration of the lambda fragments was unchanged. In one clone, the HSV-1 sequences comigrated with the lower lambda band (Fig. 1, lane 3). The molecular weight of the HSV-1 fragment was determined for each clone and the size of the deletion was calculated using a molecular weight of 16.2 kilobase pairs (kbp) for undeleted *Eco*RI fragment D. The size of the deletions varied for each clone and ranged from 2.9 to 6.7 kbp (Table 1). A map of *Eco*RI fragment D for the Patton strain of HSV-1 is shown in Fig. 2. The restriction enzyme sites were found to be very similar to those published (*Bam*HI and *Sal*I have been published for other strains) (8). Restriction enzyme fragments discussed in the rest of this paper refer to those delineated in Fig. 2. The location of the deletion in each individual clone was determined by analysis of changes in restriction enzyme patterns such as those shown in Fig. 3. The loss of one restriction enzyme fragment indicated that the deletion was contained entirely within that fragment, the loss of two fragments indicated that the deletion crossed one restriction enzyme site, and the loss of more than two fragments indicated that the deletion crossed several restriction enzyme sites. Analysis of the restriction enzyme patterns of the eight deletion clones for the enzymes *Bgl*II, *Bam*HI, *Sal*I, and *Pst*I are summarized in Table 2. The outer parameters for each deletion were established by measuring the length of the deletion away from the outermost restriction enzyme site crossed. For instance, the 2.9-kbp deletion in D1-3 resulted in the loss of *Pst*I fragment E but not *Sal*I fragment F (Table 2), so the left end of the deletion must lie between the *Pst*I site at 0.154 map units and the *Sal*I site at 0.149 map units (Fig. 2). Therefore, the furthest right the deletion could extend is 2.9 kbp from the *Pst*I site, at 0.154 map units,

TABLE 1. Size of deletion in HSV-1 phage hybrids

Clone	Deletion size (kbp)
D1	0
D1-2	5.9
D1-3	2.9
D1-4	5.4
D1-5	4.0
D1-7	5.2
D1-8	3.7
D1-9	5.0
D1-10	6.7

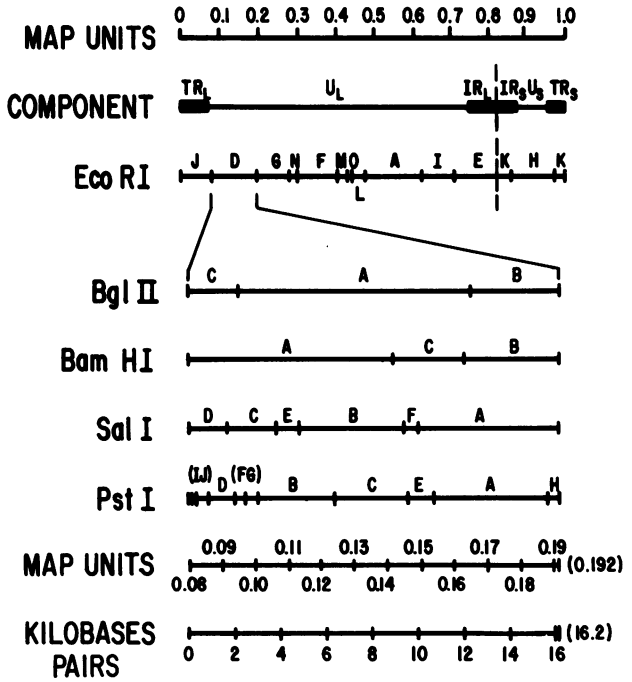

FIG. 2. Physical map of the *Eco*RI cleavage sites for HSV-1 Patton and of the *Bgl*II, *Bam*HI, *Sal*I, and *Pst*I cleavage sites for *Eco*RI fragment D. *Eco*RI fragment D lies within the long unique section of the HSV-1 Patton genome between 0.080 and 0.192 map units and is 16.2 kbp long. Restriction enzyme fragments within the *Eco*RI-D region were alphabetically labeled according to size for each enzyme. Letters in parentheses indicate areas of uncertainty in the map.

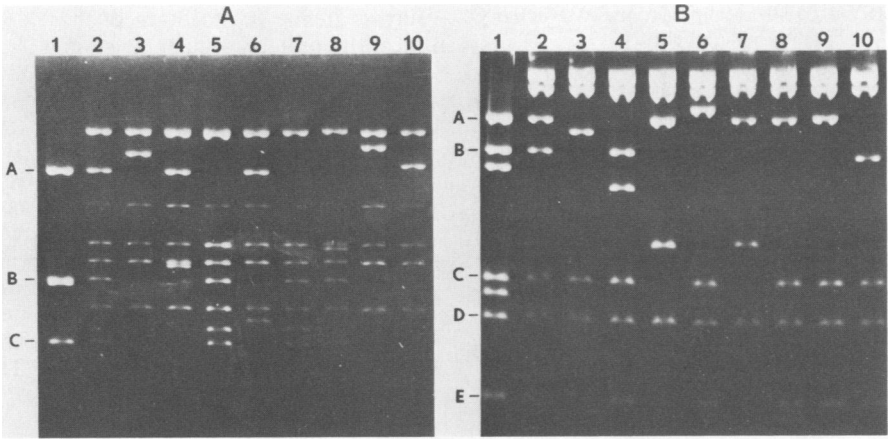

FIG. 3. Restriction enzyme analysis of deletion clones. Lambda clones containing deletions in the HSV-1 *Eco*RI-D insert were digested with *Eco*RI-*Bam*HI (A) or *Eco*RI-*Sal*I (B) and fractionated by electrophoresis in 1% agarose gels. Undeleted *Eco*RI-D cloned into both plasmid (lane 1) and lambda (lane 2) vectors were digested with the appropriate enzymes and run in the gels for reference. The clones analyzed were D1-2 (lane 3), D1-3 (lane 4), D1-4 (lane 5), D1-5 (lane 6), D1-7 (lane 7), D1-8 (lane 8), D1-9 (lane 9), and D1-10 (lane 10). The HSV-1 fragments were alphabetically labeled according to size for each enzyme (Fig. 2).

which establishes the right parameter at 0.174 map units. Similar logic was used in mapping the deletions in all the clones. No evidence for multiple deletions was found in any of the clones, although small additional deletions may have been missed. The fine mapping of the eight deletion clones is summarized in Fig. 4. The eight deletion clones were tested for their ability to rescue the alkaline exonuclease activity of *ts*1348 in transfection experiments as described above. Virus resulting from the transfection of *ts*1348 and individual deletion clones was used to infect cells at both the permissive and the nonpermissive temperature. The alkaline exonuclease activity detected in cells after infection at the nonpermissive temperature is presented in Table 3. Error

TABLE 2. Restriction enzyme fragments missing in deletion clones

Restriction enzyme and fragment		Deletion clone[a]							
		D1-2	D1-3	D1-4	D1-5	D1-7	D1-8	D1-9	D1-10
*Bgl*II									
A[b]	k[c]	−	−	−	−	−	−	−	−
B	o	−	−	+	−	+	+	+	−
C	j	+	+	−	+	+	+	+	+
*Bam*HI									
A	c	−	+	−	+	−	−	−	−
B	a	−	−	+	−	+	+	−	−
C	a	−	−	+	−	+	+	−	−
*Sal*I									
A	d	−	−	+	−	+	+	−	−
B	m	−	+	−	−	−	−	−	−
C	c	+	+	−	+	−	+	+	+
D	c	+	+	+	+	+	+	+	+
E	z	+	+	−	+	−	+	+	+
F	h[1]	−	+	+	−	+	+	−	−
*Pst*I									
A		−	−	+	−	+	+	−	−
B		+	+	−	+	−	−	+	−
C		−	+	−	−	−	−	−	−
D		+	+	−	+	+	+	+	+
E		−	−	+	−	+	+	−	−
F		+	+	−	+	+	+	+	+
G		+	+	−	+	+	+	+	+

[a] +, Restriction enzyme fragments whose migrations were unaffected by the deletion; −, fragments whose migrations were altered by the deletion and were therefore missing in the restriction enzyme digestion.

[b] A restriction enzyme map of the HSV-1 Patton *Eco*RI-D region for the four enzymes listed is shown in Fig. 1.

[c] Restriction enzyme fragments, from the Locker and Frenkel (8) study, which correspond (in whole or in part) to the fragments found in this study. Some strain variation is evident.

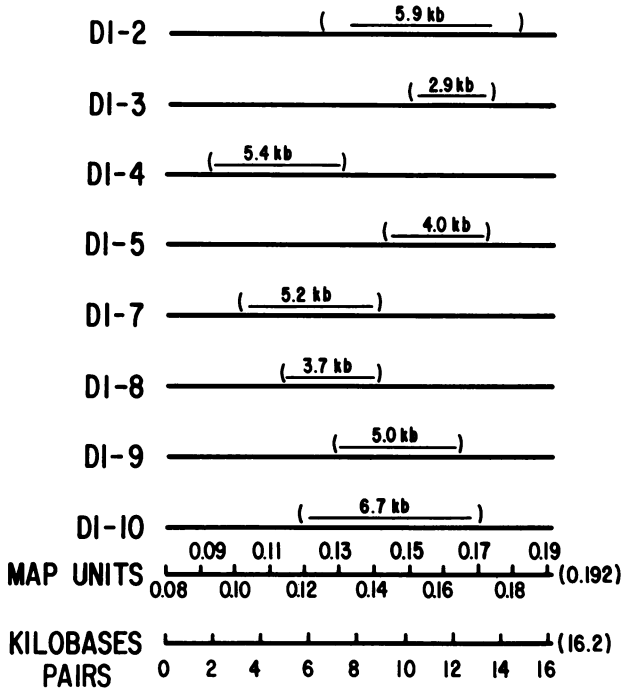

FIG. 4. Summary of the map locations of deletions in HSV-1 *Eco*RI fragment D which had been cloned into lambda gtWES. The map units and size (in kbp) for the *Eco*RI fragment of HSV-1 Patton are indicated. The limits of the map locations for the deletion in each clone are indicated in parentheses, and its size (in kbp) is indicated.

TABLE 3. Rescue of HSV-2 *ts*1348 alkaline exonuclease activity with HSV-1 deletion phage hybrids

Deletion clone[a]	Exonuclease activity (cpm)[b]	Cpm of sample/ cpm of *ts*1348
—[c]	1,270	1
D1-2	2,070	1.6
D1-3	4,020	3.2
D1-4	7,040	5.5
D1-5	20,200	15.5
D1-7	9,250	7.3
D1-8	13,900	10.9
D1-9	19,700	15.5
D1-10	5,900	4.6

[a] Extracts used in the exonuclease assay were prepared from cells at 38.5°C (nonpermissive temperature) with virus obtained from transfections with a mixture of *ts*1348 and the indicated deletion clone.

[b] The counts per minute were adjusted for alkaline exonuclease activity present in uninfected cells. Counts were corrected for differences in infectivity by equalizing the exonuclease activity detected in cells infected at the permissive temperature (32°C) using the following formula: cpm of sample at 38.5°C × (cpm of *ts*1348 at 32°C/cpm of sample at 32°C).

[c] —, Cells used to prepare the extract were infected with virus obtained from transfection of *ts*1348 alone.

due to variability in viral replication or multiplicity of infection of the virus between preparations was detected by comparing the exonuclease activity present in cells infected with each preparation at the permissive temperature. This activity would theoretically be equal for all preparations. The exonuclease activity at the nonpermissive temperature was then corrected for differences in infectivity by standardizing the activity detected at the permissive temperature (Table 3). The ability of a deletion clone to rescue the *nuc*$^-$ mutation of *ts*1348 was measured by the relative increase above the baseline of activity detected with *ts*1348 at the nonpermissive temperature. A fivefold rise in exonuclease activity was arbitrarily chosen as the amount required to denote rescue. By this criterion, the deletion clones D1-4, D1-5, D1-7, D1-8, and D1-9 were capable of rescuing the *ts*1348 exonuclease activity (Table 3). Deletion clones D1-2 and D1-3 did not rescue exonuclease activity. Therefore, the deletions in these clones must have covered the *nuc*$^-$ mutation in *ts*1348. The relative increase in activity with the D1-10 clone was on the borderline of the criteria set for rescue and therefore was not used in mapping the *ts*1348 *nuc*$^-$ locus. Analysis of the map locations of the deletions (Fig. 4) showed that the deletions in clones capable of rescue shared the same sequences with the deletions in clones D1-2 and D1-3, except in the region to the right of the deletion in clone D1-5. The furthest to the right that the D1-3 deletion could extend is 0.174 map units. Therefore, the sequences colinear with the *nuc*$^-$ mutation in the exonuclease gene of HSV-2 *ts*1348 lie between 0.169 and 0.174 map units (0.8 kbp) on the HSV-1 Patton genome.

Our previous work with the *ts*13 exonuclease activity (3, 12) suggested that the temperature-sensitive mutation occurred in the structural gene(s) for the enzyme rather than in ancillary (e.g., controlling) regions. Thus, it seems likely that the region 0.169 to 0.174 contains part of the coding sequence for the exonuclease enzyme. Preston and Cordingley (14) have mapped the coordinates of the HSV-1 exonuclease gene to between 0.145 and 0.185 map units by analyzing its expression in *X. laevis* oocytes after microinjection of cloned HSV-1 fragments. They have also suggested that the protein V185 (ICP 18), which maps in this region (9, 11), is the alkaline exonuclease polypeptide (14). Our mapping studies of the *nuc*$^-$ mutation substantiate these findings and further define the location of the exonuclease gene. More recently, transcript mapping studies (E. K. Wagner, personal communication) have been carried out in which a 2.3-kilobase RNA was identified as the message for exonuclease; this RNA mapped from about 0.160 to 0.176 map units, a region which includes the position we have proposed for the *ts*1348 exonuclease mutation. Generating the HSV-1 deletion clones has the advantage of not relying on strategically placed restriction enzyme sites which are required for subcloning. The deletions which were analyzed were both of random size and randomly located throughout *Eco*RI fragment D. Therefore, generating the deletion clones was a simple and rapid method for making differential probes for use in marker rescue experiments. These deletion clones could be used for many other purposes, such as transcription studies of the *Eco*RI-D region.

Two points of caution should be discussed in relation to the validity of the data presented here. First, deletions in DNA were mapped in such a way that small deletions would escape detection. Obviously any such undetected sequence interruptions could be lethal for rescue of exonuclease activity and might lead to a false-negative result. Second, we have experienced some difficulty in marker rescue experiments stemming from the efficiency of the process in our hands. Our earlier, similar experience (12) was put down to fragment contamination in DNA preparations which gave high and variable backgrounds. In the present work, however, all of the DNA fragments used were cloned in lambda, which should eliminate such DNA contamination problems; perhaps inherent leakiness of this exonuclease mutation is the major problem.

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