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A 15-Kilobase-Pair Region of the Human Cytomegalovirus Genome Which Includes US1 through US13 Is Dispensable for Growth in Cell Culture

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The genome of a temperature-sensitive, DNA-negative mutant of human cytomegalovirus was cloned in cosmids and analyzed by restriction endonuclease mapping and Southern blotting. The data presented show that in the mutant genome, nearly half of the short segment was deleted (14.3 to 15.1 kb; map position, 0.83 to 0.9), including the genes for a potential immediate early protein (US3) and a structural glycoprotein of 47 to 52 kDa (US6 through US11). The deleted DNA region was replaced by a 20.8- to 21.6-kb fragment that represented an inverted repetition of the retained portion of the short segment (map position, 0.92 to 1.0), suggesting that US20 through US36 were duplicated in the mutant. Northern (RNA) blots with appropriate probes of total cell RNA extracted from mutant-infected cells confirmed the absence of mRNAs originating from US3 or from US8 through US11. It is concluded that the deleted genes are dispensable for human cytomegalovirus replication in cell culture.

Mutant herpesviruses obtained by genetic engineering were recently used for functional analysis of viral gene products (13, 15). In the case of human cytomegalovirus (HCMV), this approach is hampered by intrinsic limitations of the system (e.g., species and cell type specificity of the virus, unusually long infectious cycle, and high complexity of the genome). To date, there has been only one report on the experimental basis for mutagenesis by insertion or deletion of defined HCMV genes (22). In addition, comparatively little information on the functional aspects of viral gene products has been gathered from chemically induced HCMV mutants, because availability even of these mutants is limited (3, 26).

HCMV mutant *ts*9 is a temperature-sensitive, DNA-negative mutant that was generated by nitrosoguanidinium mutagenesis of the wild-type strain AD169 (27). At the permissive temperature of 33°C, mutant virus *ts*9 produces titers comparable to those of wild-type AD169. At the nonpermissive temperature of 39.5°C, there is essentially no viral progeny yield in cultures infected by *ts*9, whereas multiplication of AD169 is only slightly reduced (12). Analysis of the protein pattern of cells infected with *ts*9 revealed that polypeptides of 140 to 160, 68, and 50 kDa are not synthesized at the nonpermissive temperature. The molecular sizes of these temperature-sensitive *ts*9 products were correlated with those of known late HCMV proteins, e.g., major capsid protein (150 kDa) and matrix protein (68 kDa), and of a DNA-binding protein (50 kDa) (5, 17, 18), respectively.

In order to possibly relate these phenotypic characteristics to structural alterations of the genome, virion DNA of *ts*9 was cloned into cosmids and analyzed. It will be shown that the organization of the short segment of HCMV mutant *ts*9 distinctly differs from that of wild-type AD169 because of an extensive deletion and structural rearrangement.

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MATERIALS AND METHODS

Cell culture, virus propagation, and virus purification. Human foreskin fibroblasts (HFF) were cultured in Eagle's minimal essential medium enriched with 10% fetal calf serum, vitamins, nonessential amino acids, glutamine, penicillin (0.5 U/ml), and gentamicin (60 μg/ml). For virus propagation, confluent monolayers of HFF (1.5×10^7 cells) were infected at a multiplicity of infection of approximately 0.01 and incubated at 33°C (*ts*9) or 37°C (AD169). The serum concentration was lowered to 2%. When >90% of the cells had developed a cytopathic effect, at 7 to 10 days postinfection, the culture medium was collected and replaced by fresh medium. Culture medium containing extracellular virus was harvested every 2 days until complete destruction of the monolayer. Cell debris was removed by low-speed centrifugation, and virus was sedimented from the supernatants in an SW28 rotor of a Beckman L8-M ultracentrifuge for 3 h at 20,000 rpm and 4°C. The virus pellets were suspended in 2.0 ml of TN buffer (50 mM Tris HCl [pH 7.4], 0.1 M NaCl), and virus was purified from dense bodies by two consecutive cycles of gradient centrifugation (23). The distinct virus band was collected, diluted four times in TN buffer, and sedimented by ultracentrifugation in an SW41 rotor at 30,000 rpm for 2 h at 4°C.

DNA extraction and purification. The virion pellet was suspended in 1.8 ml of TN buffer. After the addition of 0.2 ml of a 5% solution of *N*-lauryl sodium sarcosinate, the suspension was incubated for 60 min at 60°C for complete lysis of the viral particles. The clear solution was layered onto a 7 M CsCl gradient and centrifuged for 65 h in a Beckman 50 Ti rotor at 37,000 rpm and 20°C. Fractions with densities between 1.705 and 1.730 g/ml were pooled, dialyzed against TE buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA), and precipitated with ethanol-ammonium acetate (2 volumes of ethanol per volume of 7.5 M ammonium acetate) at −20°C overnight. The DNA was sedimented by ultracentrifugation in an SW41 rotor (Beckman) for 2 h at 30,000 rpm. Agarose gel electrophoresis was used to examine intactness and estimate the concentration of the viral DNA preparation.

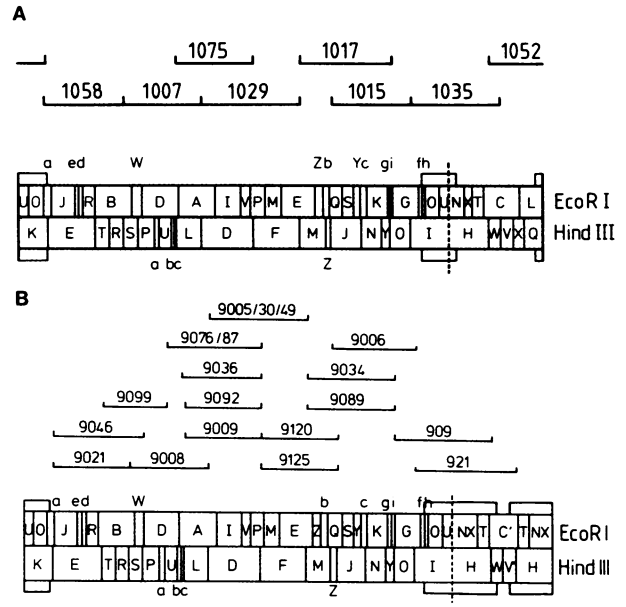

FIG. 1. (A) *Eco*RI and *Hin*dIII restriction maps of the genome of HCMV AD169 and schematic positions of overlapping genomic fragments contained in recombinant cosmid clones pCM1058, pCM1007, pCM1075, pCM1029, pCM1017, pCM1015, pCM1035, and pCM1052. (B) *Eco*RI and *Hin*dIII restriction maps of the genome of cytomegalovirus mutant *ts*9 and schematic positions of overlapping genomic fragments contained in recombinant cosmid clones pAK9021, pAK9046, pAK9099, pAK9008, pAK9076/87, pAK9036, pAK9092, pAK9009, pAK9005/30/49, pAK9120, pAK9125, pAK9034, pAK9089, pAK9006, pAK909, and pAK921.

Cosmid cloning of *ts*9 DNA. The cosmid vector pHC79 (8) was cleaved with *Hin*dIII and ligated to *ts*9 DNA that had been partially digested with *Hin*dIII to generate fragments 40 to 50 kb long. The ligated DNA was packaged in bacteriophage lambda capsids by use of a commercial packaging kit (Packagene Lambda DNA Packaging System; Promega, Heidelberg, Germany) and transduced into *Escherichia coli* DH5α according to the instructions of the supplier. Recombinant clones were selected by plating onto agar containing ampicillin. Recombinant cosmids containing more than one molecule of the vector pHC79 were eliminated on the basis of their tetracycline resistance.

Analysis of the cosmid library. Ap^r Tc^s cosmid clones were mapped to the *ts*9 genome by restriction analysis and Southern blotting. A series of cosmid clones derived from the genome of AD169 (4; generously donated by B. Fleckenstein, Erlangen, Germany) was used as probes for Southern hybridization. Cosmids pCM1007, pCM1015, pCM1017, pCM1029, pCM1035, pCM1052, pCM1058, and pCM1075 are derived from the cosmid vector pHC79 and contain the entire genome of AD169 in partially overlapping fragments (Fig. 1A).

Southern hybridization. Radioactive labeling of the probes was performed by nick translation with [^{35}S]dCTP using a commercial kit (Nick Translation Kit; BRL Life Sciences, Eggenstein, Germany). Specificity of the probes was examined by Southern blots with *Hin*dIII digests of clones pCM1007, pCM1015, pCM1017, pCM1029, pCM1035, pCM1052, and pCM1058. Southern blotting was carried out by standard procedures (19). Hybridization conditions were as follows. After being baked for 2 h at 80°C, the Southern blots were soaked in 4× SET (0.6 M NaCl, 4 mM EDTA [pH 7.8], 80 mM Tris HCl [pH 7.8]). Prehybridization was performed for 6 h at 68°C in prehybridization buffer (4× SET, 10× Denhardt's solution, 0.1% sodium dodecyl sulfate [SDS], 0.1% $Na_4P_2O_7$, 50 μg of denatured sonicated herring sperm DNA per ml) in a shaking water bath. For hybridization, the radioactively labeled probe was denatured by boiling it for 10 min in a water bath, and then it was added to the prehybridization buffer prior to hybridization overnight at 68°C. Washing steps were as follows: once for 5 min in 4× SET–0.1% SDS at room temperature, twice for 10 min in 2× SET–0.1% SDS–0.1% $Na_4P_2O_7$ at 68°C, twice for 10 min in 1× SET–0.1% SDS–0.1% $Na_4P_2O_7$ at 68°C, once for 5 min in 0.1× SET–0.1% SDS–0.1% $Na_4P_2O_7$ at 68°C, and once for 20 min in 4× SET at room temperature. The blots were air dried and exposed to X-ray film for 1 to 10 days.

Colony filter hybridization. For colony filter hybridization, specific probes were prepared by restriction digestion of pCM clones and isolation of a single restriction fragment from a preparative agarose gel using the Gene-clean procedure (Gene-clean Kit; Dianova, Hamburg, Germany). Colony filters of recombinant cosmid clones were prepared by the method of Sambrook et al. (19). Hybridization was as described above under Southern hybridization.

Northern blotting. For Northern (RNA) blotting, total cell RNA was extracted according to the method of Chomczynski and Sacchi (2). Briefly, 2×10^7 infected HFF were lysed in 4.0 ml of Sol D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% [wt/vol] *N*-lauryl sodium sarcosinate, 0.1 M 2-mercaptoethanol). After the addition of 0.5 ml of 2 M sodium acetate (pH 4.0), RNA was extracted by vigorous shaking of the mixture with 5.0 ml of equilibrated phenol and 1.0 ml of chloroform-isoamyl alcohol (24:1) for 10 s. After incubation on ice for 15 min, the emulsion was centrifuged in an SW41 rotor of a Beckman L8-M ultracentrifuge for 15 min at 10,000 rpm and 4°C. The aqueous phase was collected, and the RNA was precipitated with 5.0 ml of 2-propanol at 4°C overnight. In order to protect the RNA from degradation by contaminating RNases, RNase inhibitor (Boehringer, Mannheim, Germany) was added to the aqueous phase at a concentration of 5 U/μl according to the instructions of the supplier.

Formaldehyde agarose gel electrophoresis and Northern blotting were done by the methods of Sambrook et al. (19). Ethidium bromide staining of parallel gels was used to exclude RNA degradation during extraction. Hybridization was performed at 42°C using the identical procedure described for Southern hybridization except that the buffer for Northern hybridization contained 50% formamide and the washing temperature was 60°C.

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (20) using a commercial sequencing kit (T7 Sequencing Kit; Pharmacia, Heidelberg, Germany) with ^{35}S-labeled nucleotides. After incubation, probes were run on 6% polyacrylamide–urea gels in Tris-borate-EDTA buffer (19).

RESULTS

Cloning of the *ts*9 genome. Purified high-molecular-weight, i.e., largely intact, DNA from extracellular *ts*9 virions was partially digested with *Hin*dIII and cloned into the *Hin*dIII cleavage site of pHC79. From more than 10^3 Ap^r Tc^s bacterial transformants obtained, 90 clones were analyzed by Southern blotting. For this purpose, restriction enzyme digests of *ts*9 cosmid DNA were separated on agarose gels,

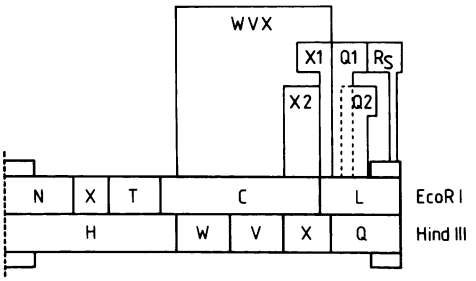

FIG. 2. *Eco*RI and *Hin*dIII restriction maps of the AD169 short segment, showing relative sizes and positions of DNA probes WVX, X2, X1, Q1, Q2, and R_s used for the hybridization studies described.

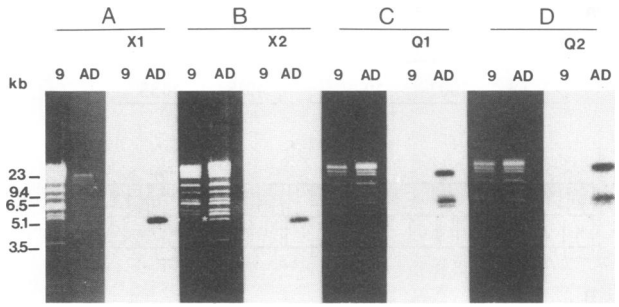

FIG. 3. Southern hybridization of virion DNA from HCMV mutant virus *ts*9 (lanes 9) and wild-type AD169 (lanes AD) with radiolabeled DNA probes X1 (A), X2 (B), Q1 (C), and Q2 (D). DNA from purified extracellular virions was digested with *Hin*dIII prior to separation of the fragments by agarose gel electrophoresis (0.8% agarose) and transfer to nitrocellulose sheets as described in Materials and Methods. Positions of marker DNA fragments are indicated on the left.

blotted onto nitrocellulose, and hybridized with radiolabeled pCM DNA derived from an AD169 cosmid library (Fig. 1A; 4). In this approach, *Hin*dIII and *Eco*RI restriction patterns of *ts*9 cosmids were compared with those of AD169 cosmids. Seventeen *ts*9-cosmids, designated pAK, were identified to represent the long segment of the mutant genome in overlapping fragments (Fig. 1B). Judging from the *Hin*dIII and *Eco*RI restriction sites, the long segment of *ts*9 was determined to be identical to that of AD169 (Fig. 1). This procedure did not, however, provide consistent data for the short segment of HCMV mutant *ts*9.

Analysis of the short segment of HCMV mutant *ts*9. To screen the cosmid library of *ts*9 for recombinant clones representing the short segment, colony filter hybridization was used. A specific probe (*Eco*RI O fragment) prepared from AD169 cosmid pCM1035 reacted with 22 of 187 recombinant bacterial colonies. Restriction analysis of DNA from these reactive clones with *Eco*RI revealed that one recognition site was apparently lost in *ts*9 DNA: the *Eco*RI fragments X (3.6 kb) and N (7.0 kb) were replaced by a fragment of 10.6 kb, which was designated NX (Fig. 1B). This mutation was verified for two separate *ts*9 clones with identical results by Southern hybridization with pCM1035 as well as with a specific *Eco*RI X-fragment probe (data not shown).

Subsequently, a probe for *Hin*dIII fragments W (5.5 kb), V (5.5 kb), and X (5.1 kb) was constructed from AD169 cosmid pCM1052 (Fig. 2). This probe selected 45 recombinant colonies of 280. In most of these positive clones, cleavage of insert DNA by *Hin*dIII yielded only two fragments, one 5.5 kb long and one >20 kb long. In several of these clones, an additional 6.5-kb fragment besides that of the vector pHC79 (6.4 kb) was found (data not shown). Consecutive examination of the *Hin*dIII restriction pattern of DNA from *ts*9 virions (Fig. 3B, lane 9, asterisk) indeed showed the absence of a 5.1-kb *Hin*dIII fragment (presumably *Hin*dIII X) and the presence of only one 5.5-kb *Hin*dIII fragment (presumably *Hin*dIII W or V). Screening of 197 additional new *ts*9 cosmid clones by colony filter hybridization with the more restricted probe X1 (the *Eco*RI-*Hin*dIII fragment from pCM1052 [1.6 kb]; Fig. 2) remained negative, an observation that substantiated the presumed deletion of *Hin*dIII fragment X.

Extent of the deletion. Consecutive experiments served to exclude cloning artifacts as well as to characterize the deletion in *ts*9 DNA. The series of adjacent specific probes constructed from pCM1052 and depicted in Fig. 2 were used in Southern hybridizations to analyze virion DNA from *ts*9 and AD169 after cleavage with *Hin*dIII. Probe X1 detected a 5.1-kb fragment of AD169 DNA, whereas again, *ts*9 DNA showed no hybridization (Fig. 3A). To define the extent of the deletion, the identical procedure for analysis of *ts*9 virion DNA was carried out with probes X2 (3.6-kb *Hin*dIII-*Eco*RI fragment from pCM1052), Q1 (2.5-kb *Hin*dIII-*Nhe*I fragment from pCM1052), and Q2 (2.3-kb *Nde*I fragment from pCM1052), which covered the adjacent genome section (Fig. 2). The following results were obtained. Probe X2 detected a 5.1-kb fragment in AD169 DNA, but again, there was no corresponding hybridization with *ts*9 DNA (Fig. 3B). Probes Q1 and Q2 both detected subsets of low-molecular-weight fragments (i.e., terminal fragments of approximately 6 to 8 kb) from one set and one larger than 20 kb (L-S junction) from the other set of genomic isomers of AD169 DNA, but failed to hybridize with *ts*9 DNA (Fig. 3C and D). Size heterogeneity observed for the low-molecular-weight fragments is in line with reported data (21). In view of their lengths, it is unlikely that failure of the probes to hybridize with *ts*9 DNA was due to a number of point mutations. It was thus concluded that the respective region in the *ts*9 genome, i.e., the *Hin*dIII X fragment as well as the *Hin*dIII Q fragment, was deleted. The structure of the deletion was further defined by analysis of a *ts*9 cosmid previously selected with probe WVX: pAK921 (see above and Fig. 1B and 2). This clone contained one *Hin*dIII fragment of more than 20 kb, two fragments of about 6.5 kb (one representing the vector molecule), and one fragment of 5.5 kb (see previous section). The *Eco*RI restriction pattern verified the presence of fragments of the L-S junction, namely, of *Eco*RI fragments f, h, O, and T and a large fragment probably representing UNX (Fig. 1B). The *Hin*dIII fragments of >20 and 5.5 kb hybridized with pCM1035 as well as with pCM1052, which verified their origin from the short segment of *ts*9 (not shown). In the case of the 6.5-kb *Hin*dIII fragment, the identical size of the vector fragment precluded unequivocal interpretation of the positive hybridization signal. For closer characterization, the 6.5- and 5.5-kb fragments were subcloned in pUC18, and the sequences of their ends (230 to 462 terminal nucleotides; Fig. 4, *ts*9) were determined and compared with the published sequence of AD169 (1, 25). By using this approach, the 5.5-kb fragment was identified as the *Hin*dIII W fragment that is adjacent to the *Hin*dIII H fragment in the short segment (Fig. 1A; Fig. 4, *ts*9 sequences 1 and 2). One end of the 6.5-kb fragment was identical to the end of the *Hin*dIII V fragment adjacent to the *Hin*dIII W fragment (Fig. 4, *ts*9 sequence 3). The opposite

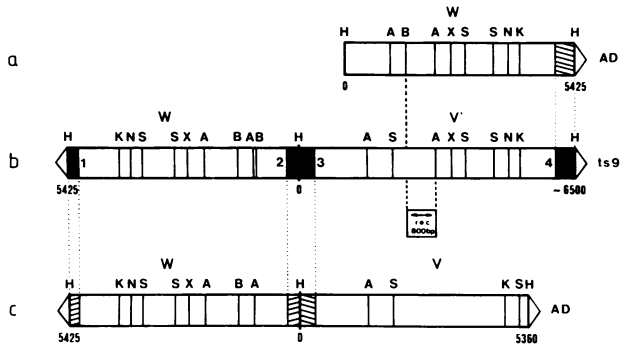

FIG. 4. Comparison of the restriction maps of *Hin*dIII fragments W and V of wild-type AD169 (AD) and W and V′ of mutant *ts*9. The *ts*9 fragments were subcloned from pAK921 and analyzed by restriction endonucleases as follows: A, *Aat*II; B, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; S, *Sma*I; X, *Xho*I. The patterns were compared with those based on sequence data published for AD169 (25). (a) Restriction map of the *Hin*dIII W fragment of AD169 in inverted orientation relative to that given in map c. (b) Restriction map of *ts*9 *Hin*dIII W and V′ fragments showing their relative orientations in the mutant genome. The *ts*9 *Hin*dIII W fragment exhibits one additional *Bgl*II site. (c) Restriction map of AD169 *Hin*dIII W and V fragments. Numbers indicate lengths of the *Hin*dIII fragments in base pairs. The box in map b labeled rec marks an 800-bp section, the putative region of fusion between *Hin*dIII W and V fragments that produces the new V′ fragment of mutant *ts*9. The solid boxes numbered 1, 2, 3, and 4 indicate portions of the *ts*9 fragments that were sequenced; the respective identical sequences of the AD169 fragment are indicated by hatched boxes. The numbers of sequenced nucleotides were 230 for sequence 1, 284 for sequence 2, 342 for sequence 3, and 462 for sequence 4.

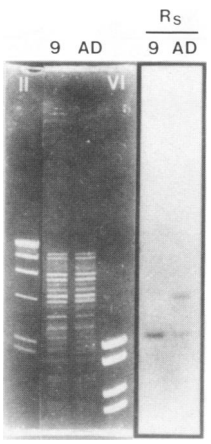

FIG. 6. Southern hybridization of virion DNA from HCMV mutant virus *ts*9 (lanes 9) and wild-type AD169 (lanes AD) with radiolabeled DNA probe R_s. DNA from purified extracellular virions was digested with *Pvu*II prior to separation of the fragments by agarose gel electrophoresis (0.8% agarose) and transfer to nitrocellulose sheets. The marker DNA fragments in lane II were identical in size to those in Fig. 3, and those in lane VI were 2.2, 1.8, 1.2, and 1.0 kb.

end of the 6.5-kb fragment (Fig. 4, *ts*9 sequence 4) was identical in inverted orientation to the end of the *Hin*dIII W fragment adjacent to the *Hin*dIII H fragment (Fig. 4, *ts*9 sequence 1). The extent of this new inverted repeat in the central region of the short segment of *ts*9 was further examined by using several restriction endonucleases to compare the 5.5- and 6.5-kb fragments (Fig. 4, restriction map). This approach suggested that the 6.5-kb fragment represented fused portions of the original W and V fragments to yield a new V′ fragment in mutant *ts*9. According to the restriction analysis, fusion occurred within a section of about 800 bp of the *ts*9 V′ fragment (Fig. 4, *ts*9, rec). From this comparative analysis for the two smaller *Hin*dIII fragments and the *Eco*RI restriction pattern of cosmid pAK921 (see above) and in regard to the described deletion, a putative structure for the short segment of *ts*9 was deduced (Fig. 5): 14.3 to 15.1 kb (*Hin*dIII X and Q plus 3.2 to 4.0 kb of *Hin*dIII V) of the short segment are deleted, and the deleted section is replaced by 20.8 to 21.6 kb representing the *Hin*dIII H fragment plus 3.2 to 4.0 kb of the *Hin*dIII W fragment in inverted orientation.

This model was further confirmed by an additional experiment. A specific hybridization probe for the wild-type short repeat R_s was constructed by isolating and labeling the 1.1-kb *Aat*II fragment from pCM1035 (Fig. 2, R_s). Southern hybridization with virion DNA from wild-type AD169 identified the two expected *Pvu*II fragments (Fig. 6, R_s, lane AD), i.e., a 2.5-kb fragment originating from within the *Hin*dIII H fragment and a 4.4-kb fragment from within the *Hin*dIII Q fragment. In contrast, in the case of *ts*9 DNA, a double band of 2.5 kb reacted with probe R_s (Fig. 6, R_s, lane 9). This result was to be expected from the structure proposed in Fig. 5.

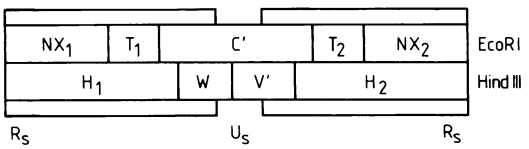

FIG. 5. Suggested *Eco*RI and *Hin*dIII restriction maps of the short segment of the genome of HCMV mutant virus *ts*9.

Transcription analysis. The region deleted in mutant *ts*9 comprises the genes for at least two viral proteins. The gene for a potential immediate early (IE) protein (US3; 1) within the *Hin*dIII Q fragment was identified by Weston (24). The *Hin*dIII X fragment was shown to contain a gene family of six open reading frames (US6 to US11; 1) that is thought to code for gp47-52, a component of glycoprotein complex II (6, 7, 10). To further establish the deletion of these genes, Northern blots of cells infected with *ts*9 or AD169 were performed with probes Q1 and X2 (Fig. 2). Probe Q1 comprises the gene for the potential IE protein, and probe X2 comprises those for open reading frames US8 to US11 (1) of the gp47-52 gene family. For transcription analysis of the IE gene, confluent monolayers of HFF were infected with AD169 or *ts*9 at a multiplicity of infection of approximately 1 and incubated in the presence of cycloheximide for 16 h at 33°C. Identically treated mock-infected HFF served as a control. After extraction, total cell RNA was run on formaldehyde-agarose gels, blotted onto nitrocellulose, and hybridized with probe Q1 (Fig. 2). In RNA from AD169-infected cells, probe Q1 detected a relatively broad band of approximately 1 kb (Fig. 7, Q1, lane AD). This result is in agreement with the data published for the potential IE gene transcripts from US3 (24). In support of the proposed deletion, no specific hybridization signal was obtained for RNA from *ts*9-infected cells (Fig. 7, Q1, lane 9).

Glycoprotein gp 47-52 has been designated a late viral product (6, 7); thus, confluent monolayers of HFF were

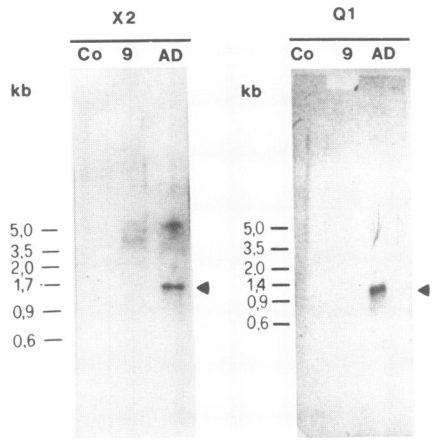

FIG. 7. Northern hybridization of RNA from uninfected (lanes Co), *ts*9-infected (lanes 9), and AD169-infected (lanes AD) fibroblasts with radiolabeled DNA probe X2 or Q1. For hybridization with probe X2, RNA was extracted from infected fibroblasts (1.5×10^7 cells each) grown at the permissive temperature for 96 h; for hybridization with probe Q1, infected fibroblasts (1.5×10^7 cells each) were cultivated in the presence of cycloheximide (200 μg/ml) for 16 h at the permissive temperature prior to extraction of RNA as described in Materials and Methods. Extracted RNA was separated by electrophoresis in formaldehyde-agarose gels (1% agarose) and subsequently transferred to nitrocellulose sheets. The positions of marker DNA fragments run in a parallel lane are indicated on the left.

again infected with wild-type AD169 or mutant virus *ts*9 at a multiplicity of infection of 1 and incubated for 4 days at 33°C. RNA from mock-infected HFF served as control. After extraction, total cell RNA was run on formaldehyde-agarose gels, blotted onto nitrocellulose, and probed with X2 (Fig. 2). As reported by Gretch and Stinski (7), the main transcript originating from the HXLF gene family is a 1.65-kb bicistronic mRNA transcribed from US11 and US10. A less-abundant 1.7-kb bicistronic mRNA is transcribed from US9 and US8. In RNA from AD169-infected cells, probe X2 detected a band of 1.6 to 1.7 kb (Fig. 7, X2, lane AD), whereas in RNAs from *ts*9-infected cells and from mock-infected HFF, no hybridization was observed (Fig. 7, X2, lane 9). This result was thus in line with the transcription data published for AD169 (7) as well as with the proposed structure of the short segment of the *ts*9 genome (Fig. 5).

DISCUSSION

The organization of the short genome segment of temperature-sensitive HCMV mutant *ts*9 originally described by Yamanishi and Rapp (27) is shown here to present an extensive, nonlethal structural alteration. Besides loss of restriction sites, the main mutation is a large deletion of almost one half of the short segment (US1 to US13; 1) comprising the genes for a potential IE protein (US3) and for gp47-52 (US6 to US11), a component of glycoprotein complex II (10). Since the deleted section (14.3 to 15.1 kb) is replaced by a large inverted repeat (20.8 to 21.6 kb) of the retained portion of the short segment including US20 to US36 (1), the original inverted repeat, R_s, of the deleted section is restored to provide terminal a′ sequences at both ends of the short segment in the correct inverted orientation as packaging signals (11). The new repeat being 5.7 to 7.3 kb larger than the deleted section implies an increase of the total genome length of mutant *ts*9 by less than 3%, which should not interfere with its packaging into nucleocapsids.

The salient implication of the mutation of the *ts*9 genome is the deletion of at least two genes, a potential IE gene (US3) and that for gp 47-52 (US6 to US11), a structural envelope protein of HCMV. In view of the fact that *ts*9 grows at the permissive temperature to titers comparable to those of wild-type AD169 (12), these genes have to be regarded as nonessential for HCMV multiplication in cultured human fibroblasts. For other herpesvirus species, e.g., herpes simplex virus type 1 (HSV-1) or pseudorabies virus, several membrane glycoproteins have been identified as being nonessential for in vitro growth (15, 17). In this context, it is of particular interest that genes in the short segments of herpesvirus genomes seem to be dispensable for replication in cell culture. Of four glycoproteins of pseudorabies virus in the short segment, i.e., gI, gp50, gp63, and gX, three are nonessential, namely, gI, gp63, and gX (15). Comparable data have been obtained with HSV-1 (14). Of the seven known HSV-1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI), four have been mapped to the short segment of the viral genome, i.e., gD, gE, gG, and gI. Three of these (gE, gG, and gI) have been shown to be nonessential for replication in vitro (14). Altogether, 11 of the 12 genes located within the short segment of HSV-1 are dispensable for virus growth in cultured cells. Glycoprotein D of HSV-1, on the other hand, has been shown to function during virus penetration, so that gD(−) mutants are unable to penetrate into cells despite binding to the cell surface (13).

The presence of luxury genes in the short segment led Longnecker and Roizman (14) to suggest that the short genome segment of herpesviruses evolved to enable the species to survive in its environmental niche in the natural host. The immunoglobulin G Fc receptor function of the HSV-1 gI-gE complex might thus represent a protective viral measure in vivo which is dispensable for replication in culture (9). Comparatively little is known, on the other hand, regarding the functions of the glycoprotein complexes of HCMV. Glycoprotein complex II of HCMV was reported to be composed of at least two different families of glycoproteins which are immunologically unrelated (10). Monoclonal antibodies made against glycoprotein complex II could be divided into two groups recognizing different glycoprotein components. One of these monoclonal antibodies, 9E10, exhibited distinct recognition of gp47-52 and was used to map the US6 to US11 gene family encoding gp47-52 (6). HCMV mutant *ts*9 should in any case represent a valuable tool for further investigation of the molecular composition of glycoprotein complex II as well as for definition of its function in virus multiplication.

Any attempt to relate the temperature-sensitive phenotype of HCMV mutant *ts*9 to the altered structure of the short genome segment must remain speculative at this point. On one hand, evidence for a regulatory function in viral DNA synthesis of potential products of the deleted open reading frames, including a potential IE protein (US3), is not available at present. On the other hand, genes that are amplified in the mutant genome by the inverted repetition of *Hin*dIII H and W fragments, e.g., ICP22 (US22; 16), may also influence the viral phenotype. Further investigation and construction of appropriate HCMV mutants (22) are indispensable for eventually better understanding the functional aspects of the molecular organization of this extremely complex agent.

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