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Growth of fastidious adenovirus serotype 40 in HRT 18 cells: Interactions with E 1 A and E 1 B deletion mutants of subgenus C adenoviruses

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Summary. Growth of fastidious adenovirus serotype 40 (Ad 40) in several cell lines was investigated. Ad 40 was able to readily propagate in human intestinal cell line, HRT 18. Coinfection assays were made in non-permissive and permissive cells between Ad 40 and Ad 5dl 312 or dl 1520, mutants deleted in E 1 A and E 1 B regions, respectively, to test the ability of Ad 40 to complement these mutants and vice versa. Ad 40 could enhance Ad 5dl 312 DNA synthesis in HRT 18 and HeLa cells, although its own DNA disappeared in the presence of this mutant in HRT 18 cells. In coinfection with dl 1520, Ad 40 DNA synthesis was inhibitied by dl 1520 in HRT 18 cells and dl 1520 DNA synthesis was inhibited by Ad 40 in 293 cells. This might reflect the presence of unusual products encoded by Ad 40 E 1 B region.

Introduction

Adenoviruses are important agents of infantile viral gastroenteritis and serotypes 40 and 41 (Ad 40 and Ad 41) are responsible for a large majority of cases [4]. In spite of this pathogenicity, Ad 40 and Ad 41 have been discovered later than most of the serotypes due to their difficulty to be propagated in vitro. Contrary to conventional human adenoviruses, the so-called fastidious adenoviruses could not be serially passaged in primary cells such as human embryonic kidney cells (HEK) or human diploid fibroblasts [1]. However, successful growth of Ad 41 in primary cells in defined conditions has been recently reported [24]. Ad 40 and Ad 41 can replicate with variable success in human cell lines such as HeLa, Hep-2, KB and A 534 cells [7, 23, 25, 37]

Replication of fastidious adenoviruses in human cell lines is thought to be

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blocked at an early stage in their growth cycle [29] and these viruses have been grown to some extent in the Ad 5-transformed human embryonic kidney cell line 293 [16, 28], which contains and expresses the Ad 5 early region E 1 [12]. This has led to the hypothesis that these viruses were defective in early regions E 1 A and E 1 B or in one of them and could be complemented by early gene functions of Ad 5 integrated in the genome of these cells [28, 19].

The Ad 2 E 1 A region gives rise to two major mRNAs (12 S and 13 S). The polypeptides encoded by these mRNAs are identical except for an internal domain of 46 aminoacids within the largest product due to differential splicing of RNAs. Adenovirus mutants in E 1 A region that express only the 13 S gene product, replicate normally, are capable of trans-activating viral gene transcription and can immortalize primary rodent cells. Adenovirus mutants expressing only the 12 S gene product do not replicate in HeLa cells at low multiplicity of infection (for review see [3, 11]).

Ad 2 E 1 B region governs the synthesis at early times of two major polypeptides. A 22 S mRNA encodes a 19 k and a 55 k polypeptides. A 13 S mRNA encodes only the 19 k polypeptide. The 55 k polypeptide is essential for a complete lytic cycle in HeLa cells and is involved in accumulation of viral mRNA during productive infection. E 1 B mutants in 55 k polypeptide are altered in expression of early mRNAs and in DNA synthesis. Mutants in 19 k cause a rapid cytopathic effect and induce degradation of host and viral DNAs [9, 27, 36].

The nucleotide sequence of early regions E 1 A and E 1 B of fastidious adenoviruses has been determined [14, 34]. There is no major difference in the structure of E 1 A and E 1 B regions of nonfastidious and fastidious adenoviruses.

An increase of the replication of fastidious adenoviruses when cultivated in the presence of nonfastidious adenoviruses has been observed. Tiemessen and Kidd [30, 31] determined that Ad 2 could complement the growth of Ad 41 in Chang conjunctival and HEF cells by measuring the rate of late antigens with monoclonal antibodies specific to Ad 41.

For Ad 40, Mautner et al. [19] found that Ad 40 could be complemented in HeLa cells by E 1 B 55 K protein of nonfastidious adenoviruses.

In this study, we investigated the possible helper function of early regions E 1 A or E 1 B of nonfastidious adenoviruses in complementing DNA replication of Ad 40. We show that Ad 40 E 1 A region is functional. Coinfection assays of Ad 40 with a nonfastidious adenovirus deleted in E 1 B region (dl 1520) led to an inhibition of the synthesis of both DNAs.

Materials and methods

Cells

Human cell lines used were 293, HRT 18, an intestinal cell line [17] and HeLa. Monolayer cultures from these cell lines were grown in Dulbecco's medium containing 10% foetal bovine serum and divided twice a week at an appropriate subculture ratio.

Viruses

Ad 5 dl 312 is a deletion mutant lacking E 1 A activity [15]. dl 1520 is a hybrid Ad 2/Ad 5 E 1 B mutant which does not synthesize E 1 B 55 K protein and shows a reduced rate of E 1 B 19 K protein synthesis [2].

Stocks of dl 1520 and Ad 5 dl 312 were prepared in 293 cells, stock of Ad 2 WT (prototype strain provided by Dr. J. F. Williams, Pittsburg, Pa.) was made in HeLa cells. These viruses were purified as previously described [10]. Stock of Ad 40 (prototype strain Dugan) was obtained in HRT 18 cells after 10 passages of the virus in this cell line. Stock of Ad 40 was maintained as a crude extract after freezing and thawing of cell cultures three times.

Titers of Ad 40, dl 1520 and Ad 5 dl 312 stocks on 293, HRT 18 and HeLa cells, were determined at 48 h post-infection, by a fluorescent focus assay as previously described [10], using as first antibody a rabbit polyclonal anti-Ad 2 raised against purified virions and as a second antibody, fluorescein conjugated sheep anti-rabbit immunoglobulin. Titers were expressed as fluorescent focus units per ml (FFU/ml).

Enzyme linked immunosorbent assay (ELISA)

Ad 40 infected cultures were freezed and thawed three times then serially diluted and tested for adenovirus antigens by ELISA as previously described [22].

Culture conditions for infection and coinfection assays

Cell monolayers of 293, HRT 18 or HeLa grown in 25 cm^2 flasks were single or double infected with virus stocks. The input virus concentration, expressed in FFU/cell, was variable. Virus inocula were adsorbed for 2 h at 37 °C. After this time, medium containing 2% foetal bovine serum was added. Each infected culture was incubated at 37 °C until total cytopathic effect (CPE). In cases of absence of CPE, infected cells were recovered after 8 days.

Extraction and digestion of viral DNAs

Viral DNAs were extracted from 5×10^6 infected cells using the Hirt [13] procedure modified by Wadell and de Jong [35]. Half of the extracted DNA was digested by Hind III restriction endonuclease and electrophoresed in a 0.8% agarose horizontal slab gel in Tris-Borate buffer at 50 V. Gels were stained in ethidium bromide and photographed under short UV light.

Isolation of RNA

Total RNA was prepared from mock-infected and from Ad 40 infected HRT 18 cells at 24 h post-infection by the acid guanidinum thiocyanate extraction method [6].

cDNA synthesis and PCR amplification

cDNA synthesis was performed using random primers (Gene Amp RNA PCR kit, Perkin Elmer Cetus). PCR amplification was carried out with oligodeoxynucleotides representing nucleotides 2485 to 2506 (5′ end) and the complementary sequence from nucleotides 3018 to 3039 (3′ end) of the Ad 40 sequence [34]. These primers were purchased from Eurogentec S. A. and purified by HPLC chromatography. The amplification reaction was performed with Taq DNA polymerase (Promega) in 40 cycles of DNA denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and elongation (72 °C, 3 min). Amplified DNAs were electrophoresed in a 2% agarose gel.

Results

Growth of Ad40, Ad5dl312, and dl1520 in 293, HRT18, and HeLa cells

Human cell lines 293, HRT 18, and HeLa were tested for their capability to support growth of Ad 40. HRT 18 is a cell line derived from human rectal adenocarcinoma [32] and was chosen because it is permissive to bovine enteric fastidious coronavirus [17]. To estimate virus growth in each cell line, the rate of Ad antigens in cultures was measured after three serial passages by ELISA. In 293 and HeLa cells, antigen levels were very low and the virus was not readily propagated. HRT 18 cell line gave a higher level of Ad 40 antigens, although Ad 40 grew more slowly than nonfastidious adenoviruses. Total cytopathic effect was observed after 6 – 7 days post-infection. A reference stock of the virus was then prepared in this cell line after several additional passages.

Reference stocks of mutants Ad 5 dl 312 [15] and dl 1520 [2] showing large deletions in transcription units E 1 A and E 1 B, respectively, were prepared in 293 cells.

Each reference stock was titrated on 293, HRT 18, and HeLa cell lines. Results are shown in Table 1. The titer of Ad 5 dl 312 mutant of HeLa cells was about 10^5-fold lower than on 293 cells. This was in agreement with the results published by Jones and Shenk [15]. The titers of Ad 5 dl 312 mutant in HRT 18 and HeLa cells were of the same order of magnitude. The titer of E 1 B mutant dl 1520 was 6-fold and 100 fold lower on HRT 18 and HeLa cells, respectively, than on 293 cells. For Ad 40, the difference between the titers obtained in the three cell lines during the first passage was less significant. Ad 40 behaved neither as an adenovirus mutated in E 1 A 13 S mRNA nor as an E 1 B mutant lacking 55 k protein.

Ad40 can complement Ad5 dl312 growth defect

To determine if a complementation could occur between Ad 40 and Ad 5 dl 312 or dl 1520, coinfection assays with Ad 40 and deletion mutants were carried out. The synthesis of viral DNAs in infection and coinfection assays was vis-

Table 1. Titers of Ad 40, Ad 5 dl 312 and dl 1520 reference stocks on 293, HRT 18 and HeLa cell lines

Viruses	Cells		
	293	HRT 18	HeLa
Ad 40	7.0×10^5	1.4×10^6	2.4×10^5
Ad 5 dl 312	6.4×10^{11}	1.7×10^7	7.0×10^6
dl 1520	3.5×10^{12}	2.1×10^{11}	3.5×10^{10}

Viruses were titrated at 48 h post-infection by fluorescent focus assay [10]. Titers are expressed in focus forming units per ml (FFU/ml)

ualized after electrophoresis on agarose gel of Hirt extracted DNAs digested by Hind III restriction enzyme.

The cells were first single infected at a multiplicity of infection (m.o.i.) of 1 FFU/cell. This m.o.i. was calculated from reference stocks titrated in the most permissive cells, i.e., HRT 18 for Ad 40, 293 for the mutants. At this m.o.i., a clear restriction pattern of Hirt extracted DNAs was seen only with the most permissive cell line (Fig. 1).

Coinfection assays of Ad 40 and deletion mutants were performed in HRT 18, 293 and HeLa cells. Figure 2 shows DNAs extracted from cells coinfected with Ad 40 and Ad 5 dl 312. The m.o.i. of Ad 40 (titrated on HRT 18) was always 1 FFU/cell. The input concentration of Ad 5 dl 312, titrated on 293 cells, varies

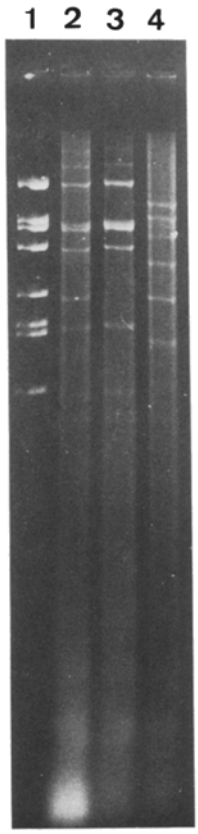

Fig. 1

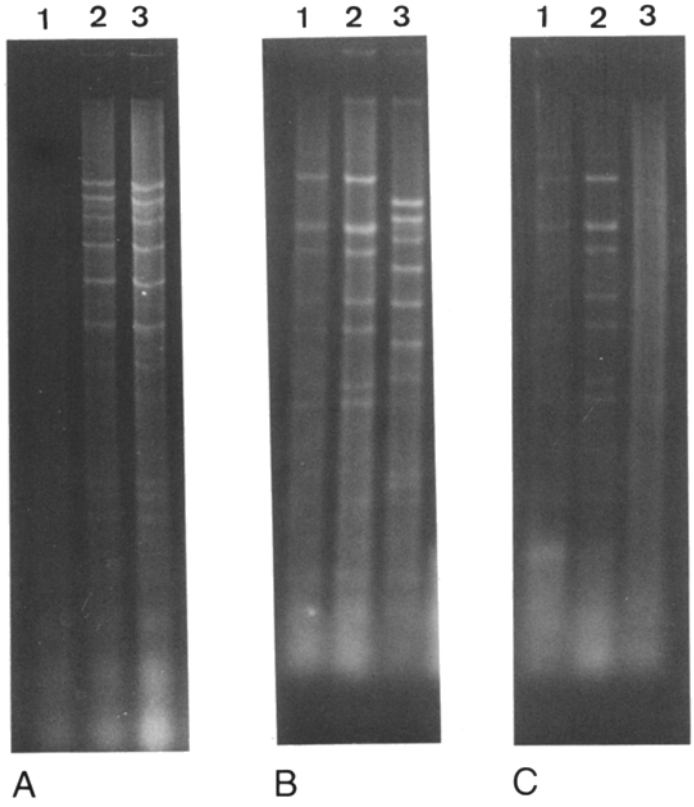

Fig. 2

Fig. 1. Hind III restriction pattern of Hirt extracted DNAs of Ad 5 dl 312, dl 1520 and Ad 40. Viruses were infected at a m.o.i. of 1 FFU/cell in permissive cell lines. *1* Ad 5 DNA (marker), *2* Ad 5 dl 312 (293 cells), *3* dl 1520 (293 cells), *4* Ad 40 (HRT 18 cells)

Fig. 2. Coinfection of deletion mutant Ad 5 dl 312 and Ad 40 analyzed by Hind III digestion of Hirt extracted DNAs. Coinfection was performed in HRT 18 (**A, B**) and HeLa (**C**) cells. The m.o.i. of Ad 40, titrated on HRT 18 cells, was 1 FFU/cell in all cases. The m.o.i. of Ad 5 dl 312, titrated on 293 cells, varies from 1 FFU/cell (**A**) to 10,000 FFU/cell (**B** and **C**). *1* Ad 5 dl 312, *2* Ad 40 + Ad 5 dl 312, *3* Ad 40

from 1 FFU/cell (Fig. 2 A) to 10,000 FFU/cell (Fig. 2 B and C). After infection of 1 FFU/cell of Ad 5 dl 312 in HRT 18 cells, the restriction pattern of this virus was not visible (Fig. 2 A, lane 1). Restriction patterns obtained in Ad 40 single infection (Fig. 2 A, lane 3) and in coinfection Ad 40 + Ad 5 dl 312 (Fig. 2 A, lane 2) were identical. At low m.o.i., no effect of one virus on DNA viral synthesis of the other virus was therefore observed.

With 10,000 FFU/cell of Ad 5 dl 312 in HRT 18, the restriction pattern of Ad 5 dl 312 was visible in single infection (Fig. 2 B, lane 1). Although the cells were washed before the viral DNA extraction, it could not be ruled out that the inoculum could be responsible for visible restriction pattern. As a control, a similar experiment was performed in which viral DNA extraction was made 1 h post infection. In this case, no visible band was seen after digestion with restriction enzyme. In coinfection assays, the restriction pattern of Ad 5 dl 312 was enhanced but Ad 40 DNA was absent (Fig. 2 B, lane 2). With 10,000 FFU/cell of Ad 5 dl 312 in HeLa cells, the same phenomenon of enhancement of Ad 5 dl 312 DNA was observed by coinfection assays (Fig. 2 C, lane 2) although the restriction pattern of Ad 40 was not visible in single infection (Fig. 2C, lane 3).

These experiments indicated that Ad 40 could complement E 1 A functions from deletion mutant Ad 5 dl 312 in HRT 18 and HeLa cells, and that this complementation was dependent on the input concentration of Ad 5dl 312. At high m.o.i. of Ad 5 dl 312, Ad 40 was able to stimulate the synthesis of Ad 5 dl 312 DNA although its own DNA disappeared in coinfection assays. In HeLa cells the restriction pattern of Ad 40 could not be visualized, but a similar enhancement of the synthesis of Ad 5 dl 312 DNA was observed when the two viruses were present.

Interactions between Ad 40 and dl 1520

To determine if Ad 40 could also stimulate the growth of a mutant deleted in the E 1 B region, coinfection assays of Ad 40 with dl 1520 were performed. The m.o.i. of Ad 40 and dl 1520 was 1 FFU/cell. A clear restriction pattern of dl 1520 was visible when the virus was infected in 293 cells (Fig. 3 A, lane 1). The bands visible on Fig. 3 B, lane 1 (dl 1520 grown in HRT 18 cells) result from leakage of this mutant (see Table 1). For Ad 40, a clear restriction pattern was observed in HRT 18 (Fig. 3 B, lane 3). In coinfection assays with dl 1520 and Ad 40 both DNAs disappeared in 293 and HRT 18 cells (Fig. 3 A and B, lanes 2).

In a similar manner, coinfection assays with Ad 2 and dl 1520 were performed in HRT 18 cells to determine if dl 1520 was able to inhibit DNA synthesis of nonfastidious adenovirus or if this inhibition was specific of fastidious Ad 40. Results of this assay are shown in Fig. 4, lane 2. No inhibition of Ad 2 DNA by dl 1520 was observed. A faint band of DNA visible under Hind III-B band of Ad 2 revealed the presence of dl 1520 DNA. As expected, this virus was therefore complemented by Ad 2.

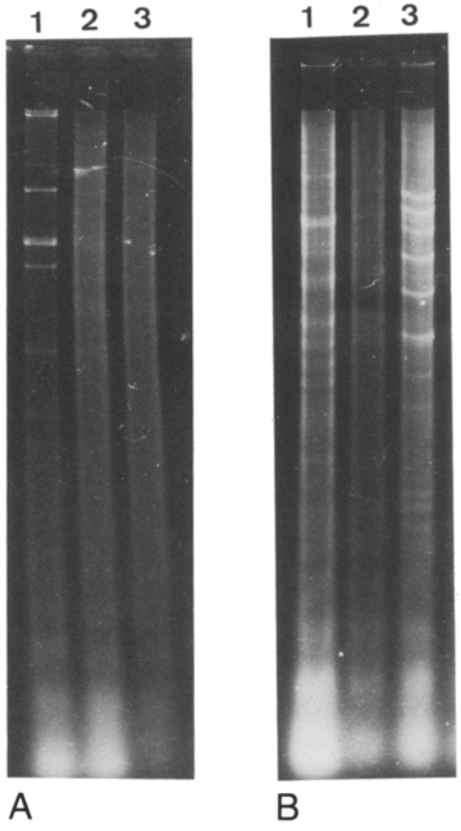

Fig. 3

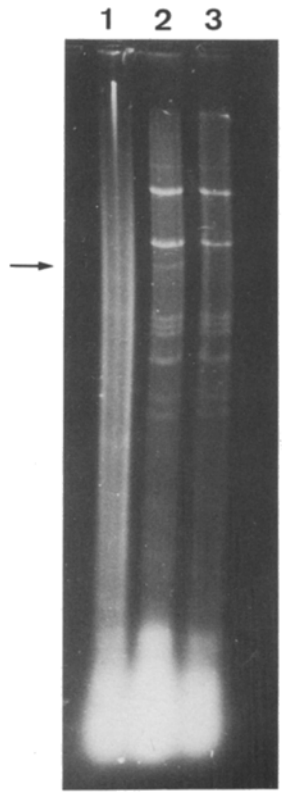

Fig. 4

Fig. 3. Coinfection of deletion mutant dl 1520 and Ad 40 analyzed by Hind III digestion of Hirt extracted DNAs. Coinfections were performed in 293 (**A**) and HRT 18 (**B**) cells. The m.o.i. of dl 1520 and Ad 40, titrated on 293 and HRT 18, respectively, was 1 FFU/cell. *1* dl 1520, *2* Ad 40 + dl1520, *3* Ad 40

Fig. 4. Coinfection of deletion mutant dl 1520 and Ad 2 in HRT 18 cells analyzed by Hind III digestion of Hirt extracted DNAs. The m.o.i. of both viruses was 1 FFU/cell. The arrow indicates a specific dl 1520 restriction fragment in double infection. *1* dl 1520, *2* dl 1520 + Ad 2, *3* Ad 2

Amplification of cDNA from E 1 B region of Ad 40

In order to determine of Ad 40 E 1 B region could transcribe a mRNA coding for 55 k protein, cDNA PCR amplification was performed from RNA extracted from Ad 40 infected HRT 18 cells. Primers were chosen based on sequence data of van Loon et al. [34] who have found an open reading frame for 55 k protein in nucleotide positions 1719 to 3147. Primers corresponding to C-terminal domain of this protein (nt 2485 to 2506 and nt 3018 to 3039) were used. Figure. 5 shows that a fragment of predicted size (522 bp) was present in Hirt extracted Ad 40 DNA (lane 2). A fragment of identical size was observed from amplified cDNA from total RNA extracted from Ad 40 HRT 18 infected cells (Fig. 5,

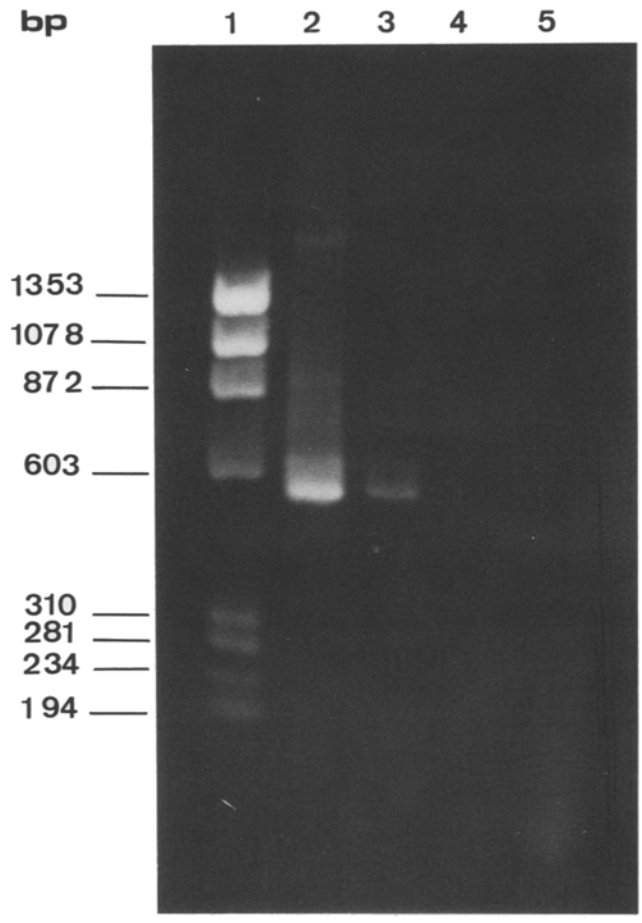

Fig. 5. PCR amplification of a fragment of Ad 40 E 1 B region. *1* Molecular weight standard, the sizes of fragments are indicated in bp on the left, *2* Hirt extracted Ad 40 DNA from HRT 18 cells, *3* cDNA amplification of Ad 40 infected HRT 18 cells, *4* PCR amplification from RNA of Ad 40 infected HRT 18 cells (without previous cDNA synthesis), *5* cDNA amplification of mock-infected HRT 18 cells

lane 3). Controls without prior cDNA synthesis (Fig. 5, lane 4) and with mock-infected cells (Fig. 5, lane 5) were negative.

Discussion

In the present study, we were able to cultivate Ad 40 in a human intestinal cell line, HRT 18, which has not been transformed in vitro by adenovirus genes. From an Ad 40 stock obtained in HRT 18 cells, there was a modest difference for antigen production during the first passage between HRT 18, HeLa and 293 cell lines (Table 1). However, the yield of Ad 40 during subsequent passages was constant for HRT 18 cells whereas it decreases for HeLa and 293 cells. The ability of Ad 40 to grow in HRT 18 cells showed that the presence of adenovirus genes integrated in the DNA of the host cell is not an absolute requirement to

allow growth of this serotype. The capability of cultivating Ad 40 in an intestinal cell line is in agreement with results from Kidd and Madeley [16] who cultivated Ad 40 in foetal intestinal organ cultures. Ad 40 grows very well in its target tissue, the intestinal epithelium and it has been detected in large amounts in stool of children with gastroenteritis [4, 5, 21, 33]. It is likely that some cellular factors from intestinal cells can promote the lytic cycle of Ad 40 as suggested by Tiemessen and Kidd [30]. However, even in HRT 18 cells, Ad 40 grows more slowly than nonfastidious adenoviruses and total cytopathic effect was observed only after 6 – 7 days post-infection. Attempts to propagate Ad 40 in HRT 18 using a high m.o.i. were unsuccessful (results not shown). This was consistent with results previously reported showing a similar Ad 40 growth inhibition at high m.o.i. in WK, A 549 and 293 cells [37].

To test the hypothesis that E 1 A or E 1 B regions of Ad 40 were defective, complementation assays were made between Ad 40 and E 1 A deletion mutant Ad 5 dl 312, and between Ad 40 and E 1 B deletion mutant dl 1520. The DNAs of both coinfecting viruses were visualized after Hirt extraction and restriction enzyme digestion.

In HRT 18 cells, Ad 40 could not complement Ad 5 dl 312 infected at low m.o.i. At high m.o.i. of Ad 5 dl 312, the amount of DNA of Ad 5 dl 312 in double infection was enhanced but Ad 40 DNA synthesis was inhibited (Fig. 2 B, lane 2). This inhibition might be the result of a phenomenon of dominance of one serotype by another as studied by Delsert and D'Halluin [8]. However, results depending on m.o.i. values should be interpreted with caution. For example, 10,000 FFU/cell of Ad 5 dl 312 titrated on 293, corresponds to 0.25 infectious units on HRT 18 cells and to 0.1 on HeLa cells (Table 1). HeLa cells infected singly with high m.o.i. of Ad 5 dl 312 supported moderate DNA synthesis (Fig. 2 C, lane 1) and residual virus growth (Table 1). This is in agreement with previous results showing some leakage for this mutant [20]. In coinfection with Ad 40, the level of Ad 5 dl 312 DNA was augmented, although Ad 40 DNA was not detected (Fig. 2 C, lane 2). E 1 A functions of Ad 40 are therefore capable of complementing Ad 5 dl 312 in cells permissive (HRT 18) and non-permissive (HeLa) to Ad 40.

Mautner et al. [19] have detected by slot blot hybridization a complementation of Ad 40 growth by Ad 5 dl 312 and vice versa in HeLa cells. Due to relatively low yields of Ad 40, these authors were not able to obtain visible restriction patterns of Hirt extracted DNA. We also used slot blot hybridization (results not shown) and found an amount of Ad 40 DNA lower in cells coinfected by Ad 5 dl 312 and Ad 40 than in cells infected with Ad 40 only. A possible explanation for this discrepancy is that the Ad 40 reference stocks have not been obtained in the same cell line.

To know if Ad 40 could complement a nonfastidious adenovirus mutated in E 1 B transcription unit, coinfection of Ad 40 and dl 1520 was performed. Surprisingly, this coinfection led to an inhibition of DNA synthesis of both viruses. As a control, cells were coinfected with dl 1520 mutant and Ad 2 wild

type. No inhibition of Ad 2 DNA synthesis was observed. Furthermore, dl 1520 was complemented by Ad 2 (Fig. 4, lane 2). We do not presently know the reasons of the mutual inhibition between Ad 40 and dl 1520. Interactions between two adenoviruses lacking E 1 B products should not lead to an inhibition of the DNA synthesis of both viruses. Several findings have suggested that Ad 40 E 1 B region is transcribed in an unusual manner [18, 26] although it is structurally similar to E 1 B region of nonfastidious adenoviruses [34]. The presence of unusual E 1 B proteins of Ad 40 associated with viral or cellular factors might explain the inhibition of dl 1520 DNA synthesis in coinfection experiments. Our PCR results show that at least one mRNA is synthesized in a region corresponding to the 55 k messenger of nonfastidious adenoviruses. To know why dl 1520 DNA synthesis is inhibited by Ad 40, further experiments are necessary to detect and characterize the Ad 40 E 1 B mRNAs and proteins.

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