Isolation and Characterization of Thermosensitive Mutants from Kilham Rat Virus, a Rodent Parvovirus

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SUMMARY

Thermosensitive mutants were isolated from nitrous acid-treated Kilham rat virus (KRV). At a restrictive temperature (39.5 °C), the mutants tested did not produce appreciable amounts of infectious particles, haemagglutinin or progeny single-stranded DNA. Virus antigen accumulation as detected by immunoperoxidase was reduced. Complementation tests revealed two distinct groups. The three members of complementation group 1 synthesized normal amounts of replicative forms but were restricted in single-stranded DNA production and capsid protein expression, exhibiting phenotypes compatible with cap mutations. The sole representative of group 2, KRV ts6, failed to accumulate replicative forms, displaying a rep- phenotype. These mutants provide new tools to test the role of viral products in the biology of autonomous parvoviruses.

INTRODUCTION

The Parvoviridae are small, unenveloped, icosahedral, single-stranded DNA viruses, having in common distinctive physical features related to their genomic structure and organization (Siegl et al., 1985). Those which infect vertebrates are divided into two genera. One, the Dependovirus group, includes defective viruses requiring for their replication a co-infecting helper virus, usually an adenovirus, hence their vernacular name of adeno-associated viruses or AAVs (Berns et al., 1985). Here we are dealing with Kilham rat virus (KRV), the type species of the other genus which contains the 'autonomous' (that is non-defective) parvoviruses. However, their DNA replication is still strictly dependent upon event(s) happening during the S phase of infected permissive cells.

Encapsidated genomes of autonomous parvoviruses are linear, single-stranded DNA molecules about 5000 nucleotides long, with short double-stranded hairpin-like structures at both extremities. Study of DNA synthesis in vivo has revealed an interesting mode of replication. Briefly, the parental single-stranded DNA, probably acting as a self-primed template, is converted into a linear duplex molecule, the origin of a whole set of essentially monomeric and dimeric linear replicative forms (m-RFs and d-RFs respectively). Progeny single-stranded DNA is strand-displaced from the RFs and simultaneously encapsidated. Nucleotide sequences established for several parvoviruses showed two major open reading frames covering approximately 90% of the genome (Astell et al., 1983 a; Rhode & Paradiso, 1983; Shade et al., 1986; Chen et al., 1986). Detailed transcription maps of the minute virus of mice (MVM) and of H-1 virus, two rodent parvoviruses closely related to KRV, identified two overlapping transcription units (Pintel et al., 1983; Lebovitz & Roeder, 1986; Jongeneel et al., 1986). Four distinct polypeptides have been characterized as translation products. Two of them, VP1 and VP2, are structural proteins (a third capsid component, VP3, is a processed form of VP2). The other two polypeptides NS1 and NS2 are non-structural proteins of which the biological functions are not yet thoroughly established (Cotmore et al., 1983; Cotmore & Tattersall, 1986).
Until now, conventional genetic studies performed on autonomous parvoviruses have been rather scarce. Several years ago, Rhode (1976) isolated more than 20 thermosensitive (ts) mutants from hydroxylamine-treated H-1 virus. The effect of their mutations upon H-1 virus DNA synthesis and intracellular virus morphogenesis was reported in detail (Rhode, 1978a; Singer & Rhode, 1978). No distinct complementation groups could be identified among these H-1 ts mutants. Most appeared to be mutated in a part of the genome coding for capsid proteins (cap- mutants), a defect which usually led to an inhibition of progeny single-stranded DNA synthesis (Rhode, 1978a). However, the existence of an RF replication function (rep function) was deduced from the results of complementation between defective H-1 mutants and some members of the rodent parvovirus subgroup (Rhode, 1978a, b, 1982). Adeno-associated viruses could not be easily subjected to standard genetic approaches. Deletion and insertion mutants were constructed in vitro from infectious AAV recombinant plasmids and tested in vivo by DNA transfection techniques. Specific regions of the AAV genome were connected to rep and cap functions which complemented each other (Hermonat et al., 1984; Tratschin et al., 1984).

In this paper, taking advantage of a convenient cell–virus system, we used classical genetic methods to isolate and characterize ts mutants from nitrous acid-treated KRV. Lethal conditional mutations are as yet unsurpassed for providing information about viral gene expression in vivo. Besides, since autonomous parvovirus biology is so strictly dependent on host cell replication, they may be invaluable for probing cellular DNA replication, transcription and recombination machineries. Among six KRV mutants recorded here, two functional complementation groups could be identified and related to different virus DNA replication deficiencies. A biological and a biochemical study of each group is presented. Their respective phenotypes are tentatively correlated to specific viral genes.

**METHODS**

*Cells and virus.* Culture conditions for rat cell lines (C1 5 RE and Fr 3T3) and KRV growth and purification were recently described (Gunther et al., 1984). The virus stock referred to here as wild-type (wt) was plaque-purified three times before being grown at 39.5 °C at a low m.o.i. Our KRV strain was obtained from Chany & Brailovsky (1967). The restriction enzyme mapping of its genome by BgII, PsI and HaeIII (not shown) revealed the sites specifically allotted to strain 171 by Mitra et al. (1982). Virus assays were performed by plaque titration on C1 5 RE cell cultures. The viral plaques were revealed by neutral red staining of monolayers on the 5th, 6th or 10th day after infection, depending on the incubation temperature (39.5 °C, 37 °C or 33 °C). Standard virus haemagglutination tests were performed at 4 °C on disposable microtitration plates with V-shaped wells (Greiner & Söhne, Nürtingen, F.R.G.) using phosphate-buffered saline (PBS) as diluent and 0.25% final concentration of guinea-pig erythrocytes. Virus antigens were detected in infected cells by an indirect immunoperoxidase technique (Wicker & Avrameas, 1969). Cell cultures on glass coverslips were fixed for 10 min at −10 °C in an acetone–methanol mixture (7:3) before being treated, first with a rabbit antiserum directed against purified KRV capsids, and second with a peroxidase-labelled anti-rabbit IgG goat antiserum (Institut Pasteur, Paris). Diaminobenzidine was used as a histochemical substrate to locate peroxidase-labelled immune complexes.

*Nitrous acid inactivation of KRV.* Wild-type KRV was inactivated by nitrous acid according to a procedure devised by Ledinko (1974). The virus stock (1.5 × 10^9 p.f.u./ml) was diluted 1:10 in a freshly prepared solution of 0.7 M NaNO₂ in 1 M-acetate buffer pH 4.6 and incubated at 21 °C in a water bath. At chosen times, aliquots were immediately diluted 1:10 in 1 M-Tris–HCl pH 8. A further 1:20 dilution was done in complete medium and the samples were kept frozen until plaque titration.

*Isolation and screening of potential ts mutants.* Restrictive and permissive temperatures were 39.5 °C and 33 °C respectively. Once the survival curve of the nitrous acid-treated virus was established, well isolated plaques were picked from the virus samples grown at 33 °C. They were directly transferred to 24-well Nunclon culture dishes (Nunc) seeded the day before with CI 5 RE cells (4 × 10^4 cells/well). Eight to 10 days later, a cytopathic effect was visible. Standard virus haemagglutination tests were performed at 4 °C on disposable microtitration plates with V-shaped wells (Greiner & Söhne, Nürtingen, F.R.G.) using phosphate-buffered saline (PBS) as diluent and 0.25% final concentration of guinea-pig erythrocytes. Virus antigens were detected in infected cells by an indirect immunoperoxidase technique (Wicker & Avrameas, 1969). Cell cultures on glass coverslips were fixed for 10 min at −10 °C in an acetone–methanol mixture (7:3) before being treated, first with a rabbit antiserum directed against purified KRV capsids, and second with a peroxidase-labelled anti-rabbit IgG goat antiserum (Institut Pasteur, Paris). Diaminobenzidine was used as a histochemical substrate to locate peroxidase-labelled immune complexes.

*One-step growth of wt and ts KRV at different temperatures.* Petri dishes (35 mm) were seeded with C1 5 RE cells at a density of 2 × 10⁵ cells per ml of complete medium. The following day they were infected at an m.o.i of 3 to 5 p.f.u. per cell. After 1 h adsorption at the planned incubation temperature, the virus inoculum was aspirated. Cultures were rinsed twice with 1 ml of prewarmed PBS and fed again with 2 ml of fresh medium before being...
incubated at 39.5 °C or at 33 °C. At fixed times, two samples were frozen. Before plaque titration or haemagglutination, the samples were thawed, monolayers were scraped into the medium and duplicate samples were pooled in 5 ml polystyrene tubes. After two more freezing and thawing cycles, cell debris was removed by low speed centrifugation. In most experiments, cell cultures established on coverslips and infected in parallel were fixed for detection of KRV antigens by the immunoperoxidase method.

**Leakiness, complementation and recombination tests.** In most cases the three tests were performed simultaneously. Cell cultures (C1 5 RE) in duplicate were infected with a mixture of two mutants (5 p.f.u./cell each) or with one mutant alone (10 p.f.u./cell); an m.o.i. equal to or less than 1 p.f.u. per cell was used in some leakiness tests. Culture conditions and virus infections were performed as above. Samples were incubated for either 24 h at 39.5 °C or 48 h at 33 °C, that is until completion of one-step growth of wt virus at the chosen temperature. Virus yields were titrated either at 39.5 °C or at 33 °C according to protocol. Leakiness percentages, complementation indices (C.I.) and recombination percentages were calculated by the following formulae in which the superscripts refer to the incubation temperature of one-step growth of a mutant alone (A) or of a mixed infection (A x B) and subscripts to the titration temperature.

\[
\% \text{ Leakiness} = \left( \frac{\text{Yield} A_{39.5}^{33.5} \text{C}}{\text{Yield} A_{33}^{33.5} \text{C}} \right) \times 100;
\]

\[
\text{C.I.} = \left[ \frac{\text{Yield} (A \times B)_{39.5}^{33.5} \text{C} - \text{Yield} (A \times B)_{39.5}^{39.5} \text{C}}{\text{Yield} A_{33}^{33.5} \text{C} + \text{Yield} B_{33}^{33.5} \text{C}} \right];
\]

\[
\% \text{ recombination} = \left[ \frac{\text{Yield} (A \times B)_{39.5}^{33.5} \text{C} - \text{Yield} A_{39.5}^{33.5} \text{C} - \text{Yield} B_{39.5}^{33.5} \text{C}}{\text{Yield} (A \times B)_{33}^{33.5} \text{C}} \right] \times 100.
\]

**Analysis of total virus DNA synthesis at 39.5 °C.** Confluent Fr 3T3 cell cultures in 60 mm Petri dishes were infected with KRV wt or ts (3 to 5 p.f.u./cell) and incubated at 39.5 °C. Ten h after infection, they were labelled with [3H]thymidine (5 μCi/ml) and DNA was extracted every 2 h according to a Hirt modified procedure, electrophoresed in 1% agarose gels (Gunther et al., 1984) and revealed by fluorography with En3Hance (New England Nuclear).

**KRV nucleoprotein extraction.** Infected Fr 3T3 cells incubated at 39.5 °C as above were labelled with [3H]thymidine from 10 to 13 h post-infection (p.i.), then re-incubated for a further 2 h in non-radioactive medium. Virus DNA was extracted according to Shoyab & Sen (1978). Briefly, cells were lysed by addition of 8 M-urea, 1% SDS and 0.01 M-phosphate buffer pH 6.8 (2 ml for 4 × 10⁶ cells) containing 1 mM-PMSF. High molecular weight DNA was pelleted by ultracentrifugation (80000 g, 1 h, 2 °C) and the supernatant was dialysed overnight against 10 mM-Tris-HCl pH 7.8, 1 mM-EDTA.

**Sucrose gradient ultracentrifugation.** Up to 0.5 ml of a nucleoprotein extract was layered on a preformed 5 to 20% sucrose gradient containing 4 M-guanidine–HCl in 50 mM-Tris-HCl pH 7.8 and 2 mM-EDTA according to Lavelle & Li (1977). After centrifugation (36000 r.p.m., 8 h, 20 °C, Beckman SW41 rotor), fractions were collected and equivalent samples (80 μl) of each were spotted onto either Whatman GF/C filters for determining total acid-precipitable radioactivity or Millipore filters (DWP 0.65 μm) according to Astell et al. (1983b), for measuring radioactivity corresponding to the nucleoprotein.

**RESULTS**

**Nitrous acid inactivation of KRV**

Fig. 1 shows a curve of inactivation of KRV by nitrous acid. From 0 to 10 min of treatment, the surviving fraction decreased linearly from 1 to 10⁻² following one-hit kinetics of inactivation; after this the curve bent slightly. This pattern of nitrous acid inactivation was observed repeatedly. It is comparable to those published for other DNA or RNA viruses (Fried, 1965; Williams et al., 1971; Ledinko, 1974; Agut et al., 1981).

**Isolation of ts mutants**

We tested a total of 493 viral plaques isolated from four different surviving fractions (5 × 10⁻² to 2.5 × 10⁻³) as shown on Table 1. Eight KRV ts mutants were isolated and properties of six of them are reported in this paper. The efficiency of plaquing (e.o.p.) at 39.5 °C compared to 33 °C was usually between 10⁻³ and 10⁻⁴, except for ts1 (less than 1.5 × 10⁻⁵) and ts5 (2 × 10⁻¹). In our conditions the e.o.p. ratio 39.5 °C/33 °C of KRV wt was at least equal to and more often greater than 1, so ts5 was definitely a mutant, but it was considered too leaky to be studied efficiently. Subcloning of KRV ts isolates allowed us to get virus clones with a lower e.o.p. ratio 39.5 °C/33 °C. Virus stocks were always checked for their e.o.p. at both temperatures before being used for experimentation.
Fig. 1. Nitrous acid inactivation of KRV wt. After various periods of treatment with nitrous acid, virus samples were plaque-titrated at 37 °C.

Table 1. Nitrous acid mutagenesis of KRV

<table>
<thead>
<tr>
<th>NO₂H treatment (min)</th>
<th>Surviving virus fraction</th>
<th>Number of plaques tested</th>
<th>ts Mutants isolated (%)</th>
<th>KRV mutant</th>
<th>E.o.p. ratio 39.5 °C/33 °C of the 1st isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>69</td>
<td>0*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>5 x 10⁻²</td>
<td>275</td>
<td>ts2, ts4, ts5, ts6</td>
<td>7.6 x 10⁻³</td>
<td>2 x 10⁻⁴, 2 x 10⁻¹, 2.6 x 10⁻⁴</td>
</tr>
<tr>
<td>10</td>
<td>7 x 10⁻³</td>
<td>116</td>
<td>ts3, ts8</td>
<td>4 x 10⁻⁴, 1.7 x 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3.5 x 10⁻³</td>
<td>86</td>
<td>ts1</td>
<td>1.5 x 10⁻⁵, 4.5 x 10⁻³</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2.5 x 10⁻³</td>
<td>16</td>
<td>ts7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Spontaneous ts mutants, that is before any mutagenic treatment, were not detected in this experiment nor in others on a total of 174 wt plaques tested.

Leakiness and reversion

Two distinctive phenomena have an impact on e.o.p. of ts mutants at restrictive temperature: leakiness and spontaneous reversion towards a wt phenotype. Leakiness of KRV ts mutants was found to be dependent on the m.o.i. At an m.o.i. of 10, ts1 and ts2 showed only 0.002% and 0.003% leakiness, the other mutants gave much higher percentages (ts3, 16%; ts6, 9%; ts4 and ts8 around 1%). At an m.o.i. equal to or less than 1, carefully subcloned KRV ts1, ts2, ts4 and ts6 mutants did not grow at 39.5 °C, but at 33 °C control inocula increased their virus titres by at least 2 orders of magnitude. At this m.o.i., KRV ts3 and ts8 were not as leaky (2.4% and 0.16%, respectively). One-step growth experiments (see below and Fig. 2) showed that up to an m.o.i. of 5, at least, KRV ts1 and ts6 were not leaky.

Concerning spontaneous reversion, we did not find any wt phenotype revertants among isolates from KRV ts plaques grown at 33 °C (15 to 41 isolates for each mutant, 123 in total). Obviously, the numbers of isolates tested, restricted for technical reasons, were too low to assess reversion rates. In contrast, isolates derived from well isolated plaques grown at 39.5 °C from a ts mutant inoculum always showed a wt phenotype, indicating that, through one round of selection pressure, wt revertants were easily detected.
Table 2. Complementation between KRV ts mutants

<table>
<thead>
<tr>
<th>ts1</th>
<th>ts2</th>
<th>ts3</th>
<th>ts4</th>
<th>ts6</th>
<th>ts8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.55</td>
<td>0.28</td>
<td>1.5</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.24</td>
<td>1.1</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.4</td>
<td>1.4</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>7</td>
<td>7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Complementation index, for calculation see Methods.
† Values in italics indicate C.I.s greater than 2, i.e. more than the minimum required to infer complementation.

Table 3. Recombination between KRV ts mutants

<table>
<thead>
<tr>
<th>ts mutant</th>
<th>Virus titres (p.f.u./ml) at 39-5 °C</th>
<th>Virus titres (p.f.u./ml) at 33 °C</th>
<th>Percent recombination*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts1 x ts1</td>
<td>2.5 x 10^1</td>
<td>4.9 x 10^6</td>
<td>0.6</td>
</tr>
<tr>
<td>ts2 x ts2</td>
<td>2.5 x 10^1</td>
<td>1.3 x 10^7</td>
<td>6</td>
</tr>
<tr>
<td>ts6 x ts6</td>
<td>5.2 x 10^3</td>
<td>3 x 10^6</td>
<td>0.7</td>
</tr>
<tr>
<td>ts8 x ts8</td>
<td>1.3 x 10^3</td>
<td>1.1 x 10^6</td>
<td>37</td>
</tr>
<tr>
<td>ts1 x ts2</td>
<td>9.3 x 10^4</td>
<td>1.7 x 10^7</td>
<td>0.6</td>
</tr>
<tr>
<td>ts1 x ts6</td>
<td>3.2 x 10^5</td>
<td>2.9 x 10^6</td>
<td>11</td>
</tr>
<tr>
<td>ts1 x ts8</td>
<td>1.8 x 10^5</td>
<td>2.9 x 10^6</td>
<td>6</td>
</tr>
<tr>
<td>ts2 x ts6</td>
<td>1.2 x 10^6</td>
<td>2.1 x 10^7</td>
<td>5.7</td>
</tr>
<tr>
<td>ts2 x ts8</td>
<td>2 x 10^5</td>
<td>7.9 x 10^6</td>
<td>2.5</td>
</tr>
<tr>
<td>ts6 x ts8</td>
<td>7.9 x 10^5</td>
<td>2.1 x 10^6</td>
<td>37</td>
</tr>
<tr>
<td>ts3 x ts3</td>
<td>9.5 x 10^4</td>
<td>1.4 x 10^7</td>
<td></td>
</tr>
<tr>
<td>ts4 x ts4</td>
<td>2.2 x 10^3</td>
<td>3.0 x 10^6</td>
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</tr>
<tr>
<td>ts6 x ts6</td>
<td>4.2 x 10^3</td>
<td>8.8 x 10^5</td>
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</tr>
<tr>
<td>ts8 x ts8</td>
<td>2.3 x 10^3</td>
<td>4.1 x 10^5</td>
<td></td>
</tr>
<tr>
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<td>4.8 x 10^4</td>
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</tr>
<tr>
<td>ts3 x ts6</td>
<td>1.7 x 10^5</td>
<td>9.8 x 10^6</td>
<td>0.7</td>
</tr>
<tr>
<td>ts3 x ts8</td>
<td>1.2 x 10^5</td>
<td>7.5 x 10^6</td>
<td>0.3</td>
</tr>
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<td>ts4 x ts6</td>
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<td>2.9 x 10^6</td>
<td>1.4</td>
</tr>
<tr>
<td>ts4 x ts8</td>
<td>3.2 x 10^4</td>
<td>5.9 x 10^6</td>
<td>0.5</td>
</tr>
<tr>
<td>ts6 x ts8</td>
<td>2.1 x 10^5</td>
<td>5.7 x 10^5</td>
<td>36</td>
</tr>
</tbody>
</table>

* Calculated as indicated in Methods.

Genetic interactions between ts mutants

Genetic interactions between the KRV ts mutants were studied through standard complementation and recombination experiments performed as reported in Methods. Possible complementation between each pair of KRV ts mutants was investigated by incubating mixedly infected cultures at restrictive temperature and titrating the virus production at permissive temperature. The C.I. was calculated to reduce the influence of leakiness and reversion or possible recombination between the two mutants. Table 2 shows the results obtained from three independent experiments, each involving at least two titrations. A group of three mutants ts1, ts2 and ts4, which did not complement each other, and ts8 were all complemented by ts6 (C.I. > 7). One mutant, ts3, was not complemented by any other mutant: the leakiness of this mutant has already been mentioned. The case of ts8 was different: this mutant was complemented by ts4 (C.I., 7.4 ± 2.3) as well as by ts6 (C.I., 8.4 ± 4.2), but the C.I. calculated from interactions between ts8 and ts1 or ts2 did not significantly exceed the value of 2 usually considered as the minimum value required to infer complementation (Ramig, 1985). From these results, it appeared that among the six mutants tested, at least two complementation groups could be identified. The first one included ts1, ts2 and ts4, one mutant, ts6, constituting a second group by itself. The classification of ts3 and ts8 remains to be established.
The possibility of genetic recombination between the KRV ts mutants was tested by titrating in parallel at both temperatures the virus production of mixedly infected cultures grown at the permissive temperature. The appearance of wt phenotype recombinants among the virus progeny was detected by the relative increase of the virus yield titrated at the restrictive temperature. It was calculated as a percentage of the total virus production titrated at the permissive temperature and corrected for leakiness or spontaneous reversion of parent mutants (see Methods). The results of two experiments are reported in Table 3. Obviously the values less than 1% observed for crosses such as ts1 x ts2, ts4 x ts8 and the ts pairs including ts3 do not imply any recombination. Three other crosses (ts1 x ts6, ts1 x ts8, ts2 x ts8) gave higher percentages (5% to 10%) which could however have been overestimated, since in this C1 5 RE cell line the e.o.p. at 39.5 °C of KRV wt phenotype was regularly higher than at 33 °C. One pair of mutants (ts6 x ts8) repeatedly gave as much as 36% to 37% recombination. That this value could, at least in part, be due to genetic recombination was supported by the fact that 18% (four of 22 tested) of the plaques grown at 33 °C from the ts6 x ts8 cross progeny were of a wt phenotype. In similar conditions, that is without previous selective pressure, we consistently failed to isolate any spontaneous revertants from each mutant alone (see above).

One-step growth characteristics

One-step growth characteristics of KRV ts1 and ts6, considered as representatives of distinct complementation groups, were compared at 33 °C and at 39.5 °C to those of KRV wt in rat C1 5 RE cells (m.o.i. 3 to 5 p.f.u./cell, Fig. 2). At 33 °C the three viruses followed very similar kinetics of replication. After an eclipse period of at least 12 h, infectious virus and haemagglutinin (HA) titres increased until 40 h p.i., extending over a 1000-fold range for the infectious virus. The amounts of virus and HA produced were similar whatever the wt or ts origin of the viral input, indicating that the mutants tested were not partly defective at permissive temperature.

At 39.5 °C, KRV wt started to replicate rapidly after an eclipse phase not exceeding 7 h, infectious wt virus and HA titration curves rose more steeply than at 33 °C, reaching a plateau at 25 h p.i. (Fig. 2a). Total wt virus and HA yields were identical at both temperatures. At the restrictive temperature, in ts1- or ts6-infected rat cell cultures, neither infectious virus nor HA production could be detected at any time p.i. (Fig. 2b and c). Growth of KRV ts1 and ts6...
Kilham rat virus ts mutants

mutants in simian virus 40-transformed human NB cells (Rhode, 1976) was also restricted at 39.5 °C (data not shown). Observations concerning other KRV ts mutants, although less detailed, led to similar conclusions (data not shown).

Thermal stability of ts HAs

The HA thermosensitivity of viruses grown at 33 °C was studied in two types of experiment. The first, similar to that described by Rhode (1976) with H-1 ts mutants, compared the thermal denaturation characteristics of HA proteins. The different KRV virus preparations, wt or ts, were heated for 20 min at given temperatures between 65 °C and 90 °C before being tested at 4 °C for retention of their HA titres. Between 75 °C and 80 °C, HAs of all the KRVs tested were irreversibly damaged, showing a sharp temperature transition of their inactivation curves similar to that already reported for H-1 virus (Greene, 1965; Rhode, 1976). However, in contrast with H-1 ts mutants, the HA proteins of none of the six KRV ts mutants were more sensitive to thermal denaturation than those of wt virus (results not shown).

Other experiments were designed to evaluate the ability of KRV wt and ts mutants to haemagglutinate guinea-pig erythrocytes at higher temperatures (24 °C, 33 °C, 37 °C or 41 °C) than the standard one (4 °C). Whatever the virus strain concerned, HA titres did not differ significantly between 4 °C and 24 °C. At higher temperatures they decreased progressively and were reduced by approximately 85% at 41 °C. No significantly reproducible differences could be observed between the six ts mutants nor between them and the wt control (not shown).

From these results, it appeared that haemagglutinating properties of virus proteins from KRV ts mutants grown at permissive temperature were not more thermosensitive than those of wt virus either in their biochemical stability (heat denaturation experiments) or in their biological activity (HA reactions performed at restrictive temperature). The failure to detect any HA production in KRV ts1- or ts6-infected cultures grown at 39.5 °C most probably reflected a restriction in synthesis or accumulation. However, synthesis at high temperature of constitutively and irreversibly inactive mutant HA proteins could not be excluded.

Capsid antigens as detected by immunoperoxidase

Capsid antigen accumulation in KRV-infected cultures was investigated by an immunoperoxidase technique using a rabbit antiserum directed against purified empty KRV capsids (see Methods). Localization and morphology of structural virus antigens in wt-infected cultures at either temperature, or in ts-infected cultures at 33 °C, were similar (Fig. 3a and b). When infection was spreading, intranuclear accumulation of virus antigens was revealed by a gradual darkening of enlarged nuclei (Fig. 3a and b). In permissive infections the progress of infection could be followed easily by assessing the percentage of immunoperoxidase-positive nuclei. By the end of the first cycle of viral growth in Cl 5 RE rat cell cultures infected at 3 to 5 p.f.u./cell, 30% to 50% of nuclei were heavily stained and c.p.e. was obvious.

At the restrictive temperature, KRV-specific staining of ts mutant-infected rat cells was strikingly reduced (Fig. 3c and d). As an example Fig. 3(b) and (d) illustrate a comparison between Cl 5 RE cell cultures simultaneously infected by KRV ts6 and incubated at 33 °C (Fig. 3b) or at 39.5 °C (Fig. 3d) for 24 h, the duration of the one-step growth of KRV wt at high temperature. At this time p.i., the first cycle of virus growth, when performed at 33 °C, was far from being ended, but 20% ± 5% of nuclei in KRV ts6-infected cultures were already heavily stained (Fig. 3b). In contrast, at 39.5 °C nuclei showed a very faint specific staining, too weak and too irregular to be easily discriminated as positive. However, a few clearly positive nuclei were still present.

A similar restriction occurred in cultures infected with other ts mutants. As an example, KRV ts1-infected cells are shown in Fig. 3(c). Whatever the KRV ts mutant considered, an accumulation of KRV antigens in the cytoplasm of infected cells was never observed at restrictive temperature.
Indirect immunoperoxidase staining of KRV capsid antigens in KRV-infected C15 RE cell cultures (24 h p.i., m.o.i. 3 to 5 p.f.u./cell). (a) KRV wt-infected cells incubated at restrictive temperature (39.5 °C), (b) ts6-infected cells incubated at permissive temperature (33 °C), (c) tsl- and (d) ts6-infected cells incubated at restrictive temperature. Cells were observed under a light phase microscope without any counterstaining. Bar marker represents 20 μm.

Viral DNA syntheses at a non-permissive temperature

DNA syntheses at 39.5 °C were followed in cell cultures infected with KRV wt or ts mutants. Fig. 4 shows the electrophoretic profile of KRV wt, tsl and ts6 DNA after a cumulative labelling with [3H]thymidine from 10 to 19 h p.i. Hirt supernatants from KRV wt-infected cell extracts showed the three characteristic KRV DNA species: m-RF, d-RF and ssDNA. As the labelling period was extended, radioactivity accumulated into duplex and ssDNA. Cell cultures infected with ts mutants clearly did not synthesize appreciable amounts of ssDNA. The radioactive staining of duplex DNA (m- and d-RF) from tsl-infected cultures was comparable to that observed at the same time in its wt counterpart. Very similar results were obtained with extracts from KRV ts3- or ts8-infected cells (data not shown). Incorporation of radioactive precursor into ts6 replicative forms was markedly reduced when compared to that of wt or tsl DNA and did not increase with the time of labelling. Such distinct DNA synthesis patterns between the two complementation groups were repeatedly observed. At 33 °C, KRV wt or ts mutant-infected cultures produced comparable amounts of m-RF, d-RF and ss-DNA, respectively (data not shown).

Sucrose gradient analysis of KRV nucleoprotein

It has been known for several years that a part of parvovirus duplex DNA is covalently linked to a terminal protein (Revie et al. 1979; Wobbe & Mitra, 1985; Chow et al., 1986). DNA extracted from KRV-infected cells and purified without any protease treatment was sedimented in a preformed sucrose gradient containing 4 M-guanidine–HCl (see Methods). Comparison between the radioactivity retained on Millipore filters in the presence of 4 M-guanidine–HCl and the total acid-insoluble radioactivity retained on Whatman GF/C filters permitted calculation of the fraction of DNA in a DNA–protein complex. Sedimentation profiles obtained from similar quantities of radioactive material from KRV wt, tsl- or ts6-infected cells grown at
39.5 °C for 16 h are presented in Fig. 5. Total acid-insoluble radioactivity from KRV wt extract revealed two peaks corresponding to m-RF and ssDNA (Fig. 5a). Radioactivity corresponding to a DNA–protein complex gave a similar profile and showed that less than one-third of duplex and ssDNA was found linked to a protein. Proteinase K treatment of the extract before sedimentation did not modify the profile of total radioactivity (data not shown) but abolished the retention of radioactivity on Millipore filters (Fig. 5a, open triangles). As shown in Fig. 5(a), part of the ssDNA appeared to be in a nucleoprotein form. DNA extracted from purified KRV and sedimented under the same conditions was not retained on Millipore filters (data not shown) indicating that, when encapsidated, KRV ssDNA was not covalently linked to protein. That newly synthesized ssDNA could be linked to protein has been suggested for only two parvoviruses: LulIII virus (Muller & Siegl, 1983) and MVM (Chow et al., 1986). In ts mutant-infected cell extracts, no ssDNA could be detected (Fig. 5b and c) confirming the result obtained by gel electrophoresis. In extracts infected with ts1 (Fig. 5b), ts3 or ts8 (data not shown) m-RF DNA and nucleoprotein behaved as their wt counterpart. Nucleoprotein and DNA from ts6-infected cell extracts sedimented as a much broader and heterogeneous peak, the slower sedimenting part of it probably being degraded DNA (Fig. 5c). Thus it appeared that RFs did not accumulate in ts6-infected cells at the non-permissive temperature (Fig. 4) and that this lack of accumulation could be partially due to some degradation (Fig. 5c). It is not yet known if parvovirus terminal protein is virus-encoded. If so, the results in Fig. 5 suggest that this function was not impaired in either of the two complementation groups.

**Discussion**

From nitrous acid-treated KRV, we isolated eight thermosensitive mutants. Among the six mutants studied here, two functional complementation groups were recognized and related to different DNA replication deficiencies. Complementation between ts mutants of the rodent parvovirus group might be suspected from current knowledge of their genome organization and...
Fig. 5. Sucrose gradient analysis of DNA–protein complexes extracted from infected cells grown at 39.5 °C. Tritium-labelled DNA was selectively extracted from cells lysed in 8 M-urea and 1% SDS (without any protease treatment) then submitted to sedimentation in a 5% to 20% sucrose gradient containing 4 M-guanidine-HCl (see Methods). Equivalent amounts of radioactive material (about $1 \times 10^6$ c.p.m.) were layered on each gradient. (○) Acid-insoluble radioactivity retained on Whatman GF/C filters; (▲) radioactivity retained on Millipore filters; (△) radioactivity retained on Millipore filters from a DNA sample treated with proteinase K and sedimented in a parallel gradient. DNA from cells infected with KRV (a) wt, (b) ts1, (c) ts6. m-RF and ss-DNA were sedimented in parallel gradients. The horizontal arrow indicates the direction of sedimentation.

viral gene products (see Introduction). However, Rhode (1976, 1978a) in an extensive search among more than 20 H-1 ts mutants was unable to identify distinct complementation groups, and notably could not find any conditional mutant deficient in RF replication. Detecting one mutant of this type among only eight KRV isolates by developing a very similar approach was unexpected. Both studies used direct mutagens: hydroxylamine for H-1 (Rhode, 1976) and nitrous acid for KRV. These chemicals act in vitro on nucleic acids by inducing primarily base transitions. When arising in single-stranded viral genomes such damage is directly expressed as point mutations upon cell infection. Nitrous acid, deaminating adenine, cytosine and guanine into hypoxanthine, thymine (uracil) or xanthine respectively, is less specific than hydroxylamine which mainly promotes cytosine to thymine transitions (Singer & Kusmierek, 1982), so that a broader range of mutations can be expected to result from the action of nitrous acid. On the other hand, differential expression of viral ts mutants may depend on the thermal response of the
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host cell used as a test system. Rhode (1978a) reported a partial defectiveness of H-1 wt when infecting simian virus 40-transformed human NB cells at 39.5 °C. This probably reflected cellular rather than viral thermosensitivity, since KRV wt yields were also reduced by one-third at 39.5 °C in these NB cells (data not shown), while they were identical at both temperatures in rat CI 5 RE cells (Fig. 2). For virus ts mutants, standard genetic analyses implied a plaque titration test working tolerably well at both temperatures. The rat cell line (CI 5 RE) we used proved to be very convenient especially at 39-5 °C, and gave us a good opportunity to carry out more extensive conventional genetic studies on parvoviruses.

To characterize the viral functions involved in the two distinct groups identified by complementation tests, experiments were performed on the time course of virus infections at both temperatures, the thermal stability of HAs, the structural antigen accumulation and the virus DNA syntheses in infected cells. As a whole, our results confirmed the thermosensitive character of each mutant studied. Clearly, at restrictive temperature no progeny infectious virus could be recovered from cultures infected by any of them. Preliminary comparative adsorption experiments (not shown) combined with results from complementation and recombination tests as well as thermal stability shown by HAs allowed us to exclude the occurrence of defects affecting the earliest events of the viral cycle (adsorption and uncoating). The possible thermosensitive deficiency of capsid proteins from KRV ts mutant was investigated through their capacities to haemagglutinate guinea-pig erythrocytes and to be detected in infected cells by immunoperoxidase using anti-KRV capsid antibodies. When produced at permissive temperature these proteins could not be distinguished from those of KRV wt according to their HA thermostability, intranuclear localization or gross morphology. At the restrictive temperature, cell cultures infected by representatives of either complementation group did not accumulate measurable amounts of HAs (one-step growth experiments). Simultaneously, the number of nuclei reacting to anti-KRV antibodies, and the staining intensity per nucleus were strongly reduced but not totally abolished. (Detectable antigens never accumulated in the cytoplasm.) Capsid protein expression of all KRV ts mutants tested was strongly inhibited at high temperature. However the technical approaches used here did not allow the phenotypic differentiation of the two complementation groups.

Virus DNA syntheses of ts mutants were restricted at high temperature and each group exhibited very distinct deficiencies. Cells infected by mutants of group 1 (and also ts3 and ts8) produced the expected amounts of apparently normal RFs at 39.5 °C but were defective in viral ssDNA synthesis (Fig. 4 and 5). These mutants displayed a virus DNA replication phenotype which has been associated with a capsid protein deficiency (cap- mutants) when observed in other parvoviruses such as H-1 ts mutants (Rhode, 1978a; Singer & Rhode, 1978) or constructed AAV-2 mutants damaged in the right part of their genome (Hermonat et al., 1984; Tratschin et al., 1984). We think that KRV ts mutants of group 1 (ts1, ts2, ts4), also present a cap genotype. As mentioned above their capsid protein expression was impaired at high temperature. Furthermore preliminary radioimmunoassay experiments revealed an 80% reduction of capsid protein synthesis at 39.5 °C in KRV ts1-infected cells when compared to KRV wt-infected controls (unpublished results). Complementation tests did not allow us to classify unambiguously KRV ts3 and ts8 mutants. Their DNA synthesis patterns, however, connected them to group 1 mutants. Further experiments are required to define their genetic status.

Rat cell cultures infected by KRV ts6, the only member of complementation group 2, failed to accumulate RFs at the restrictive temperature (Fig. 4), neither did they produce detectable single-stranded DNA. Moreover the low amounts of RFs which were detected showed an anomalous sedimentation profile in sucrose gradients (Fig. 5c). Such characteristics displayed by the ts6 mutant suggest an RF rep- phenotype. Along with complementation data they plainly differentiate this mutant from group 1 representatives. Mapping, sequencing and a more extensive characterization of the different mutants we isolated are in progress. Special attention will be paid to KRV ts6 which may be a useful tool for studying parvovirus rep function(s). To date, no thermosensitive RF rep- mutants have been recorded among parvoviruses. Adeno-associated virus recombinant plasmids deleted or point-mutated in the left part of the genome were shown to be of an RF rep- phenotype and to complement cap- AAV-2 recombinant clones.
mentioned above (Hermonat et al., 1984; Tratschin et al., 1984, 1986). That such a rep function could also be located on the left part of the autonomous parvovirus genomes was suggested from observations made on infectious MVM recombinant clones having only frameshift mutations (Merchlinsky, 1984). Two non-structural proteins (NS1 and NS2, 83K and 25K M, respectively) have been identified as products of the translation of mRNAs in vitro from several rodent parvoviruses (H-1, MVM and LuIII) and mapped in the left part of the genome (Cotmore et al., 1983; Cotmore & Tattersall, 1986). The NS1 polypeptide probably carries out several distinct functions in vitro (Pintel et al., 1983; Rhode, 1985; Cotmore & Tattersall, 1986) while the function(s) of NS2 have yet to be determined. The possibility that there could be a relationship between the RF rep deficiencies exhibited by the ts6 mutant and the expression of NS1 or NS2 is still a matter of speculation. Among different possibilities we favour at the moment the hypothesis that the defect in ts6 may be related to NS1 gene expression. Since NS1 protein transactivates the transcription of capsid mRNAs from their promoters (Rhode, 1985), a thermal restriction of it could explain why ts6 capsid proteins did not accumulate at the non-permissive temperature as revealed by immunoperoxidase and haemaggulination tests.

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