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Chimeric Theiler's Virus with Altered Tropism for the Central Nervous System

NADINE JAROUSSE,[1] LAURENCE FIETTE,[1] ROBERT A. GRANT,[2] JAMES M. HOGLE,[2] ANDRES McALLISTER,[1] THOMAS MICHIELS,[3] CHRISTINE AUBERT,[1] FREDERIC TANGY,[1] MICHEL BRAHIC,[1]* AND CLAUDIA PEÑA ROSSI[1]

Unité des Virus Lents, UA 1157 Centre National de la Recherche Scientifique, Institut Pasteur, 75724 Paris Cedex 15, France[1]; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115[2]; and Institute of Cellular Pathology, University of Louvain, UCL 75-39, B-1200 Brussels, Belgium[3]

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Theiler's virus is a neurotropic murine picornavirus which, depending on the strain, causes either an acute encephalitis or a persistent demyelinating disease. Following intracranial inoculation, the demyelinating strains infect sequentially the grey matter of the brain, the grey matter of the spinal cord, and finally the white matter of the spinal cord, where they persist and cause chronic demyelination. The neurovirulent strains cause a generally fatal encephalitis with lytic infection of neurons. The study of chimeric Theiler's viruses, obtained by recombining the genomes of demyelinating and neurovirulent strains, has shown that the viral capsid contains determinants for persistence and demyelination. In this article we describe the recombinant virus R5, in which the capsid protein VP1 and a small portion of protein 2A come from the neurovirulent GDVII strain and the rest of the genome comes from the persistent DA strain. The capsid of virus R5 also contains one mutation at amino acid 34 of VP3 (Asn→His). Virus R5 does not persist in the central nervous system (CNS) of immunocompetent SJL/J or BALB/c mice. However, it replicates efficiently and persists in the CNS of BALB/c *nu/nu* mice, showing that its growth in the CNS is not impaired. In BALB/c *nu/nu* mice, whereas virus DA causes mortality with large amounts of viral antigens in the white matter of the spinal cord, virus R5 does not kill the animals, persists in the neurons of the grey matter of the brain, and never reaches the white matter of the spinal cord. This phenotype is due to the chimerism of the capsid and/or to the mutation in VP3. These results indicate that the capsid plays an important role in the characteristic migration of Theiler's virus within the CNS.

Theiler's virus is a murine picornavirus related to encephalomyocarditis virus and mengovirus (25). Although all strains of Theiler's virus are closely related, serologically and in their nucleic acid sequence, they fall into two groups according to the disease they cause after intracranial inoculation. Most strains, including the DA strain, cause a biphasic disease of the central nervous system (CNS) in susceptible mice (15). The first phase, or early disease, is an acute encephalomyelitis which occurs during the first week following inoculation. At this stage, the virus is found exclusively in grey matter, mostly in neurons of the brain and spinal cord. The number of infected cells is small, and most of the animals survive. Soon after, the virus disappears from the grey matter and the animals enter a second phase, or late disease, during which the virus is found in the white matter of the spinal cord. Once there, the virus persists for over 1 year in oligodendrocytes, the myelin-forming cells, and also in microglial cells and macrophages (1, 8). Foci of infected glial cells are spatially associated with infiltrating mononuclear cells and numerous demyelinated axons (4). These lesions very closely resemble active plaques of multiple sclerosis. Throughout late disease, the virus persists despite a specific immune response consisting not only of neutralizing antibodies but also of $CD8^+$, class I-restricted cytotoxic T lymphocytes (26). As shown with β_2-microglobulin-deficient mice, cytotoxic T lymphocytes play a major role in the resistance to persistent infection of some inbred strains of mice but are not required for demyelination (10). Inbred strains of mice can be either susceptible, e.g., SJL/J, or resistant, e.g., C57BL/6 and BALB/c, to the persistent demyelinating infection. Persistence is controlled by several loci, including the *H-2D* locus of the major histocompatibility complex (5, 6, 18, 19, 28).

In contrast to strains with this persistent-demyelinating phenotype, the neurovirulent GDVII strain replicates acutely in neurons and, as a result, kills its host in a matter of days. It does not persist or cause demyelination in the rare survivors of this acute encephalomyelitis (16).

The genetic determinants for these opposite phenotypes have been analyzed by recombining infectious cDNA obtained from the DA and GDVII strains (21, 29, 33). These studies showed that the capsid contains the determinants for persistence and demyelination (17, 22, 32). In this article we report the phenotype of virus R5, a recombinant virus with a chimeric capsid (see Fig. 1) and a mutation in capsid protein VP3. Virus R5 replicates efficiently in the CNS. It does not persist in SJL/J or BALB/c mice. However, it does persist in large amounts in the neurons of the brains of BALB/c *nu/nu* mice but never reaches the white matter of the spinal cord. These results suggest that the capsid plays an important role in the characteristic migration of Theiler's virus in the CNS.

MATERIALS AND METHODS

Construction of chimeric genomes. The construction of plasmid pTMR5 (Fig. 1), its transcription into cRNA, and the

* Corresponding author. Mailing address: Institut Pasteur, Unité des Virus Lents, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: 33 (1) 45.68.87.70. Fax: 33 (1) 40.61.31.67.

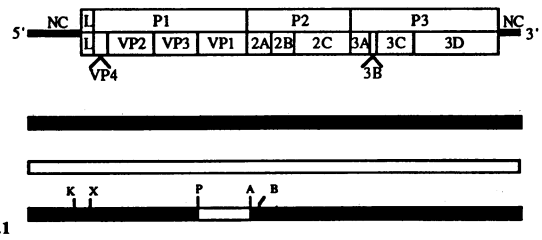

FIG. 1. Genetic map of parental DA and GDVII viruses and of chimeric viruses R5 and R5.1. The genomic organization of Theiler's virus RNA is shown by the standard nomenclature. A, B, K, P, and X represent cleavage sites of the restriction enzymes *Aat*II, *Bam*HI, *Kpn*I, *Pfl*mI, and *Xho*I, respectively. NC, noncoding region.

transfection of BHK-21 cells have been described in detail in a previous publication (32). To construct plasmid pTMR5.1, total RNA was extracted from a pellet of BHK-21 cells which had been infected with virus R5. The RNA was extracted according to a published method (7) and reversed transcribed with avian myeloblastosis virus reverse transcriptase and random hexamer primers as described elsewhere (2). A cDNA fragment, corresponding to the *Kpn*I-*Bam*HI fragment of pTMR5, was amplified by 30 cycles of PCR with primers which correspond to DA virus nucleotides 896 to 916 (5′ AGCGC CTCTGTAGGGAAGCCA 3′) and to DA virus nucleotides 3979 to 3959 (5′ CGCCCATGCACACGAGCATTC 3′). Amplified DNA was purified with glass beads and digested with *Kpn*I and *Bam*HI, and the restriction fragment was cloned in plasmid pTZ19U. The *Xho*I-*Bam*HI fragment of this recombinant plasmid, which corresponds to nucleotides 1221 to 3931 of the genome of virus R5, was recovered. This restriction fragment was exchanged for the corresponding one in plasmid pTMDA to give plasmid pTMR5.1 (Fig. 1). Plasmid pTMR5.1 was linearized by digestion with *Cla*I and transcribed with T7 RNA polymerase as described elsewhere (21, 33). BHK-21 cells were transfected with in vitro-transcribed RNA as follows. Cell monolayers which had just reached confluence were harvested with trypsin and rinsed once in ice-cold Dulbecco's modified Eagle's medium (DMEM) and once in ice-cold phosphate-buffered saline (PBS). The cell pellet was resuspended in ice-cold PBS at a density of 10^6 to 10^7 cells per ml, and 0.8 ml of the cell suspension was transferred to 0.4-cm-electrode-gap Bio-Rad cuvettes. RNA (0.5 to 2 μg) was added and incubated with the cells for 5 min at 0°C. The mixture was pulsed once at 25 μF/1 kV in a Bio-Rad Gene Pulser apparatus. The cuvette was returned to ice for 10 min. The cells were diluted 10- to 100-fold in DMEM–10% fetal calf serum and grown at 37°C on plastic dishes. The medium was changed to DMEM–1% fetal calf serum after 24 h. After a complete cytopathic effect had occurred, the culture medium was harvested and clarified by low-speed centrifugation and titers of the virus were determined on BHK-21 cells by using a standard plaque assay.

Animal studies. Female SJL/J mice, 3 to 4 weeks old, were purchased from the Institut Pasteur animal facility. Female BALB/c and BALB/c *nu/nu* mice, 3 to 4 weeks old, were purchased from IFFA CREDO. The mice were inoculated intracranially with 40 μl of PBS containing 10^4 or 10^5 PFU of virus, depending on the experiment. Mice were observed daily to record mortality. Animals were sacrificed on days 22 and 44 postinoculation, and in some cases as late as days 64 and 78 postinoculation. To perform routine histology and to detect viral antigens, mice were perfused under anesthesia with 20 ml of PBS followed by 20 ml of cold paraformaldehyde fixative as described elsewhere (1, 21). Dissection of the CNS, postfixation, paraffin embedding, and sectioning were performed as described previously (1, 21). The detection of Theiler's virus capsid antigen in paraffin sections was carried out as described previously (1, 21) by using a rabbit hyperimmune serum which binds strongly to VP1 and VP2 and weakly to VP3. Simultaneous detection of antigen by immunocytochemistry and of RNA by in situ hybridization was performed as described elsewhere (1, 3) to identify infected cells. To search for infected astrocytes or macrophages and microglial cells, the sections were reacted with, respectively, anti-glial fibrillary acidic protein hyperimmune serum and F4/80 monoclonal antibody and then hybridized with a Theiler's virus-specific ^{35}S-cRNA probe. Infected oligodendrocytes were looked for by reacting the slides with the anti-Theiler's virus immune serum described above and then hybridizing them with a proteolipid protein-specific ^{35}S-cRNA probe. For myelin studies, mice were perfused with a fixative containing 4% paraformaldehyde and 1% glutaraldehyde. Myelin staining was performed with toluidine blue on semithin sections of Epon-embedded spinal cord.

RESULTS

Description of virus R5. The genetic map of virus R5 is shown in Fig. 1. Plasmid pTMR5 contains a full-length DA virus cDNA in which a *Pfl*mI-*Aat*II restriction fragment has been replaced by its counterpart from the GDVII virus. This fragment codes for 6 amino acids of capsid protein VP3, the entire VP1 capsid protein, and 27 amino acids of protein 2A. The six amino acids from VP3 are identical in the DA and GDVII viruses. In VP1, 25 amino acids are different between the DA and GDVII viruses. Most of them are in the loops connecting the beta strands that form the beta-barrel core of VP1. These loops decorate the surface of the virus and form the bulk of the prominent star-shaped plateaus centered on the fivefold axes of the capsid (12). Four changes are present in the 27 amino acids of protein 2A.

Virus R5 is restricted to grey matter. The phenotype of the chimeric virus R5 was compared to that of the parental DA virus in immunocompetent SJL/J mice. As expected, the DA virus gave the typical biphasic disease. By 45 days postinoculation, viral antigens, inflammation, and demyelination were present in the white matter of the spinal cord (data not shown). Virus R5, however, had disappeared from the CNS by day 7 postinoculation, even in animals inoculated with 10^5 PFU. No inflammation or demyelination appeared in the white matter of the animals. Virus R5 did not kill any mice, demonstrating that the presence of the VP1 protein of GDVII virus is insufficient to confer neurovirulence.

The disappearance of virus R5 from the CNS could be due either to a replication defect or to efficient eradication by the immune response, or to both. In order to distinguish between these possibilities, the phenotypes of viruses DA and R5 were examined in an immunodeficient mouse strain (BALB/c *nu/nu*) and, as a control, in BALB/c mice which are resistant to persistent infection by the DA virus. Both DA and R5 viruses did not persist in the CNS of BALB/c mice, as demonstrated by an exhaustive search for viral antigens in frontal sections of brain and longitudinal sections of spinal cord (data not shown). In contrast, both viruses persisted in the CNS of BALB/c *nu/nu* mice, although the disease patterns and the distributions of viral antigens in the CNS were dramatically different. Virus DA, as described in previous publications (30, 31), caused a high level of mortality during the first 2 weeks following

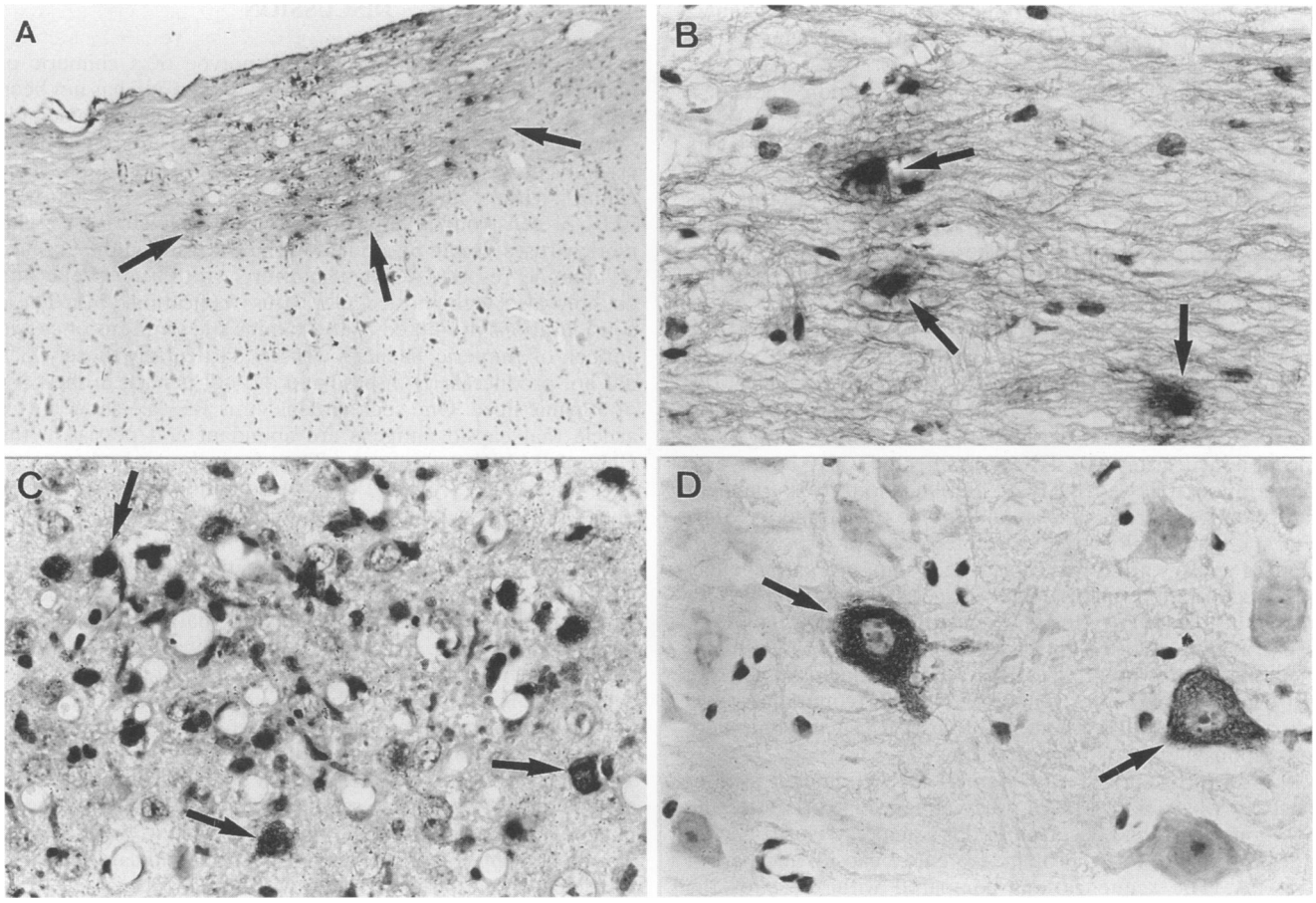

FIG. 2. Histopathological findings, 44 days postinfection, in BALB/c *nu/nu* mice inoculated with either DA virus (A and B) or R5 virus (C and D). Viral antigens were detected in brain and spinal cord sections by immunoperoxidase with a polyclonal anti-Theiler's virus rabbit serum. (A) DA virus. A longitudinal section of the spinal cord is shown. The arrows point to a focus of infection in the white matter. Original magnification, ×240. (B) DA virus. A longitudinal section of the white matter of the spinal cord is shown. The arrows point to three infected cells. Original magnification, ×600. (C) R5 virus. A cluster of infected cells in the cortex is shown. The arrows point to some of the infected cells. Original magnification, ×600. (D) R5 virus. Two infected motor neurons in anterior horns of the spinal cord are shown (arrows). Original magnification, ×600.

intracerebral inoculation. Viral antigens were found in large amounts in the white matter of the spinal cords of surviving animals at both 21 and 45 days postinoculation (Fig. 2A and B). Virus R5, on the other hand, did not cause early mortality in BALB/c *nu/nu* mice, although it caused delayed paralysis in some of the animals (Table 1). As demonstrated by the presence of viral antigens, it persisted in the CNS of 13 of 15 of these mice (Table 1). Viral antigens were still present at the latest time point examined (78 days postinoculation). In contrast to virus DA, virus R5 persisted almost exclusively in the grey matter of the brain, where large amounts of viral antigens were observed at all times postinoculation (Table 1; Fig. 2C). A small number of antigen-positive cells were observed in the grey matter of the spinal cord in some of the mice (Table 1; Fig. 2D). Antigens were virtually never found in the white matter of either the brain or the spinal cord. Most of the infected cells could be identified as neurons by their morphology. Infected neurons were often surrounded by microglial infiltration, and neuronophagia was common even at late times postinoculation. No infection of astrocytes, satellite oligodendrocytes, macrophages, or microglial cells could be demonstrated by an assay for simultaneous detection of antigen and RNA in situ as described in Materials and Methods. No demyelination was observed in these persistently infected animals (data not shown).

TABLE 1. Summary of clinical and histological findings in the CNS of BALB/c *nu/nu* mice infected with virus R5

Day postinfection	No. of animals with paralysis[a]	No. of animals with viral antigens[a] in:				No. of animals with demyelination[a]
		Brain		Spinal cord		
		Grey matter	White matter	Grey matter	White matter	
22	0/3	3/3	0/3	2/3	0/3	ND
44	1/9	7/9	0/9	2/9	0/9	0/7
64	2/2	2/2	0/2	2/2	0/2	ND
78	1/1	1/1	0/1	1/1	0/1	ND

[a] Values show the number of positive animals per total number of animals examined. ND, not done.

The phenotype of virus R5 is due to its capsid. The genetic map of virus R5 (Fig. 1) indicates that its phenotype could be due either to the chimerism of its capsid or to that of protein

2A. The latter explanation is unlikely, since the same chimerism is present in another Theiler's virus recombinant (NR4) which migrates to the white matter (13). However, chimerism of the capsid might not be the only explanation. Indeed, a cytopathic effect did not appear in every monolayer of BHK-21 cells transfected with pTMR5 transcripts. In those monolayers which produced virus, the cytopathic effect occurred late. This suggests that virion production occurred only after mutations appeared following transfection. Some of these mutations, which could have occurred anywhere in the genome, could be responsible for infectivity, and some could be responsible for the phenotype in the CNS.

In a first step, we examined whether mutations outside the capsid were responsible for the phenotype. For this purpose, we constructed virus R5.1 (Fig. 1). The construction is described in detail in Materials and Methods. Briefly, the capsid region of the genome of virus R5 (an *Xho*I-*Bam*HI fragment) was obtained from the RNA of infected cells by reverse transcription followed by PCR amplification. This fragment was exchanged for the corresponding one of plasmid pTMDA (Fig. 1). The R5.1 cRNA was infectious for every dish of BHK-21 cells which was transfected, and cytopathic effect appeared promptly. This result suggests that mutations occurred in the *Xho*I-*Bam*HI fragment and that they were responsible for the restoration of infectivity. In BALB/c *nu/nu* mice, the phenotype of virus R5.1 was indistinguishable from that described above for virus R5. Therefore, mutations outside the *Xho*I-*Bam*HI fragment of virus R5, if present, were not responsible for its phenotype.

In a second step, the *Xho*I-*Bam*HI DNA fragment used to construct plasmid pTMR5.1 was sequenced to identify the mutations which had occurred in this fragment. This fragment codes for part of the L protein, the entire capsid, and part of gene 2A. The sequence was compared with those of the parental cDNAs (pTMDA and pTMGDVII) used to construct pTMR5. Sequence analysis confirmed the expected chimerism of the R5 capsid. Two mutations were discovered; an A→C change at nucleotide 2407, which changes Asn-34 of VP3 into His, and a G→C change at nucleotide 3087, which changes Glu-28 of VP1 into Asp.

Because plasmid pTMR5.1 had been constructed with a DNA fragment cloned after amplification by PCR, a procedure prone to introduce mutations, it was important to examine whether these mutations were indeed present in the genome of virus R5. BHK-21 cells were infected with virus R5, and RNA was extracted 8 h postinfection. After reverse transcription of total RNA, the *Xho*I-*Bam*HI fragment (Fig. 1) was amplified by PCR and sequenced without cloning. Only the mutation corresponding to residue 34 of VP3 (the VP3-34 mutation) was found in the genome of virus R5. The VP1-28 mutation, therefore, must have been introduced by the *Taq* polymerase in the cloned *Xho*I-*Bam*HI fragment used to construct virus R5.1. Since viruses R5 and R5.1 had the same phenotype, the VP1-28 mutation did not contribute to the sequestration of virus R5 in the grey matter.

In summary, the phenotype of virus R5.1, which is identical to that of virus R5, shows that mutations outside the capsid and in part of protein 2A do not determine the phenotype of virus R5. Therefore, the chimerism of the capsid, a VP3-34 mutation, or the chimerism of protein 2A could be responsible for the phenotype of virus R5. It is unlikely that the chimerism of protein 2A is involved, since it is also present in another Theiler's virus recombinant (NR4) which migrates to the white matter (13). Therefore it is most likely that the phenotype of virus R5 is due to the chimerism of the capsid and/or the VP3-34 mutation.

DISCUSSION

It is difficult to interpret the phenotype of a chimeric or mutant Theiler's virus if its replication in the CNS has not been examined; e.g., capsid chimerism may cause a capsid assembly defect and therefore impaired growth (27). The growth properties of these viruses in tissue culture are of little help, since chimeric viruses may grow efficiently in the CNS but poorly in BHK-21 cells (our unpublished observation). Therefore, chimeric viruses should be studied in the CNS and in the absence of immune responses which interfere with virus spread and possibly also with intracellular virus replication (14). These considerations prompted us to examine the synthesis of capsid antigen of chimeric Theiler's viruses, particularly those which did not produce early disease and did not persist, in the CNS of athymic mice. One such virus is virus R5. We show in this article that capsid antigens are abundant and persist in the CNS of BALB/c *nu/nu* mice. Therefore, the early disappearance of virus R5 from the CNS of immunocompetent mice is due to thymus-dependent immune responses and not to a replication defect.

Possible causes for restricted migration of virus R5 in the CNS. Following intracranial inoculation, virus DA replicates first in the grey matter of the brain, particularly in neurons, and then migrates to the grey matter of the spinal cord and finally to the white matter of the spinal cord, where it persists in glial cells. Virus R5, on the other hand, remains sequestered in grey matter, mostly of the brain. It is detected only sporadically in the grey matter of the spinal cord and never in white matter. The majority of infected cells are neurons according to their morphology. Astrocytes, satellite oligodendrocytes, macrophages, and microglial cells are not infected, as shown by double-labeling experiments. Our results indicate that the restricted migration of virus R5 within the CNS is due to an altered capsid. What could be the role of the capsid in this pattern? It is conceivable that Theiler's virus uses different receptors to infect neurons and glial cells of white matter and that virus R5 does not recognize the latter. Alternatively, the migration from grey to white matter may not be due to simple diffusion but could result from an active transport mechanism, e.g., transcytosis through the neurons. Indeed, viral RNA and antigens are commonly found in neurites, including axons in the white matter of SJL/J mice infected with DA virus. Therefore, one can envision that anterograde axonal transport, or exocytosis from axons, may be impaired in virus R5 because of altered structural properties of its capsid.

Possible role of the mutation at VP3-34. Asn-34 is located in the amino-terminal extension of VP3. This extension is involved in complex interactions with VP4 and the amino-terminal extensions of VP1 and VP2 on the inner surface of the capsid (12). The structural consequences of the Asn→His change are not obvious. However, as long as the His remains unprotonated, the capsid can probably accommodate this change through minor rearrangements of the local protein conformation. Because of its position on the inside surface of the capsid, it is unlikely that VP3-34 is directly involved in either receptor or antibody binding. However, residues on the inside surface have been shown to be important determinants of host range in poliovirus and are believed to play a role in modulating the ability of the virus to undergo receptor-induced conformational changes required for cell entry (9, 23, 24). The Asn-to-His substitution at VP3-34 may be playing a similar role in the R5 chimera, either alone or in combination with other changes attributable to the chimeric capsid.

Possible link between the VP3 mutation and the chimerism of the capsid. The respective roles in the R5 phenotype of the

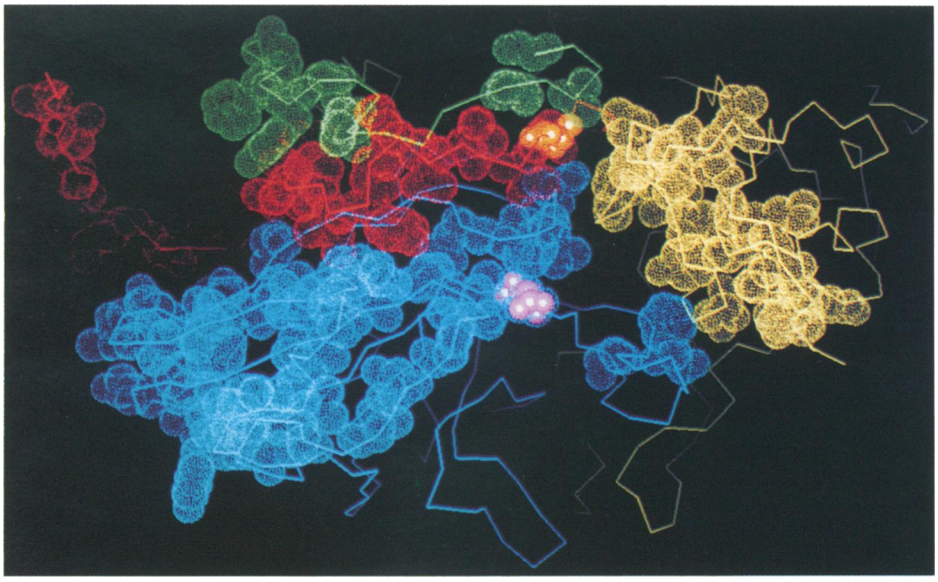

FIG. 3. Representations of one side of the interface between fivefold related protomers. Residues 218 and 219 (purple) of VP1 and residue 34 of VP3 (orange) are shown as solid spheres. Atoms at the interface between protomers are highlighted by display of their Van der Waals surfaces. Other parts of the protomer are represented by alpha carbon traces. In the backbone traces and surface representations, VP1 is blue, VP2 is yellow, VP3 is red, and VP4 is green. The outside of the capsid is at the top, and the inside surface is at the bottom. VP3-34 is located near the interface on the inside surface of the capsid. Immediately above its position, on the outer surface of the virus, are the two VP1 residues, which are at the interface. These three sites in the structure may be important in determining the altered phenotype of R5 compared with DA.

VP3 mutation and of the chimerism of the capsid (VP1 is from the GDVII virus, while the rest of the capsid is from the DA virus) are impossible to distinguish with the present data. Indeed, the two contributions may be related. The locations of the sequence differences between R5 and DA suggest that two changes in particular may play roles in conjunction with the VP3-34 substitution. These two differences, namely, substitutions of Asn (DA) to Thr (R5) at VP1-218 and Ala to Ser at VP1-219, are located at the outer surface of the virus immediately above VP3-34 (Fig. 3). Although both mutations are expected to be accommodated without major perturbations of the structure, their location suggests that they could affect the R5 phenotype by either of two mechanisms. First, these two residues may play a direct role in receptor binding. Both residues are located at the base of a depression on the virus surface which has been suggested to be a receptor-binding site (12, 20). Depending on the topology of the receptor molecule, these residues could be accessible for interaction with the receptor if a poorly ordered loop of VP3 from the neighboring protomer, residues 176 to 184, were displaced by the binding event. Second, the residues may play a role in modulating receptor-induced conformational changes. Both VP1-218 and VP1-219 are located at the interface between two adjacent protomers related by the particle fivefold axes. Mutations at this interface affect the stability and the conformational changes of poliovirus, including receptor-induced changes required for cell entry (11). In either case, the apparent requirement for the VP3-34 mutation for efficient growth of the R5 chimera in BHK cells could be explained if this mutation compensated for a primary defect due to one or both of the VP1 sequence differences.

Conclusion. In conclusion, we report the phenotype of a Theiler's virus recombinant with a capsid which is a chimera between those of the persistent DA and nonpersistent GDVII viruses and which contains a mutation in the N-terminal extension of protein VP3. The virus does not persist in immunocompetent mice but replicates efficiently and persists in the grey matter of the CNS of BALB/c *nu/nu* mice. It never reaches the white matter of the spinal cord as wild-type DA virus does. This virus provides an additional tool to study both the mechanism of clearance of Theiler's virus from the grey matter in immunocompetent resistant animals and the mechanisms of transportation of Theiler's virus within the CNS.

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