JOURNAL OF VIROLOGY, Oct. 1979, p. 140-146
0022-538X/79/10-0140/07$02.00/0

Vol. 32, No. 1

Mutant of Herpes Simplex Virus Type 2 with Temperature-Sensitive Lesions Affecting Virion Thermostability and DNase Activity: Identification of the Lethal Mutation and Physical Mapping of the *nuc*$^-$ Lesion

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Received for publication 12 March 1979

We had previously shown that a temperature-sensitive (*ts*) mutant of herpes simplex virus type 2 strain HG52, *ts*13, induced a heat-labile DNase activity in infected cells (B. Francke, H. Moss, M. C. Timbury, and J. Hay, J. Virol. **26**:209-213, 1978). Earlier work indicated that the mutant also possessed temperature-sensitive infectivity (I. W. Halliburton and M. C. Timbury, J. Gen. Virol. **30**:207-221, 1976). In this study temperature-stable revertants of *ts*13 have been isolated; examination of them revealed that *ts*13 is a double mutant, with genetically distinct temperature-sensitive lesions affecting nuclease activity and particle stability. The lethal mutation, in the cell system studied, is the latter. Revertants, which all maintain the nuclease lesion, grew well at a high temperature. Physical mapping of the nuclease lesion placed it between 0.12 and 0.21 (fractional length) on the virus genome, quite distant from the lethal mutation at 0.64 to 0.70.

One of the striking features of mammalian cells infected with herpes simplex virus (HSV) is the induction of an alkaline exonuclease activity specific for DNA (7). Recently, this nuclease activity has been extensively purified (2, 5), and some of its characteristics have been determined. Thirteen temperature-sensitive (*ts*) mutants of HSV type 2 (HSV-2) were examined for their ability to induce this nuclease activity under restrictive conditions, and one of these, HSV-2 *ts*13, failed to produce more than background levels of activity (H. Moss and J. Hay, unpublished observations). Subsequent work has shown that the HSV-2 *ts*13-induced nuclease activity is temperature sensitive both in vivo and in vitro (2), and this provides good evidence for the virus-coded nature of at least part of it. In addition to this lesion in a gene associated with the nuclease activity, stocks of HSV-2 *ts*13 have been shown to be significantly more thermolabile than wild-type parental virus, possibly due to a temperature-sensitive structural polypeptide. In view of these results, the following experiments were designed: (i) to test for the genetic relationship between these two phenotypic characteristics; (ii) if they were separable genetically, to assay for the lethality of each lesion; and (iii) to map physically the nuclease activity lesion (nuc$^-$) on the genome of HSV-2 and compare its map position with the known lethal mutation location (P. Chartrand, N. M. Wilkie, and M. C. Timbury, manuscript in preparation).

MATERIALS AND METHODS

Cells and virus. BHK-21 C13 cells were grown in Eagle medium supplemented with 10% calf serum and 10% tryptose phosphate broth. The virus used was HSV-2 strain HG52. Virus stocks were prepared by infection of 2.5-liter roller bottles at a multiplicity of infection of 10^{-2} PFU/cell for 3 to 5 days at 31°C. The efficiency of plaquing (EOP) was assayed on BHK monolayers (8). Revertants of HSV-2 *ts*13 (4) capable of growing at 38°C were selected by plating dilutions of the virus at nonpermissive temperature and picking well-separated plaques into fresh cell suspensions. After two more cycles of plaque purification at the nonpermissive temperature, clones were grown up into stocks on BHK cells as above.

Heat inactivation of virion infectivity. Virus preparations were held at 39°C in a water bath for various periods of time, cooled in ice, and diluted for assay as described previously (4).

Preparation of DNA and isolation of restriction endonuclease fragments. Virion DNA was prepared from cells infected at 31°C at a multiplicity of infection of 10^{-2} PFU/cell as described previously (11). Restriction endonuclease cleavage of DNA and purification of DNA fragments have been described in detail elsewhere (9).

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Marker rescue with restriction endonuclease fragments. The marker rescue technique has already been fully described (9); briefly, BHK cells are coinfected at the nonpermissive temperature with intact DNA of a mutant and individual restriction endonuclease fragments of the wild-type parent DNA. DNA infections are based on the calcium phosphate infectivity technique of Graham and van der Eb (3), as recently modified (10). After incubation for 3 days at 38°C the entire monolayers are harvested and disrupted by sonic treatment, and the progeny are titrated at 38 and 31°C.

Assay of alkaline DNase activity. Nuclease activity was assayed on cell extracts at pH 9, using labelled BHK DNA exactly as described previously (2). Acid-soluble radioactivity was measured after 1 h of incubation at 31°C.

RESULTS

One of the most direct ways of assessing both the relative importance for normal growth and the genetic relatedness of two phenotypic characters in a temperature-sensitive mutant is in the isolation and characterization of revertants selected to grow under nonpermissive conditions. Several such revertants were isolated, without mutagenesis, from HSV-2 *ts*13 stocks as detailed in Materials and Methods, and two (4d2 and 5d1) were grown up into working stocks. The EOPs at 38 and 31°C of these revertants are given in Table 1, and it is clear that their relative EOP (38°C/31°C) resembles that of wild-type HSV-2 rather than that of HSV-2 *ts*13.

Phenotypic characteristics of the revertants. (i) Virion thermostability. Stocks of HSV-2 wild type, HSV-2 *ts*13, and two revertants, 4d2 and 5d1, all grown at 31°C, were incubated at 39°C for up to 6 h before infectivity assay at 31°C. Whereas HSV-2 wild-type infectivity is relatively stable under these conditions, HSV-2 *ts*13 infectivity falls rapidly, as has already been shown (4). Both revertants, however, have clearly resumed the wild-type phenotype; presumably the generation of these revertants has resulted in correction of the virion thermolability (Fig. 1).

TABLE 1. *Temperature sensitivity of HSV-2 wild-type, HSV-2 ts13, and revertants of HSV-2 ts13 grown at 31°C*

Virus stock	EOP[a] (PFU/ml) assayed at:		Relative EOP (38°C/31°C)
	31°C	38°C	
HSV-2 wild type	8.2×10^8	5.6×10^8	0.68
511[b]	4.65×10^8	3.6×10^8	0.76
HSV-2 *ts*13	1.7×10^8	1×10^3	10^{-5}
4d2[c]	5×10^8	9×10^7	0.18
5d1[c]	7.5×10^8	6×10^7	0.08
4-8[d]	1.2×10^8	1.1×10^8	0.92
5-8[d]	4.7×10^8	4×10^8	0.85

[a] Virus stocks were grown at 31°C for 72 h starting with a multiplicity of infection of 0.01 PFU/cell.

[b] HSV-2 wild type plaque purified three times at 38°C.

[c] Revertants of HSV-2 *ts*13 plaque purified three times at 38°C.

[d] As footnote *c* with three additional plaque purifications at 38°C.

(ii) DNase activity defect. One of the characteristics of HSV-2 *ts*13 is its inability to induce alkaline DNase activity at 38°C (2). This is shown in Fig. 2, which also shows that revertants 4d2 and 5d1 still express the HSV-2 *ts*13 DNase activity defect and have not reverted to the wild-type phenotype. However, all four viruses tested induce normal amounts of DNase activity at the permissive temperature (31°C).

In HSV-2 *ts*13, failure to induce DNase activity at 38°C is demonstrable in vitro (2). The above result (Fig. 2) suggests that the two revertants (4d2 and 5d1) still possess the HSV-2 *ts*13 DNase activity defect; this was confirmed by testing their DNase activity in vitro (Fig. 3).

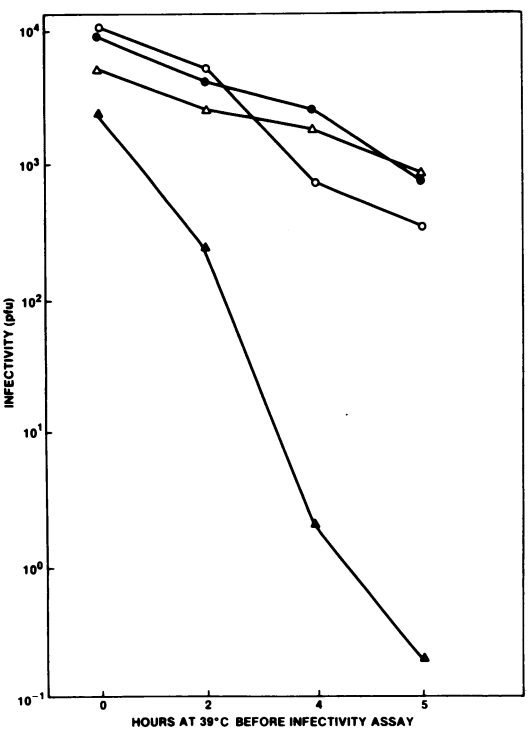

FIG. 1. *Virion thermal inactivation. Preparations of HSV-2 wild type, mutant HSV-2 ts13, and revertants 4d2 and 5d1 grown at 31°C were incubated in a water bath at 39°C for various periods of time. After cooling in ice, the residual infectivity in each preparation was assayed at 31°C. Symbols: ○, HSV-2 wild type; ●, 4d2; △, 5d1; ▲, HSV-2 ts13.*

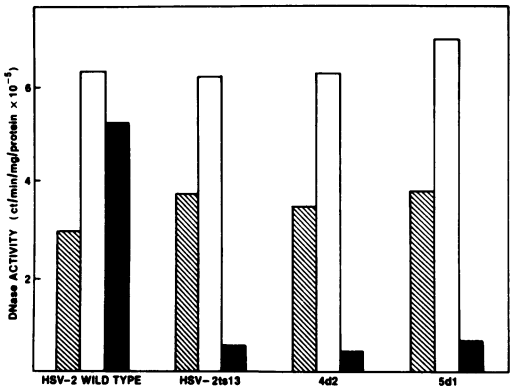

FIG. 2. *Induction of DNase activity by revertants. Cells were infected with HSV-2 wild type, HSV-2 ts13, and revertants 4d1 and 5d1 at 31 and 38°C. Extracts were assayed for DNase activity as described in the text. Symbols: ▨, 14 h at 31°C; □, 21 h at 31°C; ■, 7 h at 38°C.*

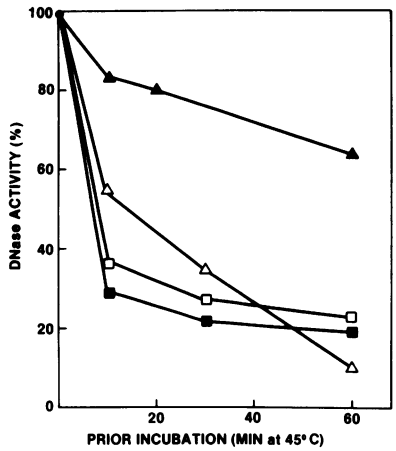

FIG. 3. *In vitro heat stability of DNase activity. Prior incubation of partially purified extracts of HSV-2 wild type-, HSV-2 ts13-, and revertants-infected cells was carried out as described previously (2) at pH 7.5 in the presence of native calf thymus DNA. After various times at 45°C, extract samples were chilled on ice and assayed (see text). 100% for each sample is DNase activity at zero time. Symbols: ▲, HSV-2 wild type; △, HSV-2 ts13; □, 4d2; ■, 5d1.*

By a technique similar to that described elsewhere (2), extracts of infected cells were partially purified by linear gradient elution from a DEAE-Sephadex column, and the in vitro stability of their DNase activities was determined. The results indicate that both revertants (4d2 and 5d1) induce DNase activity with low heat stability, similar to that of HSV-2 *ts*13 but quite unlike that of HSV-2 wild type (Fig. 3).

In an attempt to isolate revertants for the temperature-sensitive DNase activity defect, stocks of 4d2 and 5d1 were taken through a series of three further plaque purifications at 38°C. Virus stocks grown up at the end of this treatment were designated 4-8 (from 4d2) and 5-8 (from 5d1). Viruses 4-8 and 5-8 had similar EOPs at 31 and 38°C, unlike the original stock of revertant 4d2 and 5d1, which exhibited relative EOPs of 0.18 and 0.08, respectively (Table 1).

Induction of DNase activity from revertants 4-8 and 5-8 was then assessed throughout the growth cycle at 31 and 39°C. In Fig. 4 it can be seen that despite the high EOP at 38°C of 4-8 and 5-8, production of DNase activity is still as impaired as with the mutant HSV-2 *ts*13.

Physical mapping of the nuclease activity lesion (*nuc*⁻). The isolation of revertants of HSV-2 *ts*13 (4-8 and 5-8) which still induce a temperature-sensitive DNase activity allows us to use these revertant genomes in marker rescue experiments to define the physical location of the lesion.

Intact DNA from 4-8 and 5-8 was used to coinfect cells at 38°C with separated restriction endonuclease fragments of wild-type HSV-2 DNA, as described in Materials and Methods.

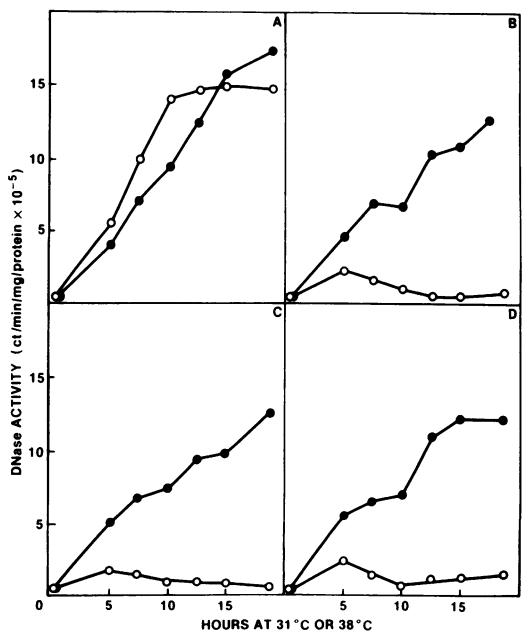

FIG. 4. *Induction of DNase activity in vitro. Cells were infected with HSV-2 wild type, HSV-2 ts13, and revertants 4-8 and 5-8 at 5 PFU/cell and incubated at 31 and 38°C. At various times, extracts were made and assayed for DNase activity as described in the text. Mock-infected cells gave background levels of activity at all times in both temperatures. (A) HSV-2 wild type; (B) 4-8; (C) HSV-2 ts13; (D) 5-8. Symbols: ●, 31°C; ○, 38°C.*

Virus progeny from these marker rescue experiments were titrated on BHK cells and used to infect 2×10^6 cells at a defined multiplicity of infection at 31 and 38°C. After 24 to 48 h the infected cells were harvested and extracts were assayed for DNase activity. All 31°C samples gave activity, including, as expected, those infected with virus derived from revertant DNA alone. On the other hand, one 39°C sample, in each case, showed higher DNase activity than any of the others. Infection of cells with wild-type virus under the same conditions gives levels of activity between two and five times those found with maximum rescue material. Figures 5, 6, and 7 give the 38°C data from experiments using, respectively, *Xba*I-, *Hin*dIII-, and *Eco*RI-derived fragments. The DNA fragments with which the highest DNase activity is seen are the same for both 4–8 and 5–8, namely, *Xba*I a, b, and c, *Hin*dIII b, and *Eco*RI j. These three fragments have overlapping DNA sequences as can be seen by comparing the restriction endonuclease cleavage maps at the bottom of Fig. 5, 6, and 7. Thus, the map position of the lesion (*nuc*$^-$) affecting the DNase activity has been narrowed down to the HSV-2 *Eco*RI j fragment, which lies between 12×10^6 and 21×10^6 daltons from the left-hand terminus of the HSV-2 genome. In Fig. 5 and 6, fragments other than those directly involved show significant levels of rescue (e.g., *Xba*I h, 5–8, Fig. 5). We have no definitive explanation for this, but we assume that leakiness or reversion is responsible.

DISCUSSION

It had already been noted by us (2) that HSV-2 *ts*13 possessed two phenotypically distinct temperature-sensitive defects, one affecting the

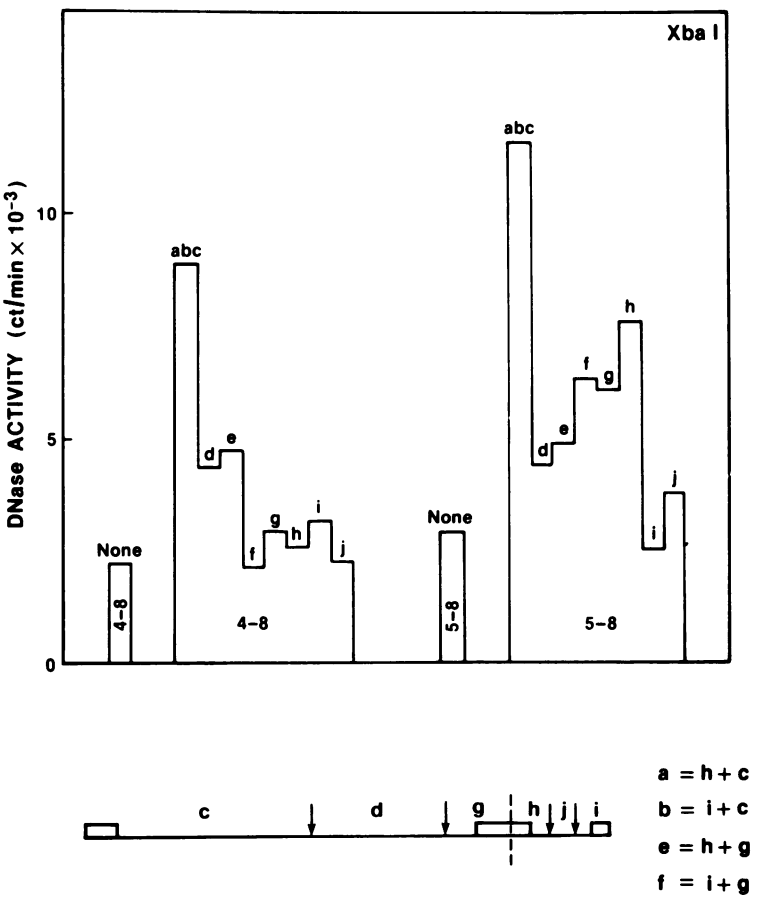

FIG. 5. *Rescue of DNase activity in vivo by XbaI fragments on HSV-2 wild-type DNA. Cells were coinfected with intact revertant DNA and separated fragments of wild-type DNA from an XbaI digest. The resulting progeny were used to infect BHK monolayers at 39°C which were harvested after 24 to 48 h and assayed for DNase activity (see text). The upper part of the figure gives the nuclease activity in each sample; the lower part shows the restriction endonuclease cleavage map of HSV-2 for XbaI taken from reference 1.*

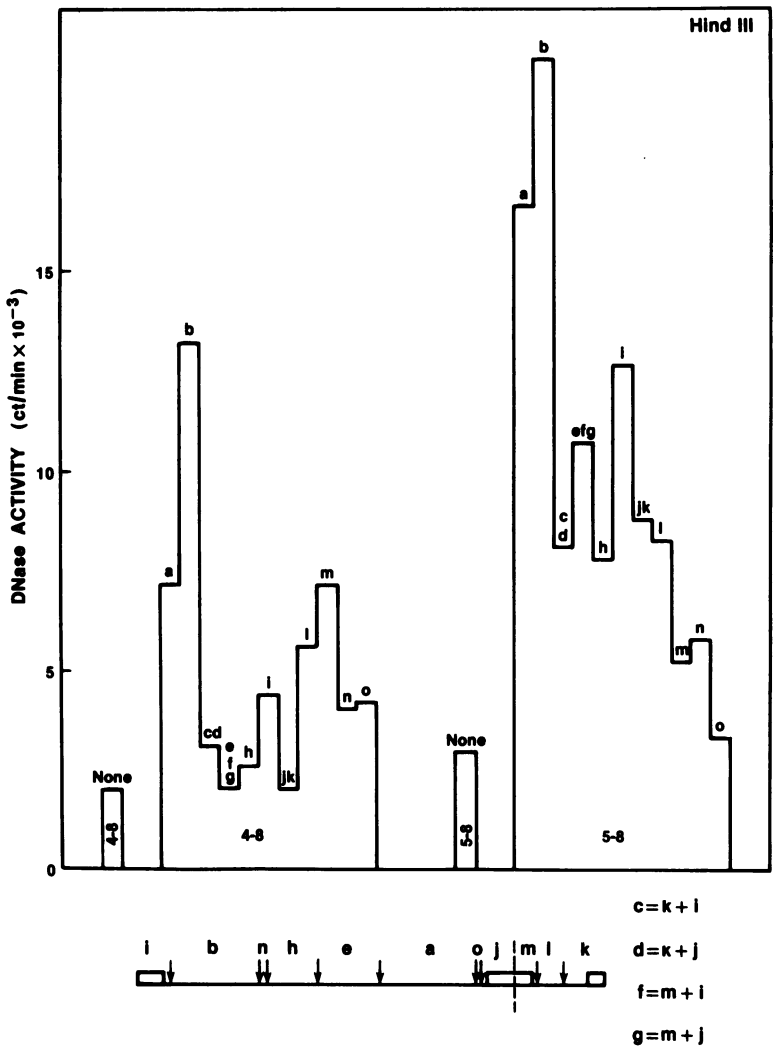

FIG. 6. *Rescue of DNase activity in vivo by HindIII fragments of HSV-2 wild-type DNA. As for Fig. 5, except that separated HindIII fragments were used.*

virion thermostability and the other affecting the capacity to induce a DNase activity. In this paper, we have shown that it is possible to isolate revertants of HSV-2 *ts*13 that can grow at the nonpermissive temperature. These revertants have lost the virion thermolability defect but not the nuclease activity defect. This indicates that the two phenotypes can be separated genetically, either because they correspond to two distinct mutations or because the reversion is due to a suppressor mutation that will correct one defect but not the other. We have mapped by marker rescue the physical location of the nuclease activity lesion (between 0.12 and 0.21, fractional length) on the HSV-2 genome and found it to be quite distant from the already known physical location for the HSV-2 *ts*13 lethal mutation (between 0.64 and 0.70, fractional length) (Chartrand et al., in preparation). The two phenotypic characters are thus the result of two distinct mutations.

We have also shown that only one of these mutations is lethal at the nonpermissive temperature under the conditions used in these experiments, that is, the mutation which results in virion thermolability. The likely mechanism for the lethal effect is the loss of infectivity due to the temperature sensitivity of one of the HG52 virus structural polypeptides, of which there are about 30 (H. S. Marsden, G. Hope, D. MacDonald, R. T. Hay, M. C. Timbury, and J. Hay, manuscript in preparation).

The second temperature-sensitive mutation, related to the DNase activity, seems to affect a

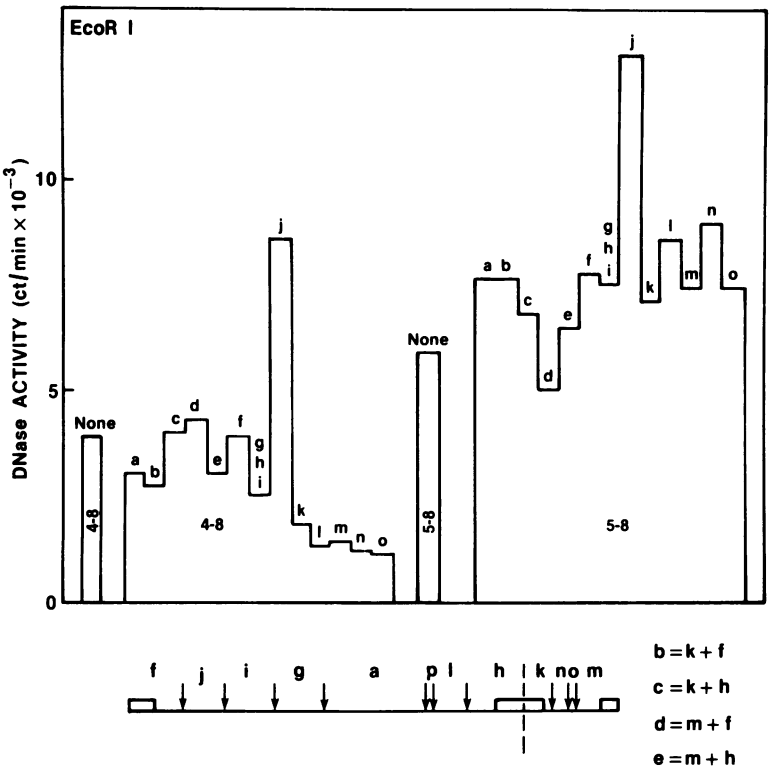

FIG. 7. *Rescue of DNase activity in vivo by EcoRI fragments of HSV-2 wild-type DNA. As for Fig. 5, except that separated EcoRI fragments were used.*

nonessential function, and we have to conclude that in exponentially growing BHK cells, HSV-2 can replicate, apparently unhindered by the lack of DNase activity. However, several points should be borne in mind. First, although assays for DNase activity show very low levels of enzyme in HSV-2 *ts*13- and revertant-infected cells, it is clear that we cannot say that all activity is lost; perhaps only very small amounts of DNase are required for normal growth of the virus. Second, rapidly growing BHK cells may be able to contribute a function, analogous to the DNase activity, which the virus can utilize. In that connection, it has been shown that the HSV-coded deoxythymidine kinase activity is an essential function in "serum-starved" cells but not in growing cells (6). Using this rationale, we have attempted to grow revertants 4–8 and 5–8 in serum-starved cells at 38°C; the results are in no way comparable to the clear-cut effects observed for deoxythymidine kinase and are, at present, somewhat variable. However, one consistent finding is that lack of DNase activity results in some reduction in DNA synthesis, and this point is currently being pursued. Third, although we assay the DNase activity as an alkaline DNA exonuclease, it may very well, inside the infected cell, turn out to have a quite different function. This putative function may not be seriously affected by the temperature-sensitive lesion which we have described. Recent experiments (M. Livingston, J. MacNab, and J. Hay, unpublished observations) with HSV-2 *ts*13 in which extracts of rat cells infected at 38°C were injected into rats confirm our earlier conclusions (2) that the HSV-2 *ts*13 gene in which the nuclease activity lesion resides synthesizes an immunologically active protein at nonpermissive temperature; this protein can induce antibodies that will neutralize the DNase activity in HSV-2 wild-type-infected cells.

ACKNOWLEDGMENTS

We thank John Subak-Sharpe for continued interest in this work and for useful suggestions.

The award of a Canadian Medical Research Council Fellowship to P. C. is gratefully acknowledged. J. H. is a member of the Medical Research Council Virology Unit.

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