Conditional Lethal Mutants of Adenovirus 2-Simian Virus 40 Hybrids

I. Host Range Mutants of Ad2⁺ND1

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Human adenovirus type 2 (Ad2) grows poorly in monkey cells, although this defect can be overcome by co-infection with simian virus 40 (SV40). The nondefective Ad2-SV40 hybrid virus, Ad2⁺ND1, replicates efficiently in both human and African green monkey kidney cells, presumably due to the insertion of SV40 sequences into the Ad2 DNA. Several mutants of Ad2⁺ND1 have been isolated that grow and plaque poorly in monkey cells, although they retain the ability to replicate and plaque efficiently in HeLa cells. One of these mutants (H39) has been examined in detail. Studies comparing the DNA of the mutant with Ad2⁺ND1 either by the cleavage patterns produced by *Escherichia coli* R·RI restriction endonuclease digestion or by heteroduplexing reveal no differences. The pattern of protein synthesis of Ad2⁺ND1 and H39 in monkey cells is quite different with the mutant resembling Ad2, which is defective in the synthesis of late proteins. However, in human cells, the proteins synthesized by H39 and the parent Ad2⁺ND1 are very similar. The production of SV40 U antigen in H39-infected cells is different from that in Ad2⁺ND1-infected cells. Finally, the growth of H39 in monkey cells can be complemented by SV40.

Genetic analysis has proved to be a useful approach for analyzing virus cell interactions. Most of the mutants of animal viruses that have been isolated are temperature sensitive. These mutants have provided a basis for constructing genetic maps of the virus genome as well as tools for dissecting the viral life cycle. It is useful to have genetic systems in which viral mutations leading to an absolutely defective as well as a temperature-sensitive phenotype can be obtained in specific regions of the genome. To isolate absolute defective mutants, one must define a permissive condition in which the altered gene product is not essential for virus growth. We describe here a system for isolating host range mutants of the nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus Ad2⁺ND1.

Ad2⁺ND1 is one of a series of hybrids isolated by Lewis and his co-workers (14, 15). Ad2⁺ND1 DNA contains an insertion of 16% of the SV40 genome close to one end of the molecule (4, 9). The segment of SV40 DNA present in Ad2⁺ND1 extends from 0.12 to 0.28 fractional units on the conventional SV40 map (Fig. 1; 19, 23, 28). Ad2⁺ND1 also contains a small deletion of 5.5% of Ad2 DNA at the site of the SV40 insertion (19).

Ad2, which replicates well in human cells, grows poorly in African green monkey kidney (AGMK) cells, and the yield of virus from infected monkey cells is about 1,000-fold less than the yield of virus from human cells (24). The cause of this restriction is not known; however, the block occurs late in infection (3, 21, 22, 24-26), and Baum et al., have shown that Ad2 does not make its full complement of late proteins in infected AGMK cells (2). Superinfection of Ad2-infected monkey cells with SV40 enhances the yield of adenovirus about 1,000-fold (24, 25). Unlike Ad2, however, Ad2⁺ND1 grows equally efficiently in human and AGMK cells (15), and this difference is presumably due to the insertion of SV40 sequences. The SV40 segment in Ad2⁺ND1 contains both early and late regions (see Fig. 1) (12, 20, 27). In lytic infection, Ad2⁺ND1 induces the synthesis of SV40 U antigen, an early SV40 antigen that is also present in SV40-transformed cells (16).

We have selected Ad2⁺ND1 host range mutants which, although still growing well in human cells, are deficient in growth in monkey cells. If Ad2⁺ND1 grows in monkey cells because it contains SV40 sequences, then viruses with mutations in the SV40 segment should comprise a class of host range mutants. We

FIG. 1. A map of the SV40 genome. The cleavage site of *E. coli* *EcoRI* endonuclease is at zero, and map distances are given as percentage lengths of SV40 in a clockwise direction. The dark line on the inner circle represents the segment of the SV40 genome present in Ad2⁺ND1. The positions of the *Haemophilus parainfluenzae* endonuclease cleavage sites are taken from Danna et al. (5) and Sharp et al. (28). The molecular weight of SV40 DNA is 3.6×10^6 (29).

describe here the isolation and preliminary characterization of three such mutants of Ad2⁺ND1.

MATERIALS AND METHODS

Cell culture. CV₁ cells (10) (from J. Mertz), a subline of AGMK cells, were used as the nonpermissive cell line for Ad2 growth. HeLa cells were obtained from J. F. Williams. Cells were cultivated in Dulbecco's modification of Eagle medium (DMEM) (6) with either 10% calf serum (HeLa) or 10% fetal bovine serum (CV₁), streptomycin (100 U/ml), and penicillin (100 U/ml).

Viruses. Adenovirus type 2, originally obtained from J. Rose, and Ad2⁺ND1, obtained from A. Lewis, were plaque purified on HeLa cells and stocks grown on HeLa cell monolayers (30). High-titer stocks were prepared as described by Pettersson et al. (22).

SV40, strain 776, was prepared as described previously (28).

Plaque titrations were performed as described by Williams (30) except that the overlay medium contained 2% agammaglobulinemic calf serum (α CS), penicillin (100 U/ml), streptomycin (100 U/ml), and mycostatin (20 U/ml). Plaque assays on CV₁ cells were performed similarly except that 0.9% Noble agar (Difco Laboratories) was used for the overlay medium and MgCl₂ was omitted. Plaques generally took 6 days to develop on HeLa cell monolayers and 7 to 8 days to develop on CV₁ monolayers. Mutant plaque titers did not increase on incubation of assay plates for 12 days.

Before all infections and plaque assays using high-titer stocks of Ad2, Ad2⁺ND1, or Ad2⁺ND1 mutants,

virus was first treated with trypsin (0.1 mg/ml)-EDTA (0.1 mM) for 30 min at 37°C to inactivate free pentons, which cause detachment of monolayer cells (21). Trypsinized virus stocks to be used without further dilution for infections were then treated with soybean trypsin inhibitor (0.2 mg/ml, Sigma Chemical Co.) for 10 min. All operations were performed at 37°C unless otherwise stated.

Mutagenesis and mutant isolation. Ad2⁺ND1 virions were treated with nitrous acid as described by Williams et al. (31). The amount of viable virus in samples treated for varying lengths of time was determined by plaque assay and preparations inactivated to a level of 10^{-4} (7 to 10 min of treatment) were used. Mutagenized virus was either directly plaqued on HeLa cells, or stocks were prepared on HeLa cell monolayers before virus was plaqued on HeLa cells.

To screen for host range mutants, individual plaques on HeLa cells were isolated by aspiration with a Pasteur pipette, inoculated into 1.5 ml of phosphate-buffered saline (PBS), and frozen and thawed three times. The virus suspension was plaque assayed on HeLa and CV₁ cells. Individual plaques contained 10^4 to 10^5 PFU of virus. Potential mutants were those viruses that gave a 10-fold lower titer on CV₁ cells than on HeLa cells. Mutants were plaque purified and tested a second time, and stocks were prepared on HeLa cell monolayers. For H39, a high-titer stock was subsequently prepared in KB suspension cultures (22). H39 derives from a mutagenized sample that was plaqued directly on HeLa cells. H140 and H162 come from a mutagenized sample that was grown in HeLa cells before being plaqued.

Growth tests. HeLa and CV₁ monolayers in 60-mm petri dishes (approximately 10^6 cells/dish) were infected with 10 PFU of trypsinized virus per cell in 0.2 ml of PBS. After the cells were incubated for 1 h at 37°C, the virus inoculum was then removed, the monolayers were washed twice with PBS, and 5 ml of DMEM plus 2% α CS was added. Cultures were incubated for 48 h, after which infected cells and medium were collected and sonically treated for 30 s using a Raytheon sonicator (model DF101), and the lysate was titered by plaque assay on HeLa cells.

Complementation. For SV40 co-infections, CV₁ monolayers in 60-mm petri dishes were preinfected with 10 PFU/cell for 24 h. Control cultures were mock-infected with 0.2 ml of PBS. Cells were then infected with Ad2, H39, or Ad2⁺ND1 at a multiplicity of infection of 10 PFU/cell as described above, and infected cells were incubated for 48 h. For adenovirus co-infections, cells were simultaneously infected with 10 PFU of the appropriate virus per cell. Infected cells and medium were collected 48 h after infection, sonically treated for 30 s, and virus was extracted with Freon 113 (trichlorofluoroethane, DuPont). Virus yields were plaque assayed on HeLa cell monolayers. Lysates from SV40 co-infections were treated for 30 min at 37°C with SV40 horse antiserum (Flow Laboratories) diluted 1:10 in PBS (1 ml of undiluted antiserum neutralized 2×10^6 PFU).

Analysis of viral DNA with *EcoRI*. The procedures for preparation of viral DNA, cleavage with *EcoRI*, and separation of DNA fragments by agarose-polyacrylamide gel electrophoresis have been de-

scribed by Pettersson et al. (22). Gels were stained with ethidium bromide as described by Sharp et al. (28).

Synthesis of viral proteins. HeLa and CV₁ monolayers in 100-mm petri plates were infected with 25 PFU of Ad2, H39, or Ad2+ND1 per cell as described above. Control cells were mock-infected with 0.4 ml of PBS. At 25 h after infection, cells were labeled for 1 h in medium containing 45 μ Ci of [³⁵S]methionine (2.2 Ci/mmol). Preparation of labeled cell extracts and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out as described by Anderson et al. (1). After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue (in 5% methanol, 7.5% acetic acid). Gels were dried under vacuum and autoradiographed with Kodak single-sided medical X-ray film (SB54T).

Immunofluorescence assays for U-antigen. For fluorescent antibody (FA) tests, cells growing on cover slips were infected with 10 PFU of virus per cell. Either 24 or 48 h after infection, the cover slips were rinsed in PBS, fixed in acetone (at -20 C) for 10 min, and stained for 1 h at 37 C with hamster T⁺U⁺ antiserum diluted 1:20 in PBS (FA titer: U, 1:160; T, 1:320, kindly provided by A. Lewis) (23). Cover slips were rinsed and counterstained for 1 h at 37 C with fluorescein-conjugated goat anti-hamster gamma-globulin (Antibodies, Inc.) diluted 1:10 in PBS and rhodamine-conjugated bovine serum albumin (Huntington Research Center) at a 1:30 dilution. Cover slips were examined at 400 $\times$ magnification under dark-field ultraviolet illumination (Zeiss). Control SV40-infected CV₁ cells showed T⁺ bright nuclear fluorescence quite distinct from the perinuclear fluorescence seen in Ad2+ND1- or H39-infected cells.

RESULTS

To obtain a mutagenized population of virus, Ad2+ND1 virions were treated with nitrous acid to a survival level of 10⁻⁴. Two hundred fifty plaques derived from mutagenized virus were screened, and three host range mutants were

found. This represents a level of approximately 1% mutants. It should be noted that, due to the method of mutant isolation (see Materials and Methods), the mutant H39 arose independently from H140 and H162. The latter two, however, might represent progeny of a single mutant virus.

Plaques and growth characteristics of mutant and wild-type virus. Wild-type Ad2 virus plaques at least 1,000-fold less efficiently on CV₁ cells, the "nonpermissive" monkey cell line, than on HeLa cells, the "permissive" human line. Ad2+ND1 plaques equally efficiently on HeLa and on CV₁ cells. The mutants H39, H140, and H162 derived from Ad2+ND1 plaque about 100-fold less efficiently on CV₁ cells than on HeLa cells (Table 1). The plaque morphology of the mutants differs from wild-type Ad2+ND1. On CV₁ cells, Ad2+ND1 gives medium-sized clear-edged plaques whereas H39, H140, and H162 form very small fuzzy-edged plaques. The plaque morphology of Ad2+ND1 and the mutants on HeLa cells is identical.

It is clearly possible to obtain a collection of mutants of Ad2+ND1 with an altered host range. We have concentrated on one of these mutants, H39, for biochemical analysis; thus, subsequent results will be discussed in terms of H39.

The plaques formed on CV₁ cells by H39 do not arise from revertant virus; virus isolated from eight plaques was retested on both HeLa and CV₁ cells and showed the same plating behavior as the parent H39 (T. Grodzicker, unpublished data). There is 100-fold less virus in these plaques than in Ad2+ND1 plaques repurified from CV₁ cells.

H39 plaques no better on CV₁ cells at 31 C

TABLE 1. *Plaque-forming ability of mutant and wild-type virus*

Expt	Virus	Temp (C)	Infectivity (PFU/ml)		Plaque morphology (CV ₁)
			HeLa	CV ₁	
1	Ad2+ND1	37	3.7 $\times$ 10 ⁸	1.3 $\times$ 10 ⁹	Medium-sized clear edged Tiny plaques fuzzy edged
	H39		4.6 $\times$ 10 ⁸	3.0 $\times$ 10 ⁷	
	H140		1.5 $\times$ 10 ⁸	2.5 $\times$ 10 ⁶	
	H162 ^a		1.5 $\times$ 10 ⁸	5.0 $\times$ 10 ⁵	
	Ad2		3.0 $\times$ 10 ⁸	<5.0 $\times$ 10 ⁵	
2	Ad2+ND1	37	10 ⁹	1.3 $\times$ 10 ⁹	
		31	1.3 $\times$ 10 ⁹	4.3 $\times$ 10 ⁸	
	H39	37	10 ⁹	5.0 $\times$ 10 ⁷	
		31	10 ⁹	6.3 $\times$ 10 ⁷	
	Ad2	37	10 ⁹	5.0 $\times$ 10 ⁵	
		31	5.0 $\times$ 10 ⁸	1.3 $\times$ 10 ⁸	

^a The lower infectivity of H140 and H162 lysates as compared to H39, Ad2, and Ad2+ND1 stocks represents only a difference in the method of preparing viral lysates and not in intrinsic properties of the virus.

than it does at 37 C; thus it is not a temperature-sensitive mutant (Table 1, experiment 2).

We next examined the yield of wild-type and mutant virus after growth in either HeLa or CV₁ cells. The results of such growth experiments are shown in Table 2. Although Ad2⁺ND1 grows equally efficiently in both cell lines, H39 grows 100 times less well in CV₁ cells than in HeLa cells. In this respect, the mutant behaves more like Ad2 than like Ad2⁺ND1.

Virion DNA and RNA synthesis in infected cells. Two explanations of the growth properties of H39 that have to be considered are: (i) that the mutant has lost the SV40 segment of its genome; and, (ii) that it adsorbs or is uncoated less well in monkey cells than the parental Ad2⁺ND1. To examine the first possibility, the fragments of Ad2⁺ND1, H39, and Ad2 DNA produced by digestion with the *Escherichia coli* restriction endonuclease R·RI (EcoRI) were compared (Fig. 2). EcoRI makes five cuts in Ad2 DNA to yield six fragments, designated A to F, in descending order of size (22). Only five fragments are produced from Ad2⁺ND1 DNA because one EcoRI site has been removed by the deletion of Ad2 DNA in Ad2⁺ND1 and there is no EcoRI site in the inserted SV40 segment. Fragments D and E are absent, and a new fragment is produced that contains the SV40 sequences. It is clear that the patterns obtained from Ad2⁺ND1 DNA and H39 DNA are the same, and thus, that the SV40 region has not been deleted. In addition, heteroduplexes between Ad2 DNA and either Ad2⁺ND1 or H39 DNA have been examined by electron microscopy (P. Sharp, unpublished data). This analysis would have detected a deletion of 200 base pairs in H39 DNA, and no detectable differences between Ad2⁺ND1-Ad2 heteroduplexes and H39-Ad2 heteroduplexes were found.

The synthesis of SV40 RNA sequences by Ad2⁺ND1 and H39 in monkey cells has also been examined (J. Sambrook, unpublished data). The pattern and amount of transcription

of SV40 sequences by Ad2⁺ND1 and H39 were found to be the same. Clearly, all steps that precede this transcription, such as adsorption, penetration, and uncoating, must proceed normally.

FIG. 2. Photograph of an 0.7% agarose, 2.2% polyacrylamide gel after separation of the specific fragments of Ad2, H39, and Ad2⁺ND1 DNA. A 0.2- μ g sample of virion DNA was digested with EcoRI as described by Pettersson et al. (22). The fragments were separated by electrophoresis through an 0.7%, 2.2% acrylamide gel for 16 h at 4 V/cm (22, 28). The gel was stained for 30 min in a solution of 0.4 μ g of ethidium bromide per ml and examined directly by short-wavelength ultraviolet illumination (28). Molecular weights of the 6 Ad2 fragments produced by EcoRI cleavage are: A, 13.6×10^6 ; B, 2.7×10^6 ; C, 2.3×10^6 ; D, 1.7×10^6 ; E, 1.4×10^6 ; F, 1.1×10^6 .

TABLE 2. Yield of virus after growth in human and monkey cells^a

Virus	Virus yield (PFU/ml)	
	From infections of HeLa	From infections of CV ₁
Ad2 ⁺ ND1	10 ⁸	9.5×10^7
H39	1.8×10^8	1.5×10^6
Ad2	1.8×10^9	2.5×10^6

^a Cells were infected with 10 PFU/cell and incubated for 48 h at 37 C. Virus yields were plaque assayed on HeLa cells.

Complementation of H39 with SV40 and Ad2. Because H39, like Ad2, grows poorly in monkey cells, we can ask if growth of H39 in monkey cells can be increased by co-infection with SV40. We can also ask if H39 is defective in a function that can be complemented by wild-type Ad2. It should be noted that complementation by Ad2 in CV₁ cells is only possible if the required Ad2 function is expressed before its block in monkey cells.

Table 3, experiment 1, shows the results of SV40 co-infections. Both Ad2 and H39, as shown before, give poor yields of virus in CV₁ cells. Although not shown here, Ad2*ND1 gives high yields of virus when grown either alone or with SV40 in CV₁ cells. The growth of both H39 and Ad2 is increased by co-infection with SV40. We consistently observe a 10- to 30-fold increase in H39 yield and a 100-fold increase in Ad2 yield in SV40 co-infected cells. We do not yet know the explanation for this difference.

Table 3, experiment 2, shows the results of Ad2 co-infections. In these experiments we are looking at the total yield of both input viruses from the co-infected cells. It is clear that co-infection with Ad2 has no effect on H39 growth.

Protein synthesis in infected cells. Ad2 does not make its full complement of late proteins in AGMK cells (2, 8). Because H39 is a mutant of Ad2*ND1 that grows poorly in monkey cells, we

wanted to see if it too is defective in the production of late proteins. Many virus-specific proteins from Ad2-infected cells have been resolved on SDS-polyacrylamide gels (1, 7, 18). HeLa and CV₁ cells were infected with 25 PFU of either Ad2, H39, or Ad2*ND1 per cell and labeled for 1 h with [³⁵S]methionine 25 h after infection. Proteins from infected cells were analyzed on SDS-polyacrylamide gels, and autoradiograms are shown in Fig. 3.

One can see that the proteins induced by all three viruses in HeLa cells are the same with one exception. A 30,000 molecular weight protein has been identified in Ad2*ND1-infected cells that is missing in Ad2-infected cells (17; C. Anderson, unpublished data). This protein is also absent in H39-infected cells.

Although Ad2*ND1 makes the same proteins in both HeLa and CV₁ cells, both Ad2 and H39 make smaller amounts of several late proteins in CV₁ cells. Clear examples of this are the reduced synthesis of both fiber (IV) protein and 27K, which is a precursor to component VI, a hexon-associated protein. Thus, H39, like Ad2, makes reduced amounts of late proteins in CV₁ cells.

U-antigen production. Ad2*ND1 induces the synthesis of one known SV40 antigen, the SV40 U-antigen, which can be detected in the perinuclear region of infected cells (16). It was of interest to examine the production of U-antigen in H39-infected cells. Infected cells, growing on cover slips, were tested for U-antigen by immunofluorescence assays (Table 4).

Although the cells were infected with equal multiplicities of virus, only 1 to 5% of H39-infected HeLa cells gave a weak positive reaction 24 h after infection, as compared to the strong positive reaction shown by 95 to 100% of Ad2*ND1-infected cells. However, 48 h after infection, essentially all infected cells show perinuclear fluorescence. Ad2-infected HeLa cells are U-antigen negative at both 24 and 48 h after infection.

U-antigen production in H39-infected CV₁ cells is always much lower than in HeLa cells. There are practically no CV₁ cells staining positively at 24 h, and even at 48 h only 1 to 5% of the cells have detectable U-antigen. The ratio of positively staining Ad2*ND1-infected CV₁ cells to positively staining H39-infected CV₁ cells is comparable with that of the yield of infectious virus from these cells. Clearly, the production of U-antigen in H39-infected cells is different than in Ad2*ND1-infected cells.

DISCUSSION

We have shown that the host range mutants

TABLE 3. *Growth of virus in monkey (CV₁) cells after co-infection with SV40 or Ad2^a*

Expt	Virus	Virus yield (PFU/ml) titered on HeLa
1	Ad2	1.5×10^6
	H39-2 ^b	9.0×10^5
	H39-7 ^b	2.2×10^6
	Ad2 + SV40	1.4×10^8
	H39-2 + SV40	3.0×10^7
	H39-7 + SV40	3.1×10^7
2	Ad2	1.1×10^6
	H39-2	4.0×10^5
	H39-7	5.0×10^5
	Ad2 + Ad2	1.8×10^6
	H39-2 + Ad2	1.2×10^6
	H39-7 + Ad2	1.2×10^6

^a For SV40 co-infections, cells were preinfected with 10 PFU of SV40 per cell for 24 h. Ad2 or H39 was then added at a multiplicity of infection of 10 PFU/cell, and infected cells were incubated for 48 h. In all other cases, cells were infected simultaneously with 10 PFU of each virus indicated per cell and incubated for 48 h. Total virus yield was plaque assayed on HeLa cells.

^b H39-2 and H39-7 are different stocks of H39.

FIG. 3. SDS-polyacrylamide gel autoradiogram of Ad2-, H39-, and Ad2+ND1-infected HeLa and CV₁ cells. The gel contained 10% acrylamide and 0.13% bisacrylamide. At 25 h after infection, petri dishes of virus or mock-infected cells were labeled for 1 h in medium containing 45 μ Ci of [³⁵S]methionine (2.2 Ci/mmol). Cells were then collected, and samples were processed as described by Anderson et al. (1). Approximately 100,000 counts/min were applied to each sample well of the gel. Nomenclature for virion components synthesized in infected cells is that assigned by Maizel et al. (18) and Everitt et al. (7). Nomenclature for non-virion virus-induced components is that given by Anderson et al. (1) and is expressed as a number based on the approximate molecular weight in SDS-polyacrylamide gels except for P-VII (molecular weight = 20,000), the precursor to the major core protein (V11). All polypeptides shown are found only late in infection with the exception of 71K, an early protein (1). ND1 protein is named as such only to indicate that it is found in Ad2+ND1-infected cells and not in Ad2-infected cells and not to imply that it is coded for by the segment of genome unique to Ad2+ND1.

of Ad2+ND1 behave as if they have lost the function presumably supplied by SV40 that enables Ad2+ND1 to grow efficiently in monkey cells. These mutants grow and plaque 100-fold less on AGMK (CV₁) cells than Ad2+ND1 while maintaining normal growth properties on HeLa cells. One of the mutants, H39, which has been

TABLE 4. Production of SV40 U-antigen in HeLa and CV₁ cells infected with mutant and wild-type virus^a

Virus	Hours post-infection	Positive cells in FA test (%)	
		HeLa	CV ₁
Ad2+ND1	24	95-100 (+) ^b	30 (+)
H39	24	1-5 ($\pm$)	<1 ($\pm$)
Ad2	24	0	0
Ad2+ND1	48	95-100 (+)	75-90 (+)
H39	48	95-100 (+)	1-5 (+)
Ad2	48	0	0

^a Cells growing on cover slips were infected at a multiplicity of 10 PFU/cell, and the number of cells producing U-antigen was determined at the times indicated by immunofluorescence.

^b Staining intensity: +, very bright; $\pm$, weak.

studied in detail, is restricted in its synthesis of late proteins and SV40 U-antigen in CV₁ cells. In HeLa cells, the permissive cell line H39 has an altered pattern of U-antigen induction as well as altered production of a 30,000 molecular weight protein found in Ad2+ND1-infected cells but not in Ad2-infected cells. Thus, the H39 mutation has pleiotropic effects even though the SV40 region in H39 has not been deleted and normal amounts of SV40 RNA are found in infected cells.

One simple explanation consistent with these results is that H39 contains a mutation in the SV40 segment of Ad2+ND1 resulting in a loss of functional activity and a decrease in the amount of detectable U-antigen. This decrease would be expected whether U-antigen is a host protein induced by SV40 or is directly coded for by SV40. It should be noted that most nonfunctional enzymes caused by missense mutations in bacteria are CRM⁺ (cross-reacting material). An alternative explanation is that H39 contains a mutation in the Ad2 portion of the genome that has a polar effect on the expression of the SV40 segment. We might also consider the possibility that the 30,000 molecular weight protein present in Ad2+ND1-infected cells, but not in H39- or Ad2-infected cells, represents the product of the gene that is mutated in H39. However, other explanations are also consistent with this result. For example, the 30,000 molecular weight protein might be a host product that is induced by Ad2+ND1 infection but not by H39 or Ad2 infection. Further studies are needed to resolve the origin of this polypeptide. We can approach analysing the defective function and type of mutation in H39 by isolating revertants including temperature-sensitive ones. We are also studying complementation and recombination

among the different host range mutants.

It is interesting to note that H39 has growth properties similar to Ad2*ND5, another of the series of hybrids isolated by Lewis and his colleagues (14). Ad2*ND5 fails to grow in AGMK cells and does not induce U-antigen production, although it contains 28% of the SV40 genome including the sequences present in Ad2*ND1 (9, 12, 13, 15).

It seems likely that mutations that, in any way, lead to an inactive gene product may occur more frequently than ones that result in the production of a temperature-sensitive protein. We have shown that it is possible to isolate host range mutants of Ad2*ND1 at a reasonable frequency. This result suggests that it is feasible to screen for mutations in other regions of SV40 that are present in other Ad2-SV40 hybrids, for example, T-antigen⁻ mutants of Ad2*ND4.

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