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Fastidious Human Adenovirus Type 40 Can Propagate Efficiently and Produce Plaques on a Human Cell Line, A549, Derived from Lung Carcinoma

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Human adenovirus type 40 (Ad40) cannot propagate in conventional established human cell lines such as KB or HeLa cells. However, it has been shown that Ad40 DNA replicates in KB18 cells which express Ad2 E1B genes, suggesting that Ad40 is defective in the E1B gene function in KB or HeLa cells. We show here that Ad40 can propagate and produce plaques on A549 cells which do not contain Ad E1B genes. Our experiments show that the levels of replication of Ad40 DNA and production of infectious Ad40 virus in A549 cells are the same as or higher than those in 293 or KB18 cells. Dot blot analysis shows that the levels of Ad40 E1A and E1B mRNAs expressed in A549 cells at early to intermediate times postinfection are at least 10-fold higher than those in KB or KB18 cells. Northern (RNA) blot analysis shows that large E1B mRNA species (approximately 24S to 26S) are synthesized prior to the onset of DNA replication in A549 cells. No E1B mRNA species are synthesized in KB or KB18 cells at early times postinfection, and no differences in the expression of E1B mRNAs are seen between KB and KB18 cells. The experiment suggests that A549 cells have a cellular factor(s) which activates Ad40 E1B mRNA synthesis and that the E1B mRNA synthesis helps Ad40 propagation. In contrast, Ad40 can propagate in KB18 cells by using Ad2 E1B gene products that are constitutively expressed in this cell line. Furthermore, this result shows that Ad40 cannot propagate in KB cells because of the failure in the expression of E1B genes at early times postinfection.

Human adenovirus type 40 (Ad40) and Ad41 are referred to as enteric adenoviruses (EAds). They have been demonstrated to be one of the causes of viral-associated diarrhea in children (5, 6). Ad40 and Ad41 are closely related antigenically (5, 29) and share nucleotide sequences in terms of DNA-DNA hybridization (30) but have distinct restriction endonuclease cleavage patterns of viral genomic DNAs (17, 34). Nucleotide sequences of Ad40 and Ad41 left-terminal DNA regions, including E1 regions (15, 16, 34) and the DNA regions encoding DNA-binding protein and 23K protease (35), have been recently determined. Open reading frames in the E1A and E1B regions of both Ad40 and Ad41 corresponding to the polypeptides encoded by the Ad2 or Ad5 E1A and E1B regions, respectively, have been found. EAds, particularly Ad40, are fastidious since they cannot propagate in conventional established human cell lines or in human embryonic kidney cells. Moreover, Ad40 strains isolated from some locales have been demonstrated to grow in 293 cells (a human embryonic kidney cell line that constitutively expresses Ad5 E1A and E1B) (8). However, those isolated from other locales have been shown to be unable to grow in 293 cells (32).

Another difficulty in the study of EAds is that virus preparations isolated from patients with diarrhea frequently contain more than one kind of virus, including different types of Ad (4, 18, 36, 37). Thus, the possibility exists that the presence of different viruses interferes with or stimulates the replication of fastidious EAds in tissue culture.

We have isolated an Ad40 strain (Sapporo strain) which can grow in 293 cells and does not contain other Ads in terms of DNA restriction analysis (10). Recently, Mautner et al. (25) showed that the E1B 55K function of Ad2 or Ad5 and Ad12 complements the defect in Ad40 DNA replication in KB or HeLa cells. However, the molecular basis of Ad40 DNA replication in the presence of other Ad E1B genes remains to be established. Furthermore, it is not known whether infectious Ad40 virus is produced in KB or HeLa cells in the presence of other Ad E1B genes. During the course of these studies, we found that Ad40 can grow and produce plaques on A549 cells, a human cell line established from lung carcinoma, which do not have E1B gene function. Thus, the growth properties of Ad40 in A549 cells were analyzed and compared with those in 293, KB18 (a KB cell line which constitutively expresses Ad2 E1B genes) (3), KB8 (a KB cell line which constitutively expresses Ad2 E1A genes) (3), and KB cells. Our experiments using plaque-purified Ad40 show that the efficiency of Ad40 DNA replication as well as infectious Ad40 virion production in A549 cells is the same as or higher than those in 293 and KB18 cells that have Ad E1B gene function. The experiments reported herein describe for the first time the quantitative analysis of Ad40 growth by using plaque-purified virus.

The questions arise as to how Ad40 can propagate in A549 or KB18 cells and why Ad40 cannot propagate in KB cells. Our experiments show that Ad40 E1B large (24S to 26S) mRNAs which could code for the 55K protein are synthesized prior to the onset of DNA replication and that these as well as smaller E1B mRNA species are not synthesized at early times postinfection (p.i.) in KB18 and KB cells. It is thus suggested that the growth of Ad40 in A549 cells is helped by Ad40 E1B mRNA synthesis which is probably enhanced by a cellular factor(s) in A549 cells. In contrast, the growth of Ad40 in KB18 cells is helped by Ad2 E1B

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genes which are constitutively expressed in KB18 cells. Failure in the propagation of Ad40 in KB cells is due to a failure in the expression of E1B genes at early times p.i.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of KB, KB8, KB18, 293, and A549 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Wild-type (wt) Ad2 and H5*dl*337 (an Ad5 mutant which lacks a 147-bp sequence within the E1B 19K protein region) (22) were propagated in KB cells, and their titers were determined by plaque formation on KB cells as described previously (13). H5*dl*338 (an Ad5 mutant which lacks a 522-bp sequence within the E1B 55K protein region) (22) and H5*dl*339 (an Ad5 mutant which lacks 147- and 522-bp sequences within the 19K and 55K regions, respectively) (22) were propagated in 293 cells, and their titers were determined on 293 cells.

Preparation of Ad40 virus and plaque production. Ad40 (Sapporo strain) was isolated from stool specimens of one of the infants involved in an outbreak of gastroenteritis in a welfare nursery in Sapporo, Japan (10). A prototype strain which was used for this study was passaged one time in 293 cells. Stool suspensions (160 μl) were inoculated to 293 cells in a 25-cm^2 flask (10^6 cells). Cells were harvested at 7 days p.i., and virus in 1.2 ml of medium was harvested.

A549 cells plated in 35-mm-diameter dishes (4×10^5 cells per dish) were infected with various dilutions of virus stocks. Cells were over laid with 5 ml of 0.9% agar containing 25 mM $MgCl_2$ (12) and incubated for 10 days. At 10 days p.i., cells were stained with neutral red and incubated until plaques were produced. In most experiments, plaques were counted at the 18th day p.i. Plaque-purified Ad40 was propagated by passaging it several times in 293 cells or A549 cells.

Extraction and analysis of LMW DNA. Low-molecular-weight (LMW) DNA was isolated from monolayer cultures of virus-infected KB, KB18, 293, or A549 cells by the method of Hirt (14). The DNA present in the Hirt supernatant from 5×10^4 to 12×10^4 cells was digested with *Bam*HI and subjected to 1% agarose gel electrophoresis. DNAs were visualized by staining with ethidium bromide. In some experiments, DNAs were transferred to nylon membrane filters (Biodyne A) and were detected by Southern hybridization (24) probed with ^{32}P-labeled Ad40 DNA fragments. Ad40 *Bam*HI DNA fragments (A to F) that had been cloned into bacterial plasmids (15) were used in this experiment.

Analysis of Ad40 DNA synthesis induced by Ad5 E1B mutants. KB cells were infected with Ad40 at a multiplicity of infection (MOI) of 0.3 PFU per cell in the presence of various dilutions (0.01, 0.1, or 1 PFU per cell) of wt Ad2, H5*dl*337, H5*dl*338, or H5*dl*339. At 48 h p.i., cells were harvested and the LMW DNA was isolated. The DNA from approximately 2×10^4 cells was digested with *Bam*HI and analyzed by Southern blot hybridization. The autoradiograms were scanned with a scanning densitometer.

Preparation and analysis of RNA. KB, KB18, and A549 cells were infected with Ad40 at an MOI of approximately 0.3 PFU per cell and harvested at 0, 16, and 40 h p.i. Cytoplasmic RNA was isolated from cells as previously described (9, 19). Two and six micrograms of RNA were applied on nylon membrane filters (Schleicher & Schuell, Inc.) and were hybridized with ^{32}P-labeled Ad40 E1A and E1B DNA with ^{32}P specific activities of about 7×10^8 and 5×10^8 cpm/μg, respectively. Ad40-specific E1A and E1B mRNA species were identified by electrophoresis of denatured cytoplasmic RNA (33 μg per lane) on agarose-formaldehyde gels (11), transferring RNAs to nylon membranes, and hybridization with ^{32}P-labeled Ad40 E1A and E1B DNA probes. Hybridization, washing, and stripping of the membranes were carried out in sodium dodecyl sulfate buffer according to the instructions of the manufacturer. The DNA fragments (nucleotides 1 to 1211 and 1845 to 3933) specific for the Ad40 E1A and E1B regions were obtained by digestion with restriction enzymes of cloned *Bam*HI-F and *Bam*HI-D fragments (15), respectively, and by purification on agarose gels. The relative amount of E1A and E1B RNA was determined by scanning densitometry of spots or RNA bands on the fluorogram. The control experiment showed that Ad2 E1B mRNAs do not hybridize to the Ad40 E1B DNA probe under the conditions used here.

RESULTS

Growth properties of Ad40 isolated from stool specimens of a patient. Isolated Ad40 was passaged and propagated in 293 cells. Monolayer cultures of KB, KB8, KB18, and 293 cells were inoculated with the 293 cell lysate. After 3 days of incubation, LMW DNA was isolated and the *Bam*HI digest was analyzed by Southern blot hybridization (data not shown). DNA fragments which are consistent with those of Ad40 DNA reported by Takiff et al. (30) were obtained in the analysis of KB18 and 293 cells but not in the analysis of KB or KB8 cells. DNA fragments derived from other Ads were not detected when agarose gels were stained with ethidium bromide. It is thus suggested that the virus stock does not contain other types of Ad and that the Ad40 strain can replicate in KB18 and 293 cells but not in KB or KB8 cells.

Serial passages of Ad40 in KB18, 293, and A549 cells. It has been shown that Ad40 strains isolated from some locales were unable to propagate in 293 cells (32). To know whether Ad40 used in this study is able to grow in serial passages, Ad40 grown in 293 cells was used to infect KB, KB18, and 293 cells. In the course of this experiment, we found that Ad40 could propagate in A549 cells. Thus we also tested serial passages of Ad40 in A549 cells. At 3 days p.i., LMW DNA was isolated, digested with *Bam*HI, and analyzed by agarose gel electrophoresis (Fig. 1, lanes 1). Virus for the next passages was also prepared and was used to infect the same cell lines in the second, third, and fourth cultures. DNA was similarly isolated from the passage cultures and analyzed (lanes 2, 3, and 4). Complete Ad40 DNA fragments were observed in passage cultures of KB18, 293, and A549 cells but not in KB cells. We conclude from this result that Ad40 used in this study can propagate serially in KB18, 293, and A549 cells.

Plaque formation of Ad40 on A549 cells. Plaque formation of Ads has not been reported. Thus, experiments on Ad40 and 41 have been done by using viruses without plaque purification. We found that Ad40 is able to produce plaques on A549 cells. The plaque formation was performed by adding 25 mM $MgCl_2$ in the overlay medium as described previously (12). Figure 2 shows plaque morphologies of Ad40 which was propagated in KB (panel A), KB18 (panel B), 293 (panel C), and A549 (panel D) cells. Yields of Ad40 grown on these cell lines are summarized in Table 1. KB, KB18, 293, and A549 cells were infected with Ad40 at an MOI of 0.3 PFU per cell. At 48 and 72 h p.i., cells were harvested and virus was titrated by plaque formation on A549 cells. Under the infection conditions, wt cytopathic effect (CPE) (aggregation of large round cells) was fully produced at 72 h p.i. on KB18 and 293 cells. This result

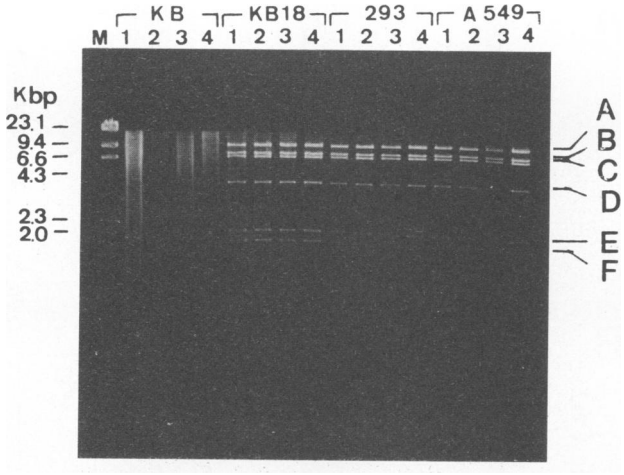

FIG. 1. Serial passages of Ad40 in KB18, 293, and A549 cells. Ad40 passed in 293 cells was used to infect KB, KB18, 293, and A549 cells (each 10^6 cells). LMW DNA was isolated from 1.25×10^5 cells after 3 days of incubation, digested with *Bam*HI, and analyzed by agarose gel electrophoresis (lanes 1). Virus was prepared from 10^5 cells of the same cell culture and used to infect the next cultures of the same cell line (each 10^6 cells). After 3 days of incubation, DNA was similarly isolated the second-, third-, and fourth-passage cultures and analyzed (lanes 2, 3, and 4). Lane M shows molecular size markers.

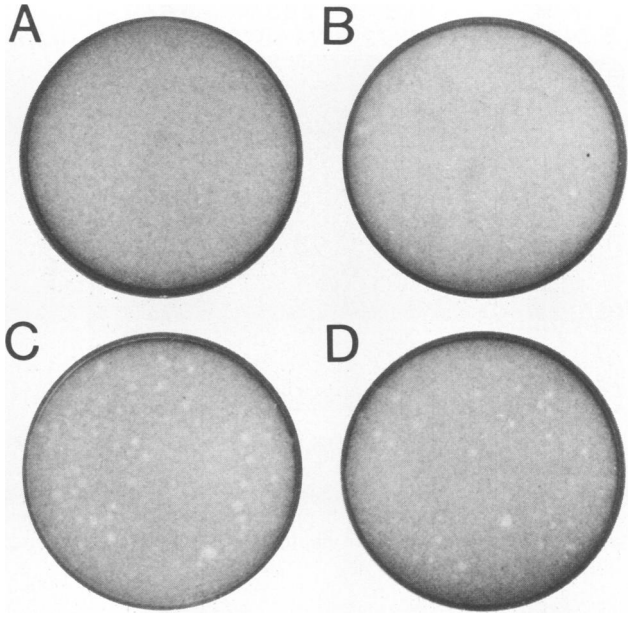

FIG. 2. Plaque formation of Ad40 on A549 cells. Ad40 propagated in A549 cells was used to infect KB (A), KB18 (B), 293 (C), and A549 (D) cells. After 3 days of incubation, 0.1 ml of 1:200 dilutions of the virus preparations was used for plaque formation on A549 cell monolayers (35-mm-diameter dish). Cells were overlaid with 5 ml of 0.9% agar containing 25 mM $MgCl_2$ and incubated for 10 days. At 10 days p.i., cells were stained with neutral red and incubated until plaques were produced. Photographs were taken at the 18th day p.i.

shows that Ad40 replicated in KB18, 293, and A549 cells but not in KB cells.

The efficiency of plaque formation of Ad40 on A549 cells appears to be lower than that of Ad2 or Ad5. This may be because, in part, the Ad40 production at 72 h p.i. may not yet have plateaued. However, the experiments described below suggest that the lower yield of Ad40 is due to the lower efficiency of plaque formation. (i) Ad2 did not produce a CPE in KB18 cells at 2 or 3 days p.i. when infected at an MOI of 0.3 PFU per cell, although Ad40 produced a complete CPE at an MOI of 0.3 PFU per cell (see below). (ii) The yield of Ad40 DNA in the analysis of LMW DNA on agarose gels from permissive cells infected with Ad40 at an MOI of 0.3 PFU per cell at 48 or 72 h p.i. was about the same as that from these cells infected with Ad2 at an MOI of 20 PFU per cell. Much less Ad2 DNA was obtained from the cells infected with Ad2 at an MOI of 0.3 PFU per cell at 48 or 72 h p.i. (data not shown). Furthermore, it has been shown that the efficiency of plaque formation of Ad12 on A549 cells is highly dependent on the concentration of $MgCl_2$ and synthesis of E1B 55K (12). However, the relationship between the number of viral particles produced and the number of plaques formed on A549 cells remains to be established. We propagated plaque-purified Ad40 in 293 or A549 cells and used it for following experiments.

CPE produced by Ad40. It is known that wt Ads produce an Ad CPE (wt CPE) in human cell lines. In contrast, *cyt* or *lp* mutants which have a mutation(s) in the E1B 19K coding region produce an unusual CPE (*cyt* CPE, a scattering of small disrupted cells).

Plaque-purified Ad40 was used to infect KB, KB18, 293, and A549 cells. The amount of virus infected was adjusted to produce a CPE in 293 cells in 2 or 3 days p.i. (equivalent to 0.3 PFU per cell). Ad40 produced wt CPE in KB18 and 293 cells. Appearance of the CPE in A549 cells was slightly delayed and seemed to be incompletely wt in terms of the aggregation of cells. In contrast, when KB cells were infected, small disrupted cells that did not aggregate were produced; the CPE was similar to that of *cyt* mutant-infected KB or HeLa cells. The same result was obtained when Ad40 without plaque purification was used for infection.

Growth properties of plaque-purified Ad40. To know whether plaque-purified Ad40 has the same properties as the original virus does, replication of viral DNA in Ad40-infected KB, KB18, 293, and A549 cells was studied. At 3, 24, 48, and 72 h p.i., LMW DNA was isolated and analyzed by agarose gel electrophoresis (Fig. 3). The level of DNA replication in A549 cells at 72 h p.i. was almost the same as those in KB18 and 293 cells. However, the initiation of DNA replication in A549 cells appeared to be delayed compared with that in KB18 or 293 cells. The level of DNA replication in KB cells was much lower than that in other cell lines. In addition, DNA degradation was produced by infection of KB cells with plaque-purified Ad40, as was observed with Ad40

TABLE 1. Infectivity of Ad40 grown in various cell lines on A549 cells

Cell line	Infectivity (PFU/ml)[a]	
	48 h	72 h
KB	1.0×10^1	4.4×10^2
KB18	1.7×10^3	1.1×10^4
293	1.1×10^4	6.3×10^4
A549	7.7×10^3	8.3×10^4

[a] Final plaque counts were made at 18 days p.i. Samples (5×10^5 cells) harvested in 1 ml of medium at 48 and 72 h p.i. were frozen and thawed five times to release the virus and assayed for infectivity on A549 cells.

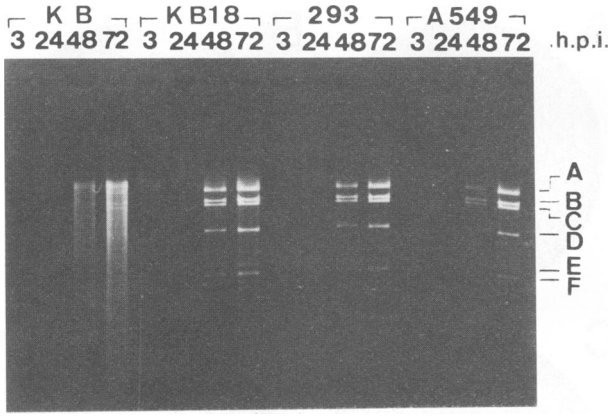

FIG. 3. The levels of Ad40 DNA replication in KB, KB18, 293, and A549 cells. The indicated cells were infected with plaque-purified Ad40 at an MOI of 0.3 PFU per cell. LMW DNA was isolated at 3, 24, 48, and 72 h p.i., digested with *Bam*HI, and analyzed by agarose gel electrophoresis as shown in Fig. 1. Fragments are indicated at the right.

without plaque purification (Fig. 1). Thus, plaque purification of Ad40 does not affect viral DNA replication during productive infection.

Enhancement of Ad40 DNA replication by the Ad5 E1B 55K protein. Ad40 DNA replicated in KB18 but not in KB or KB8 cells, suggesting that Ad2 E1B genes complement the defect in Ad40 DNA replication. To locate this ability more precisely, Ad40 DNA synthesis in KB cells coinfected with Ad40 and H5*dl*337, H5*dl*338, or H5*dl*339 was analyzed (Fig. 4). H5*dl*337 produces Ad5 E1B 55K but not 19K protein in infected KB cells. In contrast, H5*dl*338 produces the E1B 19K protein but not the 55K protein. H5*dl*339 produces neither the 55K nor the 19K protein. Lane 1 in Fig. 4A shows the level of Ad40 DNA in Ad40-infected KB cells. The level of Ad40 DNA synthesis in lane 1 was 4 to 5% of that in lane 4 (100%). In the presence of wt Ad2 (lanes 2, 3, and 4) or H5*dl*337 (lanes 5, 6, and 7), replication of Ad40 DNA increased. When Ad40 coinfected cells with these viruses at an MOI of 1 PFU per cell, Ad40 DNA replication was at least 20-fold higher than that of Ad40 alone (compare lanes 4 and 7 with lane 1). However, infection with larger amounts of wt Ad2 or H5*dl*337 inhibited Ad40 DNA replication (data not shown), probably because of higher levels of replication of wt Ad2 or H5*dl*337 DNA. Although H5*dl*338 or H5*dl*339 slightly increased Ad40 DNA replication, the effect was not marked. It is of interest to note that H5*dl*337 DNA was degraded in KB cells by coinfection with Ad40 (data not shown), although Ad40 DNA did not degrade (lanes 6 and 7). Furthermore, we note that DNA fragments derived from wt Ad2 or H5*dl*337 were more abundant than those from Ad40 in lanes 4 and 7 when detected by staining with ethidium bromide. However, Ad40 DNA probe did not hybridize to the Ad2 or Ad5 DNA fragments under the hybridization conditions. A few faint fragments were detected between fragments D and E and below fragment F. These should be, however, derived from Ad40 DNA, because these DNA fragments were detected in some experiments with single infection with Ad40. At the present time, we do not know where these DNA fragments are located in Ad40 DNA.

Analysis of E1A- and E1B-specific mRNAs in Ad40-infected cells. Analysis of Ad40 E1B mRNA in Ad40-infected cells by means of dot or Northern (RNA) blot hybridization has not been reported. We tried to detect E1b-specific RNA in Ad40-infected A549, KB, and KB18 cells by dot blot hybridization. Cytoplasmic RNAs were isolated from Ad40-infected cells at 0, 16, and 40 h p.i. Ad40 E1A- and E1B-specific RNAs were detected in A549, KB, and KB18 cells at 16 or 40 h p.i. (Fig. 5A and B). The levels of E1A and E1B RNA in A549 cells at 40 h p.i. were about 10-fold higher than those in KB or KB18 cells, although they were the same as those in KB or KB18 cells at 16 h p.i. This result indicates that the levels of E1A and E1B mRNAs increased between 16 and 40 h p.i. prior to the onset of viral DNA replication in A549 cells (Fig. 3). In contrast, they did not increase in KB or KB18 cells between 16 and 40 h p.i. This result suggests that the low level of Ad40 55K in KB cells is insufficient for DNA replication.

Two major E1B mRNAs, 22S and 13S RNAs encoding the 55K and 19K proteins and the 19K protein, respectively, are synthesized at early times p.i. in Ad2-infected KB cells. To know whether the large E1B mRNA is synthesized in Ad40-infected A549 cells at early times p.i., we analyzed cytoplasmic RNA from Ad40-infected cells by Northern blot

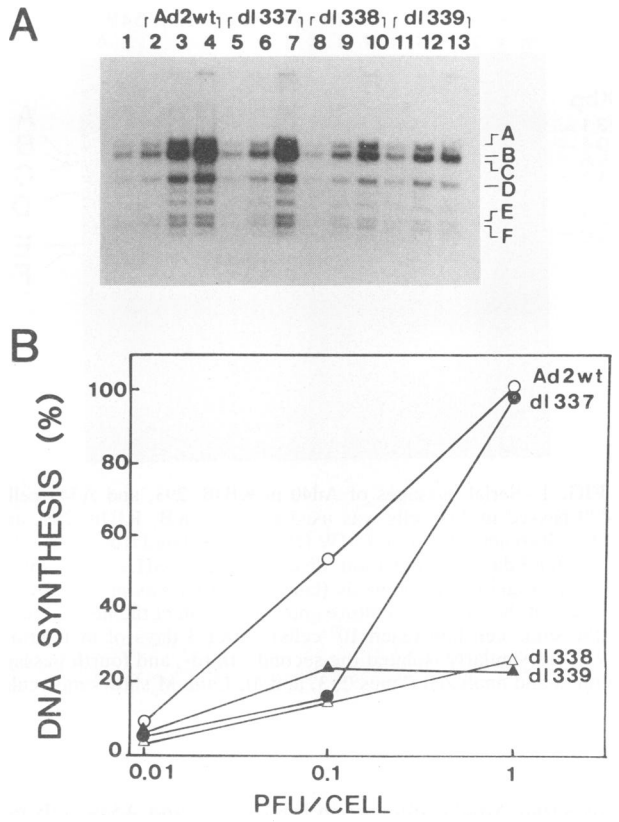

FIG. 4. Enhancement of Ad40 DNA replication by coinfection with wt Ad2, H5*dl*337, H5*dl*338, or H5*dl*339. (A) KB cells were infected with plaque-purified Ad40 at an MOI of 0.3 PFU per cell in the presence of 0.01 (lanes 2, 5, 8, and 11), 0.1 (lanes 3, 6, 9, and 12), or 1 (lanes 4, 7, 10, and 13) PFU of wt Ad2 (designated Ad2wt at the top), H5*dl*337 (dl337), H5*dl*338 (dl338), or H5*dl*339 (dl339) per cell. At 48 h p.i., cells were harvested and LMW DNA was analyzed by Southern blot hybridization. Fragments are indicated at the right. (B) The level of Ad40 DNA replication was estimated by densitometry scanning of autoradiograms of DNA fragments. The peaks were cut out, and the masses of each lane were normalized to a value of 100% for lane 4.

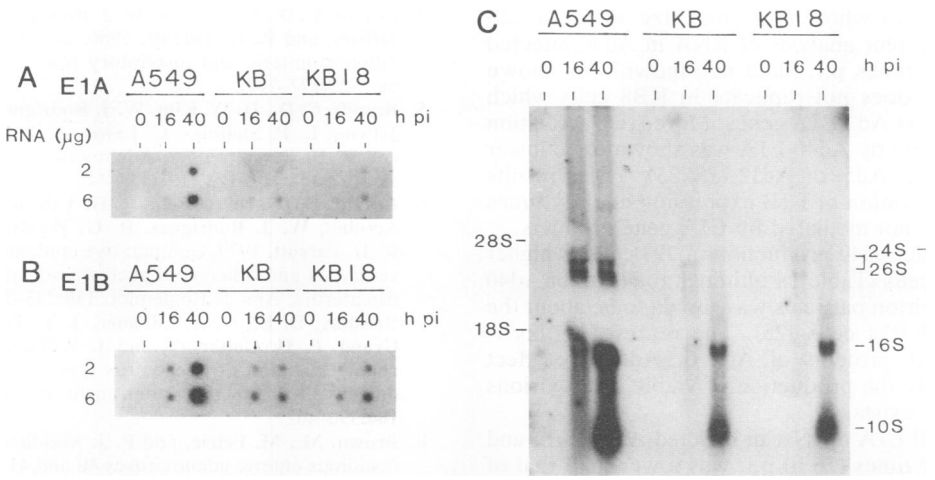

FIG. 5. Quantification of Ad40 E1A and E1B RNA in infected cells. A549, KB, and KB18 cells were infected with plaque-purified Ad40 at an MOI of 0.3 PFU per cell. Cytoplasmic RNA was isolated at 0, 16, and 40 h p.i. (A and B) Two and six micrograms of cytoplasmic RNA were spotted on nylon filter membranes and hybridized to ^{32}P-labeled Ad40 E1A and E1B DNA probes. The specific activity of ^{32}P was almost the same in E1A and E1B DNA probes. The level of E1A and E1B RNA synthesis was estimated by densitometry scanning of the autoradiograms. (C) Cytoplasmic RNA was isolated as described for panels A and B. RNA (33 μg per lane) was separated by electrophoresis on an agarose-formaldehyde gel, transferred to nylon filter membranes, and hybridized with ^{32}P-labeled Ad40 E1B DNA probe.

analysis (Fig. 5C). Two large RNAs (approximately 24S to 26S) and a small RNA (approximately 16S) were synthesized in Ad40-infected A549 cells at early times (16 h) p.i. No distinct RNAs were detected between the large and small RNAs in two different RNA preparations. In contrast, E1B mRNA species were scarce or not detectable in Ad40-infected KB and KB18 cells. However, small RNA species (16S and 10S) were synthesized in KB and KB18 cells at late times (40 h) p.i. This result suggests that Ad40 DNA replication in A549 cells is mediated by the synthesis of E1B-specific mRNAs at early times p.i. and that Ad40 E1B mRNA synthesis in KB18 is not affecting viral DNA replication but rather that there is insufficient E1B mRNA and protein to effectively facilitate viral DNA replication. Thus, it is clear that Ad40 cannot propagate in KB cells because of the failure in expression of the E1B genes at early times p.i. We previously showed that Ad2 E1B mRNAs (22S and 13S) were detected in the analysis of RNA of KB18 cells when hybridized with Ad2 E1B DNA probe under hybridization conditions similar to those used here (19). We did not, however, detect any dots or bands in KB18 RNA at 0 h p.i. with Ad40 DNA probe (Fig. 5B and C), showing that Ad40 E1B DNA did not hybridize to Ad2 E1B mRNAs.

DISCUSSION

Ad40 is a ubiquitous Ad and grows well in intestinal cells of young children. Despite the presence of up to 10^{11} virus particles per g of stool from young children with diarrhea (31), it does not grow in conventional established human cell lines, such as KB or HeLa cells. Its growth in 293 cells has suggested that Ad40 has properties similar to Ad host range mutants in E1 function(s). However, the defects in propagation have not been localized. We show here that infectious Ad40 virions are produced in KB18 (possess Ad2 E1B genes) but not in KB8 (possess Ad2 E1A genes) cells. This result indicates that the E1B function complements the growth of Ad40.

In lytic infection of KB or HeLa cells with Ad2 or Ad5, the Ad2 or Ad5 E1B 55K protein has been shown to be required for preferential transport of viral mRNAs during the late phase (2, 21, 26, 27, 38), for efficient synthesis of viral late proteins (20), and for efficient shutoff of host protein synthesis (1) but not for viral DNA replication (1, 38). In contrast, Ad12 E1B 55K protein is required for Ad12 DNA synthesis in KB, HeLa, and A549 cells (7, 12, 23, 28). As shown here and by Mautner et al. (25), Ad40 requires the Ad2, Ad5, or Ad12 E1B 55K function for its DNA replication in KB or HeLa cells, showing the property in DNA replication similar to that of Ad12. The question arises as to whether Ad40 E1B mRNAs are synthesized in KB or HeLa cells.

We report here that Ad40 can propagate in A549 cells which do not have E1B genes. Our experiments reported herein indicate that the levels of Ad40 DNA replication and viable Ad40 virion production in A549 cells are the same as or higher than those in 293 or KB18 cells. The result suggests that a cellular factor(s) in A549 cells can enhance Ad40 E1B gene expression and/or can help Ad40 DNA replication in the absence of the Ad E1B 55K protein. Our experiments on E1B mRNA indicate that Ad40-specific E1B mRNA species are synthesized at early times p.i. in A549 cells but not in KB or KB18 cells. We thus conclude that there is a cellular factor(s) which can activate Ad40 E1B genes and/or help maturation and processing of the E1B mRNA species in A549 cells.

The possibility that A549 cells contain a cellular factor(s) which could help Ad40 DNA replication is not completely excluded. However, Ad12 mutants defective in the E1B 55K function have been shown to be defective in Ad12 DNA replication in A549 cells, although wt Ad12 or Ad12 E1B 19K mutants can grow well in A549 cells (7, 12). The notion suggests that a cellular factor(s) which helps E1B mRNA synthesis plays a more important role to help Ad40 growth in A549 cells than the cellular factor(s) which helps Ad DNA replication.

It is well known that Ad E1A gene products *trans* activate Ad E1B gene transcription. However, expression of Ad40 E1A mRNA synthesis in A549 cells at early times p.i. is very weak (Fig. 5A). Furthermore, it is difficult to detect E1A-

specific mRNA species whose molecular size would be 12S or 13S by Northern blot analysis of RNA in Ad40-infected A549 cells at early times p.i. (data not shown). As shown above, Ad40 DNA does not replicate in KB8 cells which constitutively express Ad2 E1A genes. Moreover, activation of Ad early promoters by Ad40 E1A was shown to be lower than by E1A of Ad2, Ad5, or Ad12 (15, 33). These results suggest that the activation of E1B expression at early times p.i. in A549 cells is not mediated by E1A gene products.

The level of viable Ad40 production in 293 cells is higher than that in KB18 cells (Table 1), although the level of Ad40 DNA packed into virion particles was shown to be about the same in 293 and KB18 cells (25). The reason for this is unknown. The E1A proteins of Ad2 or Ad5 may affect directly or indirectly the production of viable Ad40 virions when the E1B 55K exists.

The level of Ad40 E1A mRNA in infected A549, KB, and KB18 cells at early times (16 h) p.i. was lower than that of E1B mRNA (Fig. 5). Northern blot analysis of RNA in A549 cells shows that two large RNA species (24S to 26S) and one small RNA species (16S) were hybridized to the E1A DNA probe (data not shown). When membrane filters which had been used for hybridization with E1B DNA probe were used for hybridization with E1A DNA probe after stripping off the E1B DNA, the two RNA bands corresponding to 24S to 26S were shown to match completely the RNAs hybridized with E1B DNA probe. The RNA species appear to be too large to be a class of E1A transcripts. Furthermore, we were unable to detect distinct RNA bands which hybridized with E1A probe in KB or KB18 cells at early times p.i. This result suggests that the E1B RNAs corresponding to 24S to 26S in A549 cells may contain at least in part a novel class of E1A-E1B cotranscripts originating from the E1A promoter. The 24S to 26S mRNAs are present in KB18 cells, though in reduced levels compared with those in A549 cells. Thus, the possibility that a novel class of E1A-E1B cotranscripts has an important role in Ad40 growth in A549 cells is not excluded.

In the course of this experiment, we observed that another EAd, Ad41, can also propagate and produce plaques on A549 cells (data not shown). Our findings that Ad40 and Ad41 can grow in A549 cells and that Ad40-specific E1A and E1B mRNAs are synthesized in A549 cells prior to the onset of DNA replication but not in KB or KB18 cells may provide further insight into the growth mechanism of fastidious EAds as well as the regulation mechanism of enhancers and promoters in E1 region of Ads.

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