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Host Range Mutants of Human Rhinovirus in Which Nonstructural Proteins Are Altered

FAY H. YIN* AND NANCY B. LOMAX

Central Research and Development Department, E. I. du Pont de Nemours & Co., Inc., Experimental Station, Wilmington, Delaware 19898

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Human rhinovirus type 2 did not replicate in nonpermissive mouse cells; the restriction was not in adsorption but in the early events of virus replication. Mutants which had been adapted to grow in mouse cells had the following characteristics: (i) no change in the structural protein, (ii) a larger nonstructural protein and its precursor protein, and (iii) an altered viral RNA synthesis. The altered nonstructural proteins correlated with a change in host range of the virus and may be involved in viral RNA synthesis.

Human rhinovirus (HRV) is a primary cause of mild upper respiratory infection in humans (24). Although rhinovirus infection, as the "common cold," is known to spread easily throughout human populations, only higher primates are susceptible (7, 20); thus, the virus possesses a narrow host range. This narrow host range is reflected in tissue culture in that the HRV replicates only in cells of primate origin.

We have successfully adapted HRV type 2 (HRV2) to grow in a nonpermissive cell line, L cells, which are of mouse origin. The only selective pressure used in adaptation was that of the nonpermissive host.

Host cell restriction of a virus can be the result of restriction at the cell surface or at any intracellular viral event. Restriction at the cell surface is generally due to the absence of appropriate receptors on the nonpermissive cells (11), whereas intracellular restriction can include any viral event in which host factors are involved. Host range mutants of picornaviruses have been previously reported (23); changes in nonstructural proteins have also been described for host range mutants of other viruses (1, 21). However, to our knowledge, no host range mutant of picornavirus has been reported in which nonstructural proteins have been altered.

We have studied the restriction of L cells on HRV2 (HRV2/H) and have also analyzed the biochemical changes of the mouse cell-adapted HRV2 (HRV2/L). The L-cell restriction on HRV2/H is found to occur at an intracellular level. The adapted virus, HRV2/L, which can overcome L-cell restriction, has altered nonstructural proteins, the functions of which have yet to be identified. Using different isolates of HRV2/L, we have shown that the alteration of these nonstructural proteins covaries with the host range of HRV2.

MATERIALS AND METHODS

Cells and viruses. HeLa cells (Flow Laboratories, Rockville, Md.) used with this work were handled as described by Korant et al. (15).

L cells (strain LM) were originally obtained from the American Type Culture Collection and have been propagated in our laboratory since 1974. A subculture of L cells which best supported the replication of HRV2/L was selected and used throughout this study. Karyotyping of this subculture of L cells was performed by the American Type Culture Collection in 1977, and again in 1981 by P. Moorhead, University of Pennyslvania, to confirm the mouse origin of this cell line. L cells were grown in minimum essential medium with Earle salts (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 7% heat-inactivated fetal bovine serum (GIBCO).

HRV2 (strain HGP) was originally obtained from R. R. Grunert (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The propagation and concentration of virus were as described previously (15).

Adaptation of HRV2/H to growth in L cells. L-cell monolayers were infected with HRV2/H at a multiplicity of infection (MOI) of 1 to 10. After a 45-min adsorption period, unadsorbed virus was removed with extensive washing. After 28 h of incubation, the infected cells were frozen and thawed twice. The resulting medium was cleared of cell debris by low-speed centrifugation, and the virus-containing supernatant was then assayed on HeLa cells by plaque titration. Usually a typical starting inoculum contained about 10^7 PFU/ml; after propagation in L cells, the titer decreased to about 10^4 PFU/ml. This resulting virus from L cells was then propagated back in HeLa cells to boost the virus yield. The alternative propagation between HeLa and L cells had to be continued about 40 times before the virus began to form mini plaques on L cells. From that point on, the virus was serially propagated in L cells. During the serial propa-

gation, the virus gradually improved in its plaquing ability on L cells, and after 10 serial passages, the plaque diameter on L cells stabilized at 1 to 2 mm. The L-cell-adapted virus, after three cycles of single-plaque purification, was designated HRV2/L.

Additional isolates of HRV2/L were obtained by performing a second adaptation experiment, again starting with plaque-purified HRV2/H. Individual plaques of adapted virus were picked from the second adaptation experiment and arbitrarily numbered HRV2/L-1, HRV2/L-9, HRV2/L-21, HRV2/L-23, etc.

Purification of viral RNA and infectious RNA assay. Viral RNA was isolated from metrizamide gradient-purified virions. The virion preparations were disrupted in 10 mM Tris-hydrochloride–1 mM EDTA–0.1% lithium dodecyl sulfate (pH 7.5) and then digested with protease K (0.1 mg/ml) for 15 min at 37°C; viral RNA was chromatographed on an oligodeoxythymidylic acid-cellulose column as described by Aviv and Leder (2).

The infectious RNA assay was performed on HeLa cell and L-cell monolayers (9). Viral RNA in a buffer containing 0.14 M LiCl, 1 mM $MgCl_2$, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 1.2 mg of DEAE-dextran per ml was absorbed on cells for 30 min and overlaid with agar. Plaques were scored after 3 days of incubation at 34°C.

Neutralization. The preparation of antiserum against HRV2/H and the procedure for neutralization were as described before (25).

Assay of viral inhibitors. Effective doses of viral inhibitor were determined by yield reduction assays. HeLa cell monolayers, grown in 60-mm dishes, were infected with HRV2/H or HRV2/L at an MOI of 1 to 2. After absorption at 34°C for 30 min, growth medium, containing the desired concentration of test inhibitor, was pipetted onto each plate. Plates were incubated at 34°C for 24 h, after which the infected cells were frozen and thawed twice and the viral yields were determined by plaque assay on HeLa cells.

Determination of pH and heat stability. Growth medium without $NaHCO_3$, but with 10 mM HEPES buffer, was adjusted to the desired pH with NaOH or HCL. Higher-titer virus was diluted into pH-adjusted medium and incubated at room temperature for 1 h. Virus was then titrated by plaque assay on HeLa cells.

Heat stability of the two viruses was determined by incubating high-titer virus at 45°C in medium containing 1% fetal bovine serum. Virus samples were taken out every 30 min and immediately diluted into cold medium. Virus titers of all samples were obtained by plaque assay.

TABLE 1. Virus yields of HRV2/H and HRV2/L

Virus and growth condition	Avg yield (PFU/cell)	
	HeLa cells	L cells
One-cycle growth		
HRV2/H	87	0.01
HRV2/L	21	22
Multicycle growth		
HRV2/H	89	0.01
HRV2/L	24	27

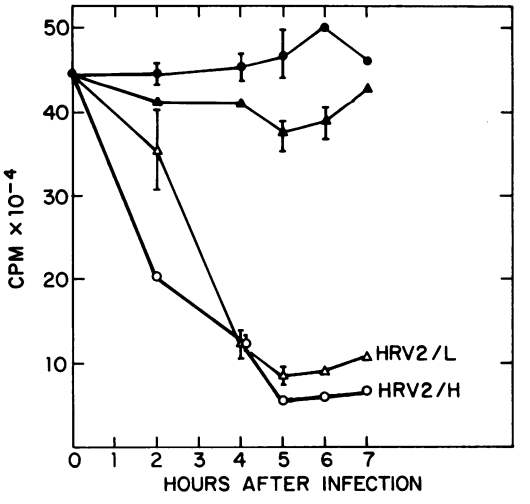

FIG. 1. Inhibition of L-cell protein synthesis. Duplicate plates of L-cell monolayers containing about 10^5 cells per plate were infected with HRV2/H or HRV2/L at an MOI of 50. [^{35}S]methionine at a final concentration of 100 μCi/ml was added at various times after infection. After 1 h of labeling, the cells were scraped, pelleted, and solubilized in 1% SDS. Trichloroacetic acid-insoluble counts were determined. Symbols: ●, uninfected L cells; △, L cells infected with HRV2/L; ○, L cells infected with HRV2/H; ▲, L cells infected with HRV2/H that was irradiated with UV light (254 nm; 280 μW/cm^2 at a distance of 15 cm) for 10 min, which decreased titers to 0.1% of the original.

Labeling of viral protein. Conditions were optimized for labeling of viral proteins in the absence of host protein synthesis. HeLa cell monolayers were infected with either HRV2/H or HRV2/L at an MOI of at least 50. After adsorption, maintenance medium, which contained 1% heat-inactivated fetal bovine serum and Cosmegen (water-soluble actinomycin D; Merck Sharpe & Dohme, West Point, Pa.) at 5 μg/ml, was added to the monolayers. Five hours after infection, the cells were gently washed with phosphate-buffered saline, and medium lacking methionine was added. Viral proteins were labeled by adding 50 to 100 μCi of [^{35}S]methionine (New England Nuclear, Boston, Mass.) per ml to the infected cells at 7 to 8 h after infection, unless otherwise described.

SDS-polyacrylamide gel electrophoresis (PAGE) of ^{35}S-labeled viral proteins. Virus-infected cells were solubilized in 1% sodium dodecyl sulfate (SDS) and heated to 100°C for 3 min in the presence of 10 mM 2-mercaptoethanol. The denatured proteins were then analyzed on either 10% or gradient SDS-polyacrylamide slab gels, using the discontinuous system described by Laemmli (17). Molecular-weight markers used were 150,000, 93,000, 69,000, 46,000, 30,000, 18,000, and 12,000. Fluorography was performed according to the procedure of Bonner and Laskey (3).

Limited proteolysis. The procedure of Cleveland et al. (6) for limited proteolysis was used. Essentially, ^{35}S-labeled viral polypeptides were cut out of dried but

TABLE 2. Neutralization of HRV2/H and HRV2/L

Dilution of anti-HRV2/H	Reduction of PFU (%)					
	HRV2/H	HRV2/L	Isolate of HRV2/L			
			1	9	21	23
1/20,000	98.5	100	100	100	100	100
1/40,000	95	100	100	100		100
1/80,000	83	88	96	87	95	93
1/160,000	60	66	77	78	78	81

unstained polyacrylamide gels, using autoradiography as a guide. The gel slices were rehydrated, and the individual polypeptides were electroeluted into 25 mM NH_4HCO_3 buffer (pH 8.5) containing 0.02% SDS, using a sample concentrator (ISCO model 1750).

For limited proteolysis, buffer containing the eluted polypeptide was adjusted to 50 mM NH_4HCO_3–70 mM NaCl–0.1% SDS–1 mM EDTA–10% glycerol. Bovine serum albumin was added to yield a final concentration of 1.3 mg/ml. Generally, two reactions were run side by side for a given polypeptide, using two different concentrations of protease. The reactions were initiated by the addition of *Staphylococcus* V8 protease (Miles Laboratories, Elkhart, Ind.) to desired concentrations and incubated at 37°C for 30 min. The proteolysis was stopped by adding SDS and 2-mercaptoethanol to final concentrations of 2.5% and 10 mM, respectively, and heating the mixture for 3 min at 100°C. The resulting peptide products were immediately analyzed on a 9 to 18% gradient SDS-polyacrylamide gel. Fluorography was performed as indicated previously.

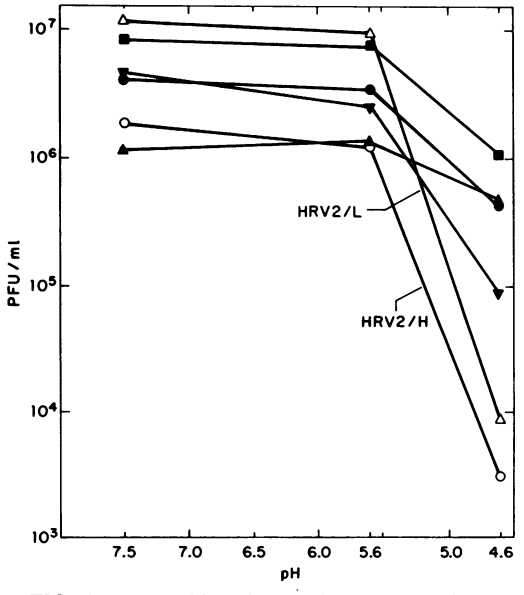

FIG. 2. pH stability of HRV2/H and HRV2/L. The experiment was performed as described in the text. Symbols: ○, HRV2/H; △, HRV2/L; ■, ▲, ●, ▼, isolates HRV2/L-1, HRV2/L-9, HRV2/L-21, and HRV2/L-23.

Pactamycin mapping. For pactamycin mapping, the procedure of Butterworth and Rueckert (5) was followed closely. Spinner suspensions of HeLa cells were infected with virus at an MOI of 100 to 200. At 6.15 h postinfection, pactamycin was added to a final concentration of 2×10^{-7} M; 4 min later, [^{35}S]methionine was added for 14 min. Whole-cell lysate was prepared and analyzed by slab gel electrophoresis; densitometer tracings of the resulting gel were used for calculations.

Antiviral compounds. Experimental antiviral triazinoindole and bisbenzimidazole were obtained from

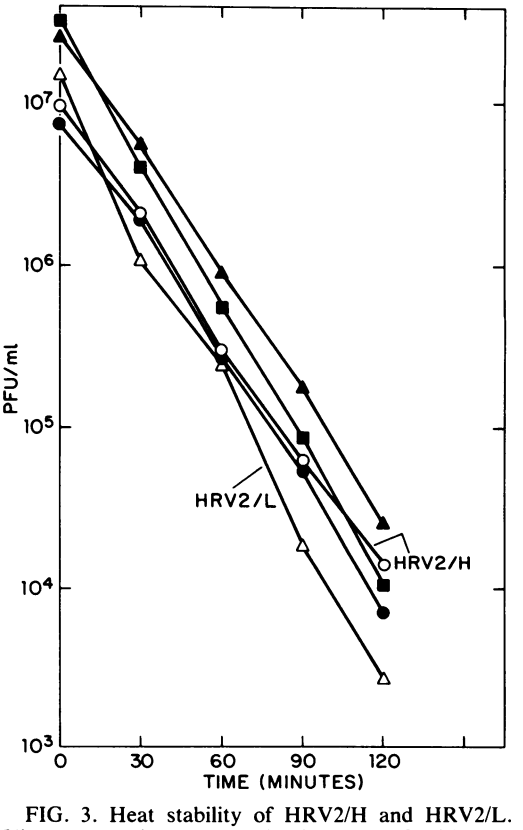

FIG. 3. Heat stability of HRV2/H and HRV2/L. Virus preparations were maintained at 45°C with samples withdrawn every 30 min, and virus titer was determined by plaque assay on HeLa cells. Symbols: ○, HRV2/H; △, HRV2/L; ●, ▲, ■, isolates HRV2/L-1, HRV2/L-9, and HRV2/-21.

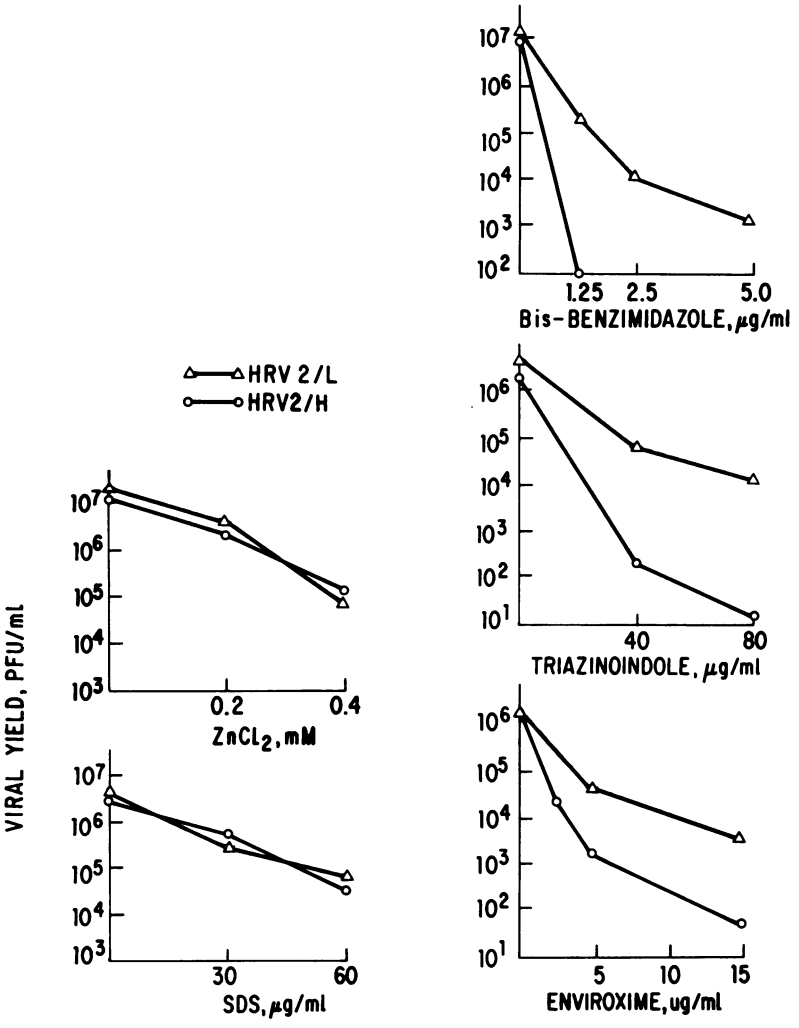

FIG. 4. Inhibition of HRV2/H and HRV2/L with antiviral compounds. The experiment was performed as described in the text. Viral yield of each virus in the presence of various concentrations of inhibitors was determined by plaque assay.

C. E. Hoffman of E. I. du Pont de Nemours & Co., Inc. Enviroxime was kindly provided by D. C. Delong of the Lilly Research Laboratories, Indianapolis, Ind.

RESULTS

Growth characteristics of HRV2/H and HRV2/L in HeLa and L cells. Kinetics of one-cycle growth have been determined to be the same for both HRV2/H and HRV2/L. Viral yields of both viruses were determined in HeLa and L cells under one-cycle growth conditions for which the MOI was 2 to 10 and growth was for 18 h at 34°C; yields were also determined for multicycle growth for which the MOI was 0.1 and growth was for 41 h at 34°C. Table 1 shows that HRV2/H fails to replicate in L cells, whereas HRV2/L, although propagated in L cells serially in our laboratory for more than 40 passages, still maintains its ability to grow and form plaques on HeLa cells. In addition, there has been no change of the viral yields of HRV2/L in HeLa cells as the virus was continually propagated. The viral yields of HRV2/L are consistently the same in both HeLa and L cells and are always about one-fourth of that of HRV2/H.

Restriction of nonpermissive cells. L cells possess approximately the same number of receptor sites for HRV2/H as do HeLa cells, as shown by Lonberg-Holm, using radioactively labeled HRV2/H (K. K. Lonberg-Holm, unpublished data). A purified preparation of virion RNA, in an infectious RNA assay described in Materials and Methods, yielded 2×10^7 PFU/ml on HeLa cells and $<10^2$ PFU/ml on L cells. Thus, the failure of HRV2/H to replicate in L cells is not due to the lack of receptor sites on L cells.

Further evidence for the adsorption and penetration of HRV2 into L cells is demonstrated by the ability of HRV2/H to inhibit the synthesis of host cell proteins. HRV2/H at an MOI of 50 was adsorbed onto L cells for 0.5 h, and the residual virus was washed off. The L-cell proteins were then labeled with [^{35}S]methionine for 1 h at various times after infection, and trichloroacetic acid-insoluble radioactivity was determined. Figure 1 shows that HRV2/H and HRV2/L, at the same MOI, inhibited L-cell protein synthesis equally, in both amount and rate of inhibition. Also, when HRV2/H was inactivated by UV light, no host cell inhibition was observed (Fig. 1). Therefore, the inhibition of L-cell protein synthesis by HRV2/H is caused by viral events.

Although HRV2/H infection inhibited L-cell protein to <10% of noninfected controls, the infection yielded no discernible viral proteins or viral RNA. [^{35}S]methionine-labeled cytoplasmic extract of HRV2/H-infected cells, as analyzed by SDS-PAGE, contained protein patterns similar to those of the uninfected cells. Similarly, no virion RNA, as estimated by trichloroacetic acid-insoluble [^{3}H]uridine label on a sucrose gradient, was detected in HRV2/H-infected L cells (data not shown).

Thus, the restriction of L cells on the replication of HRV2/H is not in adsorption or penetration, but in early replicative events, before amplification of viral protein or viral RNA synthesis.

Comparative studies of HRV2/H and HRV2/L. All comparative studies were performed with HRV2/H and HRV2/L; the results were further confirmed by using additional isolates of HRV2/L. The following comparisons were examined: capsid structure, viral RNA replication, and viral protein synthesis. Since HRV2/L replicates equally well in HeLa and L cells, when replicative events were examined, all infections were carried out in HeLa cells to eliminate the differential effects contributed by different host cells. The viral yields of both viruses were routinely determined by plaque assay on HeLa cells.

Capsid structure. Capsid structures of both HRV2/H and HRV2/L were examined by comparing virion antigenicity and pH and heat stability. Table 2 shows that HRV2/H, HRV2/L, and the various isolates of HRV2/L were neutralized to the same degree by antiserum made to HRV2/H, suggesting that there is no detectable change of HRV2/L in its antigenicity.

Rhinoviruses differ from the other picornaviruses in the lability at low pH. HRV2/L retained the same pH lability as that of HRV2/H (Fig. 2), and the various isolates of HRV2/L were also inactivated at low pH, although to a lesser extent than HRV2/L. Figure 3 indicates the lack of change in the heat stability of HRV2/L and the various isolates. Thus, we conclude that, in terms of the parameters measured, the capsid structure of HRV2/L has not altered.

Viral RNA replication. Both HRV2/H and HRV2/L replications are blocked by a variety of inhibitors. Although the mode of action has not yet been completely elucidated, some inhibitors are known to interfere with viral RNA replications. Three compounds, triazinoindole (SKF 30097), bisbenzimidazole (Abbott 36683), and Enviroxime (Lilly 122771-72 or AR 366), belong to this group (8, 19; C. Y. E. Wu, J. D. Nelson, B. R. Warren, and D. C. Delong, Abstr. Annu. Meet., Am. Soc. Microbiol. 1978, S129, p. 234). All three compounds, at a given concentration, were found to inhibit HRV2/L less than HRV2/H. In contrast to the viral RNA inhibitors, $ZnCl_2$ and SDS, which inhibit HRV2 replication by interacting with structural proteins (4, 18), inhibited both HRV2/H and HRV2/L equally well (Fig. 4).

These studies with inhibitors indicate that the process of viral RNA replication of HRV2/L has altered, although in an unknown manner, and the alteration has resulted in the decreased sensitivity of HRV2/L to viral RNA inhibitors.

Viral protein synthesis. Viral proteins made by HRV2/H and HRV2/L were labeled with [^{35}S]methionine under conditions in which there was no host cell protein synthesis. Figure 5 shows the slab gel electrophoresis pattern of viral proteins obtained from HRV2/H and HRV2/L-infected cells. The nomenclature of these viral proteins essentially follows that of poliovirus (13). The three primary cleavage groups of polypeptides are 1A, X, and 1B. The only difference in nomenclature between poliovirus and HRV2 polypeptides lies in the X region; HRV2 proteins 58K, X (or 43K), and 38K correspond to poliovirus proteins 3b, 5b, and X. The gene order of HRV2 polypeptides is shown in the insert. Among the large viral proteins, one major difference between HRV2/H and HRV2/L was observed. HRV2/H was found to make a viral protein with a molecular weight of 43,000, whereas HRV2/L made a corresponding protein with a molecular weight of 45,000. This alteration was observed whether the viral proteins of HRV2/L were made in HeLa or L cells (lanes 1 and 3, Fig. 5). Of the low-molecular-weight viral proteins, a 9K protein was consistently found among proteins made by HRV2/L but not in proteins made by HRV2/H.

To establish that the 45K protein made by HRV2/L was the equivalent of the 43K protein of HRV2/H, pactamycin mapping of viral proteins was performed as described in Materials and Methods. The gene order of each viral protein was obtained from the ratio of the amount of that protein labeled in pactamycin-

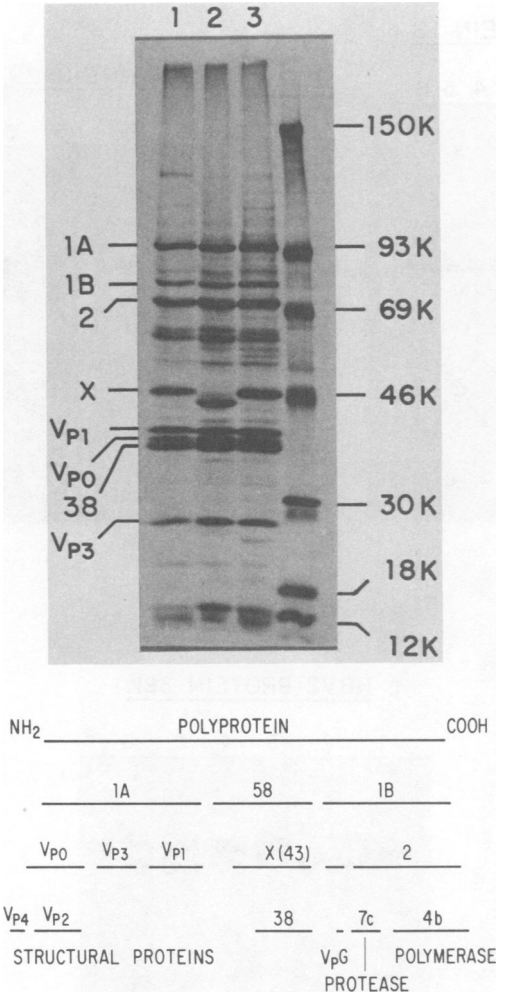

FIG. 5. Viral proteins of HRV2/H and HRV2/L. Infection of HeLa cell monolayers and labeling of viral protein were as described in the text. Viral proteins were labeled at 5 to 6 h after infection and analyzed on a 10% acrylamide gel. Lane 1, Viral proteins of HRV2/L in HeLa cells; lane 2, viral proteins of HRV2/H made in HeLa cells; lane 3, viral protein of HRV2/L made in L cells. Molecular-weight marker proteins were labeled. The gene order of HRV2 polypeptides is shown in the lower portion of the figure.

treated sample to that amount of protein labeled in the untreated control sample. Both 45K of HRV2/L and 43K of HRV2/H mapped in the middle region of the genome and, therefore, were concluded to be the equivalent of each other, or the X protein (data not shown).

To further confirm that X(45) of HRV2/L and X(43K) of HRV2/H are equivalent proteins, and also to determine whether any additional differences exist among pairs of other major proteins of the two viruses, limited proteolysis of the major precursor polypeptides (1A, 1B, 2, X, and 38K) was performed as described in Materials and Methods. Figure 6 shows SDS-PAGE slab gel patterns for each pair of equivalent proteins, before and after proteolysis, at two concentrations of V8 protease. No detectable differences in peptide patterns were observed between the pairs of digested proteins 1A, 1B, 2, and 38K. However, the pattern of digested X(45K) of HRV2/L differed from that of X(43K) of HRV2/H in yielding three cleavage products with higher mobilities (Fig. 6D).

These findings from the limited proteolysis of isolated polypeptides by V8 protease confirmed that X(43K) and X(45K) are related proteins with minor differences. The V8 digests also indicated no detectable differences between the other pairs of equivalent proteins.

Covariation of X(45K) and 9K protein with host range. Figure 7 is an autoradiogram of the viral proteins in HeLa cells infected by various host range mutant isolates of HRV2/L.

To be certain that host cell protein synthesis was completely inhibited, the labeling condition was changed; [^{35}S]methionine was added from 8.5 to 14 h after infection. It was clear that X(45K) was made by all HRV2/L isolates and was absent only among the proteins made by HRV2/H (see lanes 1 and 10). Thus, it can be concluded that X(45) is a viral protein which covaries with the host range of the virion.

In addition, all HRV2/L isolates made a 9K protein which was absent in HRV2/H-infected cells (Fig. 8). Thus, the 9K protein also covaried with the host range of the virion.

Precursor proteins of X(43K) and X(45K) also have different molecular weights. Although $ZnCl_2$ is known to be an inhibitor of HRV protein cleavage, it inhibits cleavage of the HRV2 structural precursor polyprotein to a greater extent than it affects the cleavage of nonstructural precursor proteins (4). Thus, when the viral proteins are labeled in the presence of $ZnCl_2$, it is possible to observe the precursor of X(43K) clearly on the slab gel. Figure 9 shows such an experiment in which the structural proteins were not labeled and proteins of the X region could be clearly identified. It was found that in HRV2/H-infected cells the precursor of X(43K) is 58K and in HRV2/L-infected cells the precursor of X(45K) is 60K. Thus, the difference of molecular weights between proteins 43K and 45K is also evident in the corresponding precursor proteins.

DISCUSSION

HRV2/H is unable to replicate in mouse cells. The host restriction occurs at an intracellular step of an early viral event.

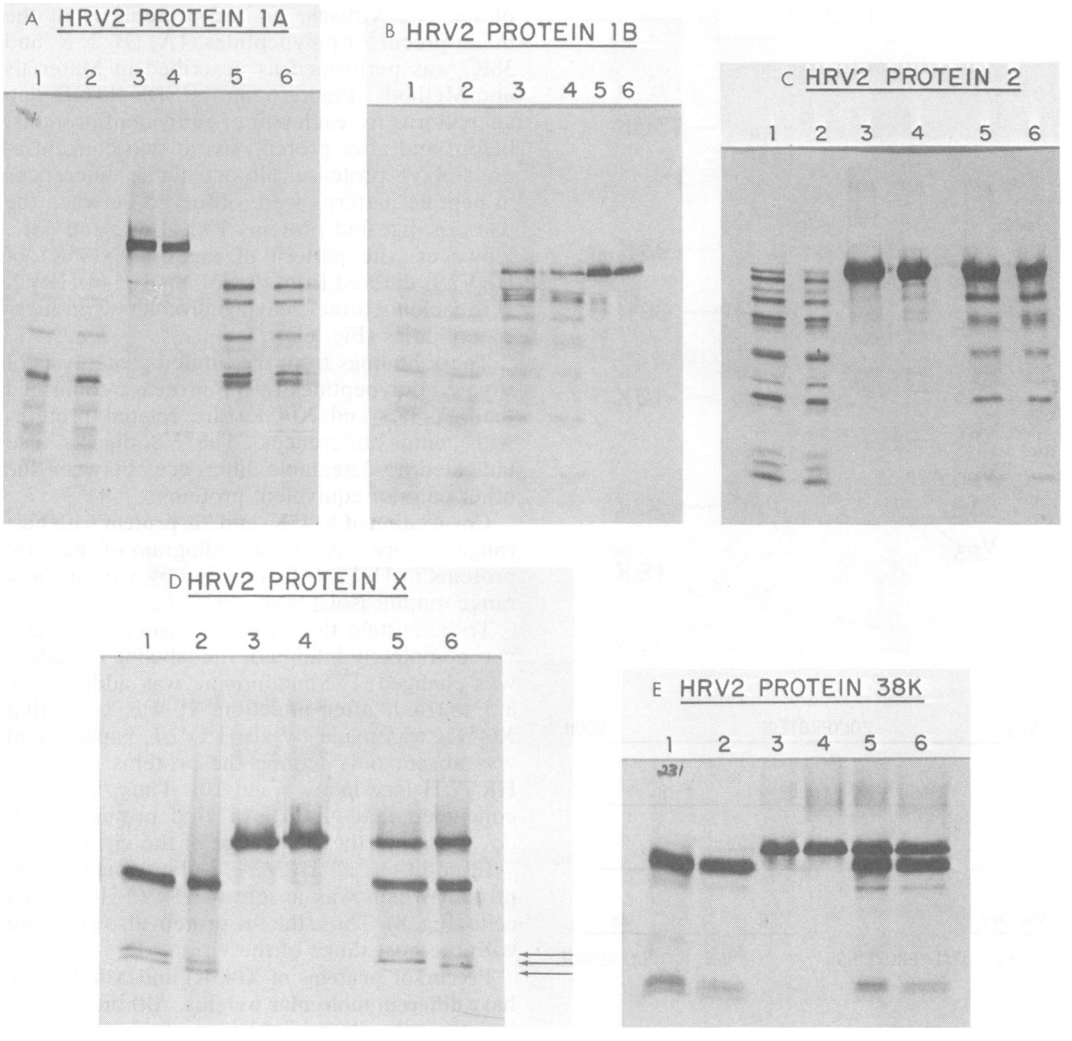

FIG. 6. Limited proteolysis of pairs of equivalent proteins made by HRV2/H and HRV2/L. Elution of viral protein and limited proteolysis were described in the text. (A) 1A protein of HRV2/H and HRV2/L, respectively, was digested with 286 μg of V8 protease per ml (lanes 1 and 2), no protease (lanes 3 and 4), and 72 μg of V8 protease per ml (lanes 5 and 6). (B) 1B protein made by HRV2/H and HRV2/L was digested with 143 μg of V8 protease (lanes 1 and 2) per ml, 36 μg of V8 protease per ml (lanes 3 and 4), and no protease (lanes 5 and 6). (C) 2 protein made by HRV2/H and HRV2/L was digested with 143 μg of V8 protease per ml (lanes 1 and 2), no protease (lanes 3 and 4), and 36 μg of V8 protease per ml (lanes 5 and 6). (D) X(43K) of HRV2/H and X(45K) of HRV2/L were digested with 286 μg of V8 protease per ml (lanes 1 and 2), no protease (lanes 3 and 4), and 72 μg of V8 protease per ml (lanes 5 and 6). Arrows indicate region on gel where digested polypeptides of X(43K) of HRV2/H and X(45K) of HRV2/L showed different mobilities. (E) 38K protein of HRV2/H and HRV2/L was digested with 286 μg of protease per ml (lanes 1 and 2), no protease (lanes 3 and 4), and 72 μg of protease per ml (lanes 5 and 6).

Host range mutants of HRV2 were obtained by adaptation, using only the selective pressure of the nonpermissive host. No change in the capsid structure of the mouse cell-adapted virus HRV2/L was observed since both virions possess similar biological properties of heat and pH stability and antigenicity. In addition, no biochemical differences between the virion polypeptides could be detected by one-dimensional SDS-PAGE or limited proteolysis.

HRV2/H and HRV2/L were found to differ in a process involving viral RNA synthesis, as demonstrated by the decreased sensitivity of HRV2/L to three viral RNA inhibitors. A nonstructural viral protein coded for by HRV2/L, X(45K), and its precursor, 60K, were of differ-

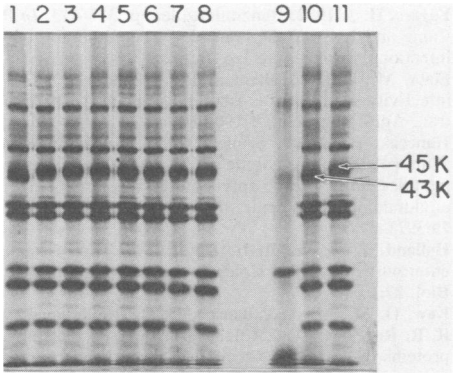

FIG. 7. Viral proteins made by various isolates of HRV2/L as compared to those made by HRV2/H. [^{35}S]methionine at 200 μCi/ml was added to infected HeLa cells at 8.5 to 14 h after infection. Viral proteins were analyzed on a 10% polyacrylamide gel. Lanes 1 and 10, Proteins made by HRV2/H; lanes 2 and 11, proteins made by HRV2/L; lane 9, marker proteins; lanes 3, 4, 5, 6, 7, and 8, proteins made by isolates of HRV2/L.

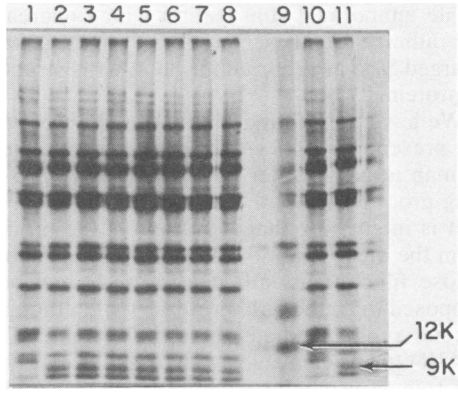

FIG. 8. Viral protein made by various isolates of HRV2/L as compared to that made by HRV2/H. The labeling condition was the same as described in the legend to Fig. 7. Viral proteins were analyzed on 9 to 18% acrylamide gels. Lanes 1 and 10, Proteins made by HRV2/H; lanes 2 and 11, proteins made by HRV2/L; lane 9, marker proteins including 12K protein; lanes 3, 4, 5, 6, 7, and 8, proteins made by isolates of HRV2/L.

ent molecular weights than the corresponding viral proteins coded by HRV2/H. HRV2/L also made a 9K protein which was absent in HRV2/H-infected cells.

We were unsuccessful in obtaining back mutants of HRV2/H from HRV2/L because of the lack of selective pressure. After 30 passages in HeLa cells, HRV2/L still codes for X(45K) and 9K protein. With the lack of back mutants, it was impossible to correlate directly the host range change with the alterations of X(45K) and 9K proteins. Nevertheless, we believe that the alterations of X(45K) and 9K proteins covaried with the host range of HRV2, since all isolates of HRV2/L possessed these two identical alterations and no other detectable changes. The possibility of these isolates being the progeny of the same infectious virus particle could be safely ruled out because the isolates were obtained from two separate adaptation experiments, performed at different times, using slightly different procedures. Furthermore, one mutant in particular forms plaques more efficiently on L cells than on HeLa cells, possessing a biological property distinct from other mutants. This argues strongly that the mutant isolates are not merely siblings of each other.

A most likely hypothesis of host range mutation of HRV2 based on these findings is that the polyprotein of HRV2/H either is unable to be cleaved by the protease in the nonpermissive L-cell host or is cleaved incorrectly and therefore fails to function. The host range mutant HRV2/L is different in that its polyprotein can be cleaved by L-cell protease, although at a different cleavage site in the precursor, yielding a product of X(45K) instead of X(43K). Alternatively, the change of the electrophoretic mobility of a protein(s) in SDS-PAGE could be the result of a

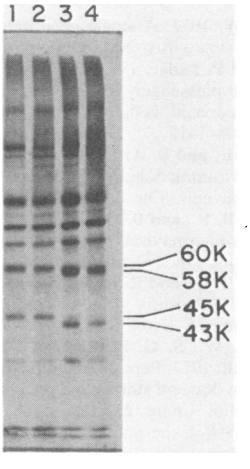

FIG. 9. SDS-PAGE of viral proteins made by HRV2/H and HRV2/L in the presence of $ZnCl_2$. Infection of HeLa cell monolayers was as described in the text. At 7 h and 45 min after infection, medium lacking methionine was placed on the infected cells. At 8 h and 45 min, $ZnCl_2$ was added to a final concentration of 0.6 mM. Five minutes later, cells were labeled with 375 μCi of [^{35}S]methionine per ml for 20 min. ^{35}S-labeled products were analyzed by PAGE on an 8% gel. Lanes 1 and 2, Proteins made by HRV2/L, duplicate samples; lanes 3 and 4, proteins made by HRV2/H, duplicate samples.

single amino acid substitution which decreases the affinity of these proteins for negatively charged SDS as reported for polio virus structural proteins (12).

We have no information on the 9K protein at the present time; its small size makes it difficult to map its gene order by the pactamycin mapping procedure.

It is interesting that X protein is the product from the only region of the picornaviral genome whose function is still unknown. It had been proposed to be the viral protease (14), although a recent report indicated that 7C instead of X(43K) (or its cleavage product) plays an essential role in proteolytic activity (10). Guanidine sensitivity has been mapped in this X protein in an aphthovirus (22), thus indicating the possible involvement of this protein with viral RNA synthesis. Our data indicate that the X region may be involved with viral RNA synthesis, although the detail of its function is yet to be defined, and also that a host factor(s) plays a role in its function. One of the many possible roles of this membrane protein could be the binding and compartmentalization of the various components of viral RNA synthesis, such as polymerase, VpG, and host factors.

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