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# Alkaline DNase Activity in Cells Infected with a Temperature-Sensitive Mutant of Herpes Simplex Virus Type 2

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BHK cells infected with the temperature-sensitive mutant *ts*13 of herpes simplex virus type 2 at a nonpermissive temperature lack the alkaline nuclease activity, which is induced by the mutant at a permissive temperature and by wild-type virus at either temperature. For *ts*13, enzyme activity could be induced by a temperature shift to permissive conditions, but not in the presence of cycloheximide. After a shift from permissive to nonpermissive conditions in the presence of cycloheximide, the activity was stable in wild-type, but not in mutant-infected, cells. After extensive purification, the wild-type nuclease was fourfold more heat stable in the presence of substrate than was the mutant enzyme. Mixtures of both purified enzymes showed the predicted intermediate stabilities. The results strongly suggest that the enzyme is virus coded and that the mutant possesses a lesion in the structural gene of the enzyme.

After infection of cells in culture, herpes simplex virus (HSV) induces a number of enzymatic activities related to DNA metabolism. Among them are a new DNA polymerase and a nuclease (5). A partial purification of the nucleolytic activity (7) indicated that it was a DNA-specific alkaline exonuclease and that its induction and properties differentiated it from known cellular nucleases. Preliminary experiments (4; H. Moss and J. Hay, unpublished data) showed that a DNase with properties very similar to those reported by Morrison and Keir (7) for HSV type 1 was induced in cells infected with wild-type HSV type 2 (HSV-2) and that, of 13 HSV-2 temperature-sensitive mutants studied (9), only 1, *ts*13, failed to induce the activity at a nonpermissive temperature. A large proportion of the temperature-sensitive mutants isolated for HSV (1, 8) exhibit a DNA-negative phenotype. The *ts*13 mutant of HSV-2 is capable of synthesizing viral DNA at a nonpermissive temperature (2); yet the amounts of DNA made vary considerably and are generally lower than those for wild-type infections (J. Hay and B. Francke, unpublished data). The study presented here was undertaken to determine whether *ts*13 represents the structural gene for the viral alkaline nuclease, as a basis for further investigation of the potential role of this enzyme during viral replication.

## MATERIALS AND METHODS

**Cells and virus.** BHK-21 (C13) cells (MacPherson and Stoker [6]) were grown in Dulbecco-modified Eagle medium with 10% (vol/vol) calf serum (Colorado Serum Co.) and 10% (vol/vol) tryptose phosphate broth (Difco Laboratories) on 5-cm plastic petri dishes.

The virus used was HSV-2, strain HG 52 (wild type), and a plaque-isolated temperature-sensitive mutant, *ts*13, obtained from a bromodeoxyuridine-mutagenized stock of HG 52 (9).

Virus stocks were prepared by infection of BHK cells at a multiplicity of $10^{-2}$ PFU/cell and titrated on monolayers of BHK cells under an agarose overlay.

For experimental purposes, BHK cells in 5-cm petri dishes were infected with virus at 5 PFU/cell, with an adsorption period of 1 h at 31°C or 30 min at 39°C, after which time 5 ml of fresh medium was added. For temperature-shift experiments, the medium was replaced with 5 ml of medium prewarmed to the respective temperature. For experiments involving inhibition of protein synthesis by cycloheximide, the drug was present at 50 μg/ml. At this concentration, the incorporation of [$^{35}$S]methionine by infected cells was reduced to <2% of that of untreated control cultures.

**Alkaline exonuclease assays from crude extracts.** Extracts were prepared from $10^7$ cells as described by Morrison and Keir (7) by sonic treatment in an ultrasonic water bath (Bransonic), yielding 250 μl of crude extract. The assay mixture (150 μl) contained the following components (final concentrations): 50 mM Tris-hydrochloride (pH 9.0), 2 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 125 μg of $^3$H-labeled BHK DNA ($1.6 \times 10^3$ cpm/μg) per ml, and 50 μl of crude extract. Degradation of the labeled DNA was monitored by precipitating the undigested DNA with 5% trichloroacetic acid and counting the precipitate after collection onto glass-fiber filters (Whatman GF/C) in toluene containing 5 g of 2,5-diphenyloxazole per liter or by counting the soluble nucleotides as described by Morrison and Keir (7). Both methods gave identical results. For each nuclease determina-

tion, a time course with at least three time points, sampled in duplicate, was carried out. The results are presented as micrograms of DNA digested in 1 min by 1 mg of protein.

**Purification of alkaline nuclease.** A total of $10^9$ cells were infected with wild type or *ts*13 for 18 h at 32°C. Cytoplasmic extracts were prepared after scraping the cells into the medium and pelleting them at 1,200 × *g*. The pellets were washed in 0.15 M NaCl–0.02 M Tris-hydrochloride (pH 7.5) at 4°C and suspended in 3 volumes of 10 mM Tris-hydrochloride (pH 8.0)–1 mM $MgCl_2$–1 mM dithiothreitol. Nonidet P-40 was added to 0.2% (vol/vol), and the whole was homogenized with 50 strokes in a Dounce homogenizer. After centrifugation for 30 min at 30,000 rpm and 4°C in a Beckman 60Ti rotor, the supernatant was used as starting material for purification. The extract was chromatographed on columns of DEAE-Sephadex (Pharmacia Fine Chemicals, Inc.) and phosphocellulose (Whatman) and centrifuged on a glycerol gradient to give between 1- and >2,000-fold purification with 20% (*ts*13) to 30% (wild type) recovery of activity. The method of purification and the properties of the purified enzyme will be published elsewhere in detail (Francke et al., manuscript in preparation).

**Heat inactivation of the purified nuclease.** The preincubation mixture contained the following: 500 μg of bovine serum albumin per ml, 10 mM β-mercaptoethanol, 1 mM EDTA, 10 mM Tris (pH 7.5, unless otherwise indicated), 625 μg of BHK DNA (if present) per ml, and enough purified enzyme to degrade 6 μg of DNA in 1 h at 32°C. Preincubation was carried out at 45°C and was terminated by chilling the mixture to 4°C. To assay for residual activity, the mixture was diluted fivefold, restoring the conditions of the standard nuclease assay described above. Since the protein content of the purified enzymes was not measurable, these results are presented as the percentage of residual activity after preincubation for the times indicated, with the activity of a sample not exposed to 45°C as 100%. If kept at 4°C, the preincubation mixture had no effect on the enzymatic activity, a control that was performed with each of the inactivation experiments described below.

## RESULTS

One of the distinguishing features of the HSV-induced nuclease is its high pH optimum (7). When either uninfected or *ts*13-infected cells were assayed for nuclease activity between pH 5.5 and 9.0, the results shown in Fig. 1 were obtained. The crude extracts used in this experiment show activity at acid pH and at alkaline pH, yet little activity at pH 7. The acid nuclease appears to be of cellular origin, since it is found in uninfected cells, whereas the alkaline nuclease appears to be virus induced. The absence of the latter from cells infected with *ts*13 at a nonpermissive temperature (39°C) is unique for this mutant. All other mutants of HSV-2 expressed some alkaline nuclease after infection at 39°C (Moss and Hay, unpublished data). Figure 1 shows that the enzymatic activity was not temperature sensitive during the in vitro assay, at least up to 1 h. The rate at which the substrate DNA was degraded in vitro at 39°C was about twice as fast as at 31°C, and extracts from cells infected with *ts*13 at 31°C did not differ from wild-type-infected cell extracts in this respect (data not shown). The apparent lack of in vitro temperature sensitivity of the *ts*13-induced activity will be discussed below.

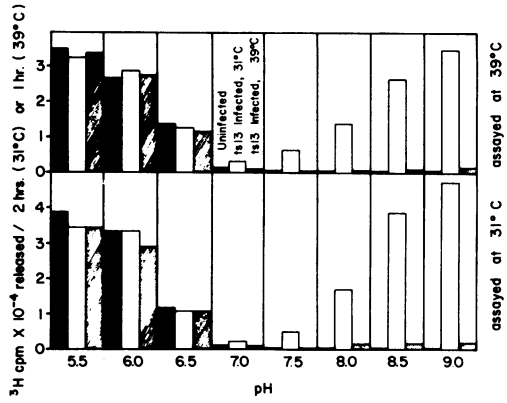


FIG. 1. *Effect of pH on the nuclease activity in extracts from ts13 or mock-infected BHK cells. Extracts were prepared and assayed for nuclease activity as described in the text, except that Tris-chloride buffer was replaced with 50 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid adjusted to the pH indicated with KOH and brought to equimolar $K^+$ with KCl. Symbols: ■, uninfected cell extract; □, ts13-infected cell extract (31°C infection for 18 h); ▨, ts13-infected cell extract (39°C infection for 9 h).*

In contrast, when *ts*13-infected cells were shifted to a nonpermissive temperature in vivo, the alkaline nuclease activity behaved in a temperature-sensitive fashion (Fig. 2). Both wild type and *ts*13 induced similar amounts of activity at 31°C. A shift to 39°C had little or no effect on wild type, but resulted in a reduction of the activity in *ts*13-infected cells (Fig. 2a and c). The kinetics of the turnoff of the nuclease was variable, depending most markedly on the timing of the shift to 39°C (see also Fig. 3). For a reduction to 50% of the preshift level, between 30 and 90 min at 39°C was required, whereas after 3 h the activity reached background levels in all cases. In the reverse experiment, shifting to a permissive temperature after 9 h at 31°C, *ts*13-infected cells were capable of expressing the nuclease. The kinetics of the turn-on exhibited no noticeable lag period. Again, a shift from 39 to 31°C had little effect on the activity present in wild-type-infected cells (Fig. 2b and d). To test whether the appearance of alkaline nuclease in *ts*13-infected cells after a shift to a permissive temperature was due to de novo synthesis or to the activation of an inactive enzyme, the exper-

iment shown in Fig. 3 was performed. *ts*13-infected cells, lacking the activity due to a shift to 39°C for 3 h, were capable of producing the nuclease again at 31°C at any time during the replicative cycle. At later times, induction was poorer and delayed, probably reflecting the state of the infected cells at this time. However, at no time did the activity return after a shift to 31°C if cycloheximide was present, indicating that its appearance required the synthesis of new protein and that the preexisting activity became irreversibly inactivated during the period at 39°C. The accelerated appearance of nuclease in cells kept at 39°C from the time of infection and shifted to 31°C at 3 h suggests that the mutation does not involve any steps in early transcriptional control but rather affects the enzyme itself. To compare the heat lability of the mutant-induced nuclease with the wild-type enzyme after shifting infected cells to a nonpermissive temperature, the experiment shown in Fig. 4 was

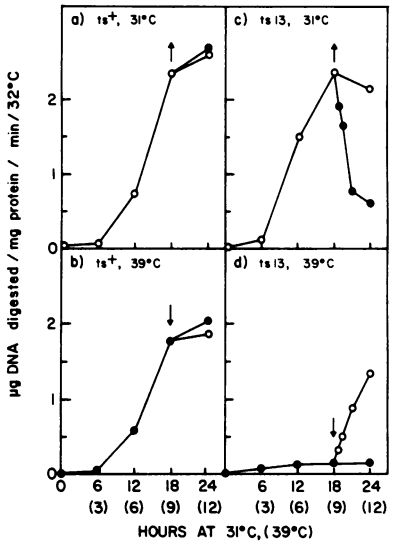


FIG. 2. *Effect of temperature on the induction of alkaline nuclease activity. Four series of dishes were infected with wild-type HSV-2 or with ts13 and incubated at 31°C (○) or 39°C (●). At the times indicated, samples were taken for extract preparation and DNase assay at 31°C, as described in the text, at pH 9.0. At times indicated by arrows, samples were shifted up to 39°C (↑) or shifted down to 31°C (↓).*

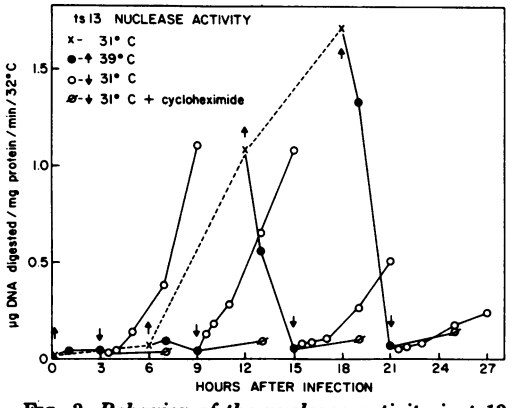


FIG. 3. *Behavior of the nuclease activity in ts13-infected cells. ts13-infected cells were shifted to 39°C at 0 h or at 6, 12, and 18 h after infection at 31°C (indicated by ↑). At 3 h after the shift-up, each set was shifted back to 31°C (indicated by ↓), and the recovery of the nuclease activity was followed in the absence or presence of 50 µg of cycloheximide per ml, added at the time of shift-down. Experimental details were as described in the legend to Fig. 2.*

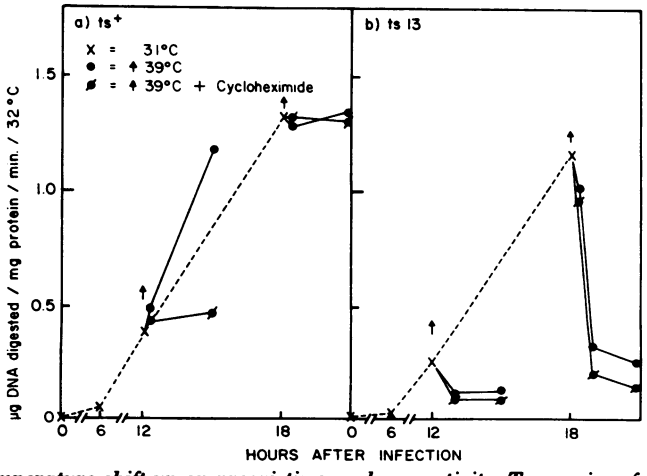


FIG. 4. *Effect of temperature shift-up on preexisting nuclease activity. Two series of dishes were infected with wild type or ts13 at 31°C. At 12 and 18 h, the cultures were shifted to 39°C (indicated by ↑), and half of them received cycloheximide at the time of the shift-up. Experimental details were as described in the legend to Fig. 2.*

performed. When protein synthesis was inhibited at the time of shift to 39°C in wild-type-infected cells, the level of nuclease activity indicated a heat-stable enzyme (Fig. 4a). The *ts*13-induced activity was heat labile, as noted earlier, which was slightly exaggerated by the presence of cycloheximide (Fig. 4b). This experiment also argues against a potential inhibitor of preexisting nuclease induced by a shift to 39°C, which could have gone unnoticed in the type of experiment shown in Fig. 2 due to continued de novo synthesis in wild-type-infected cells.

The results reported so far are compatible with the notion that the *ts*13 mutation corresponds to the structural gene for the viral alkaline nuclease. Since crude extracts from *ts*13-infected cells failed to show temperature sensitivity when assayed in vitro at 39°C (see Fig. 1), the heat lability of purified enzyme preparations from wild-type- and *ts*13-infected cells was compared. During the purification (as outlined above) the acid exonuclease activity was removed during the first chromatography step (only the alkaline activity bound to DEAE-Sephadex). Both enzyme preparations were more than 2,000-fold purified over a soluble cytoplasmic extract, and the final preparations contained too little protein to measure. The nuclease activities for both preparations were linearly proportional to the volume of enzyme used per assay (data not shown). Table 1 shows that preincubation of the enzymes by themselves at 45°C resulted in a rapid loss of activity, with no measurable difference between wild type and *ts*13, but in the presence of substrate (DNA), the wild-type activity was 3.5 to 4 times more stable than the *ts*13 enzyme. Interestingly, the stabilizing effect of the substrate was only observed at close to neutral pH (7.5) and not at the pH optimum for enzymatic activity (9.0). For a reproducible difference in $t_{1/2}$ at 45°C between the two preparations, it was further necessary to keep the salt concentration at less than 30 mM KCl. Bovine serum albumin, although used in the experiments shown, was not required for the stability of the enzyme, nor did it affect the $t_{1/2}$ at 45°C or the enzymatic activity at 31°C (data not shown). Because potential contaminants in the enzyme preparations, causing the different heat labilities, could not be excluded, the mixing experiment shown in Fig. 5 was performed. Both preparations were adjusted to equal activities per volume and then preincubated at 45°C in the presence of DNA by themselves and as 2:1 enzyme mixtures of wild type to mutant or mutant to wild type. The experimental points for the mixtures fell closely on the theoretical lines constricted from the best-fit lines for the two enzymes alone. It, there-

TABLE 1. *Inactivation of wild-type (ts⁺) and ts13 nuclease activities at 45°C*[a]

| Inactivation conditions | | $t_{1/2}$ (min) | |
|---|---|---|---|
| pH | DNA | *ts*⁺ | *ts*13 |
| 9.0 | − | 8 (6.5)[b] | 9 (16.6)[c] |
| | + | 16 (61.7) | 16 (78.7) |
| 7.5 | − | 9 (77) | 8 (112) |
| | + | 136 (79) | 36 (108) |

[a] The preparation of purified enzymes and preincubation conditions were as described in the text. Preincubation was for 15, 30, 60, 90, and 120 min at 45°C, and $t_{1/2}$ was determined graphically after plotting $\log_{10}$ of residual activity against time.

[b] Numbers in parentheses are initial enzyme activities (at zero time) expressed as counts per minute × $10^{-2}$.

[c] Even at 0°C, the enzyme activities were unstable in the absence of DNA at pH 9.0.

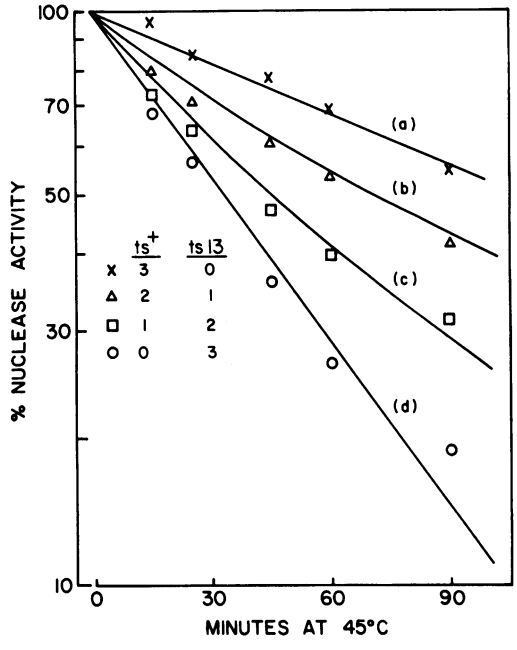


FIG. 5. *Heat stability of wild-type- and ts13-purified alkaline nucleases. Preincubation at 45°C was in the presence of DNA at pH 7.5 as described in the text. Each preincubation mixture (200 μl) contained 15 μl of enzyme. Both enzymes had been adjusted to equal activity per volume before mixing. The 100% values (i.e., not exposed to 45°C) were as follows (in $^3H$ counts per minute × $10^{-3}$ acid soluble after 60 min of incubation at 32°C under nuclease assay conditions): 13.5 (a), 13.7 (b), 13.2 (c), and 13.4 (d). (a) and (d) were drawn to best fit the experimental points × and ○, respectively. (b) and (c) represent theoretical curves derived from (a) and (d) by adding the two input activities at 1/3 or 2/3, respectively, corrected for the surviving activity for each time point during the inactivation.*

fore, appears that the increased heat lability of the *ts*13 nuclease in the presence of DNA at pH 7.5 is a property of the enzyme itself and not caused by a contaminant. The failure of crude extracts from *ts*13-infected cells to show temperature sensitivity during an in vitro assay is possibly a consequence of the high pH required for the assay.

## DISCUSSION

The data given here show that a temperature-sensitive mutant of HSV-2 induces an alkaline nuclease activity that is temperature sensitive both in vitro and in vivo. Shifting infected cells in vivo from a permissive to a nonpermissive temperature results in irreversible inactivation of the enzyme's activity, as documented by the requirement for protein synthesis for the reappearance of activity after shifting back to a permissive temperature. In addition, the wild-type activity is stable in the absence of protein synthesis at a nonpermissive temperature, whereas the mutant activity is not. Incubation of purified enzymes in vitro at 45°C shows a fourfold-faster inactivation rate for the *ts*13-induced enzyme over the wild-type enzyme. These findings provide the first strong evidence that the nuclease activity is coded directly by the virus genome, although they do not rule out the possibility that part of the enzyme protein is contributed by the cell. The accelerated appearance of activity at a permissive temperature after a period of 3 h at a nonpermissive temperature indicates that early steps in the infectious cycle are not affected by the mutation, as would be expected for a lesion in the structural gene of a late function.

Halliburton and Timbury (2) have reported that *ts*13 makes significantly less virus DNA than does wild type at a nonpermissive temperature, but is not DNA negative. Our own studies on DNA synthesis agree with this assessment and would suggest that HSV-2 can replicate its DNA, at least to some extent, in the absence of the great majority of this nuclease activity. Several possibilities arise from this result. Perhaps the virus needs only trace amounts of exonuclease activity for its replication, or, alternatively, such DNA as is synthesized in *ts*13-infected cells at a nonpermissive temperature is defective in some way. The DNase activity could also represent a dispensable viral function, at least in the system used to isolate and test the virus mutant.

Earlier observations on *ts*13 (3) revealed that the infectivity of virus preparations made at a permissive temperature was sensitive to heat under conditions of wild-type stability. Thus, in this mutant, we can define two temperature-sensitive functions, one related to the virus particle and likely to be a structural polypeptide and the other the virus nuclease activity. It is conceivable that these two changes in phenotype are the result of a single genetic change. If, however, we are dealing with a double temperature-sensitive mutant, then we cannot at present say which of these is lethal. We have recently isolated revertants of *ts*13, and their examination should help to resolve some of these issues.

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