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Adenovirus Types 2 and 5 Functions Elicit Replication and Late Expression of Adenovirus Type 12 DNA in Hamster Cells

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Human adenovirus type 12 (Ad12) cannot replicate in hamster cells. There is a complete block of viral DNA replication and of the expression of late viral genes. Early viral functions are expressed. In contrast, hamster cells are permissive for human adenovirus type 2 (Ad2). Some of the Ad12-specific functions are insufficient to support viral replication in hamster cells, or else cellular functions are missing or inhibitory for Ad12 replication. It was shown that the block in the replication and late expression of the Ad12 genome in hamster cells could, at least in part, be complemented by Ad2 and adenovirus type 5 (Ad5) functions. When hamster cells were coinfected with Ad2 (or Ad5) and Ad12, both Ad2 (Ad5) and Ad12 DNA replicated. Ad2 (Ad5) virions were produced in double-infected hamster cells. The assembly of intact Ad12 virions was not detectable by the techniques used here. The analysis was further refined by Ad12 superinfecting Ad2- or Ad5-transformed cells which carried in an integrated form defined fragments of the Ad2 or Ad5 genome. Persistence and continued expression of the left terminus of the Ad2 or Ad5 DNA in these cells has been documented and helped to support replication and late expression of Ad12 DNA. It remains to be determined which of the E1 functions of Ad2 or Ad5 were responsible for the helper effect. Investigations on the biochemical mechanism of this complementation will entail studies on very complex viral and possibly cellular functions involved in the control of viral gene expression.

Human adenovirus type 12 (Ad12) is highly oncogenic in hamsters (44) and transforms hamster cells in culture (29). Ad12 fails to replicate in BHK-21 or primary hamster cells. The Ad12-hamster cell system provides an excellent example of an absolutely abortive virus-host interaction. It has been established that Ad12 DNA cannot replicate in hamster cells (9–12, 16, 40, 46). This conclusion has been based on the results of DNA-DNA hybridization experiments using more and more sensitive techniques. Ad12 virions do infect hamster cells; the viral DNA reaches the nucleus, and a portion of the parental viral DNA molecules is integrated into the host genome (9, 11). The early functions of Ad12 DNA are expressed (27, 28, 30), whereas RNA sequences homologous to the late Ad12 genes cannot be detected in hamster cells (14, 28). It could be demonstrated by in vitro translation of hybrid-selected RNAs that, in Ad12-infected BHK-21 cells, all of the early viral mRNAs are synthesized as in productively infected cells, except for a 34,000-molecular-weight (34K) protein from the E1A region of Ad12 DNA (14). It is not known whether this 34K protein is involved in eliciting late expression and replication of Ad12 DNA in hamster cells.

In contrast to Ad12, human adenovirus type 2 (Ad2) can replicate and grow to substantial titers in hamster cells (10, 32, 42). We therefore asked whether Ad2 or Ad12 DNA can replicate in Ad2 and Ad12 double-infected BHK-21 cells. It was also interesting to investigate to what extent the expression of Ad12 late functions could be promoted in hamster cells by simultaneously expressed Ad2 functions.

In a series of experiments, Ad2- or adenovirus type 5 (Ad5)-transformed hamster cells carrying various parts of the Ad2 or Ad5 genome in an integrated form were superinfected with Ad12. The persistence and integration patterns of Ad2 DNA in the Ad2-transformed hamster cell lines HE1, HE3, and HE5 (45) and of Ad5 DNA in the Ad5-transformed hamster cell line BHK297-131 (47) have been described earlier. In the complementation experiments described here we also used the Ad2-transformed hamster cell line HE7 (7). The integration patterns of Ad2 DNA in cell line HE7 have not previously been determined and are presented here. The results of the current investigations demonstrated that hamster cells carrying the left 18.7% of Ad5 DNA have the capacity to elicit replication and late gene expression of Ad12 DNA in hamster cells. The production of newly synthesized Ad12 virions could not be detected in double-infected or in Ad2- or Ad5-transformed hamster cells.

MATERIALS AND METHODS

Cells and virus. The following cell lines were propagated in Dulbecco modified Eagle medium (3) supplemented with 7 to 10% fetal bovine serum: BHK-21 cells, subline B3 (41), and the Ad2-transformed hamster cell lines HE1, HE3, HE5, and HE7 (7), as well as human HeLa cells (ATCC, CCL 2). Human KB cells (ATCC, CCL 17) were grown in Eagle medium containing 10% newborn calf serum. The Ad5-transformed cell line BHK297-131 (47), a gift of J. Sussenbach, Utrecht, The Netherlands, was grown in the Dulbecco modified Eagle medium containing 7% fetal calf serum. Cell lines HE1, HE3, HE5, and HE7 were gifts of A. M. Lewis, Jr., National Institutes of Health, Bethesda, Md., and J. L. Cook, Denver, Colo.

Ad2 and Ad12 were replicated and grown to high titers on suspension or monolayer cultures of human KB or HeLa cells (10). For the propagation of Ad12, extracellular virus inocula (cell culture media) from Ad12-infected human embryonic kidney cells were used (5). Primary human embryonic kidney cells were the gift of P. Gallimore, Birmingham, United Kingdom.

Identification of cell lines. Since several adenovirus-

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transformed hamster cell lines were used, the identities of cell lines were ascertained by determining the characteristic integration patterns of Ad2 DNA (45) and karyotypes or by analyzing the total DNA by cleavage with *Eco*RI. Hamster-specific patterns of repetitive DNA bands proved highly characteristic. Southern blots (38) of DNA preparations from individual cell cultures were hybridized to the ^{32}P-labeled hamster cell clone pF19 from HE5 cells (17) which yielded hamster-specific hybridization signals.

Double infections of BHK-21 (B3) hamster cells with Ad2 and Ad12. B3 cells growing in monolayer cultures were infected with Ad2 and Ad12 at multiplicities of 50 and 150 PFU per cell, respectively. At time periods after infection as indicated for individual experiments, the total nuclear DNA was extracted from Nonidet P-40 nuclei by using standard methods including RNase treatment and omitting the CsCl step (43).

Infection of Ad2-transformed hamster cell lines with Ad12. The Ad2-transformed hamster cell line HE1, HE3, HE5, or HE7 or the Ad5-transformed hamster cell line BHK297-131 was superinfected with CsCl-purified Ad12 at multiplicities of 100 to 150 PFU per cell. The total nuclear DNA was extracted by modified standard procedures (43). In some of the experiments, the total RNA (2) or the cytoplasmic RNA (35) was extracted and analyzed for Ad12-specific RNA sequences (24, 25).

Test for replication of Ad12 virions in Ad2 and Ad12 double-infected BHK-21 or HeLa cells. Double-infected BHK-21 (B3) hamster cells were labeled with [^{32}P]orthophosphate (30 μCi/ml) starting 2 h after infection. At 72 h, the total intracellular virus was harvested and purified. Similarly, human HeLa cells were double infected. It had been shown previously that Ad2 virions (ρ = 1.334 g/cm^3) and Ad12 virions (ρ = 1.328 g/cm^3) differed in buoyant densities so that they were separable by equilibrium sedimentation in CsCl density gradients (10). At the end of equilibrium centrifugation (SW60 rotor in a Beckman ultracentrifuge; 20 h, 40,000 rpm, 4°C), the tubes were punctured and 2 or 3 drop fractions were collected. Of each fraction, 20 μl was mixed with 200 μl of TE buffer (0.01 M Tris hydrochloride [pH 7.5], 1 mM EDTA), and the DNA from virions was extracted or mock extracted from fractions devoid of virus (6, 11). Subsequently, the identities of the ^{32}P-labeled Ad2 or Ad12 DNA were ascertained by cleavage with *Sma*I, separation of fragments by electrophoresis on 0.7% agarose gels, and autoradiography of the dried gels. In some experiments, unlabeled Ad2 or Ad12 DNA was visualized by ethidium bromide staining of the gels and by UV photography.

Cloning of the terminal *Hin*dIII fragments of Ad2 DNA. The terminal *Hin*dIII fragments G (left terminal) and K (right terminal) were cloned into the *Hin*dIII site of *Hin*dIII-cut and phosphatase-pretreated pBR322 plasmid by published methods (19, 20, 48).

Cloning of the *Hin*dIII fragments of Ad5 DNA. Ad5 DNA was cut with *Hin*dIII, and the fragments generated were cloned into *Hin*dIII-cut pBR322 vector DNA that was pretreated with calf intestine phosphatase. The terminal Ad5 DNA fragments were cloned as described above.

Most of the standard methods used here have been described earlier: preparation of adenovirus DNA and cloned viral DNA fragments (13, 48), extraction of DNA (43) or RNA (24, 25), restriction analyses of DNA (39, 43), Southern blotting (38), DNA-DNA or DNA-RNA hybridization (49), analyses of RNA by electrophoresis and blotting (1, 34), and labeling of DNA or DNA fragments with ^{32}P by nick translation (31).

RESULTS

Experimental approaches designed to test replication and late expression of the Ad12 genome in hamster cells infected with or transformed by Ad2 or Ad5. At various times after double infection of hamster cells with Ad2 and Ad12, the total intranuclear DNA was cleaved with *Sma*I. The distinctly different *Sma*I patterns of Ad2 and Ad12 DNAs (cf. Fig. 2b and 3a) permitted us to distinguish unequivocally between the two types of viral DNA. Increases of Ad2 or Ad12 DNA due to their replication in hamster cells were visualized by Southern blotting and hybridization to either Ad2 or Ad12 DNA probes. Since Ad2 and Ad12 DNAs exhibited considerable cross-homology, distinctions between the two DNA molecules could not simply be based on the use of the appropriate probes but had to be determined by exploiting differences in the *Sma*I restriction patterns. Ad12 DNA replication could also be ascertained by using the right terminal *Bam*HI-E fragment of Ad12 DNA as a hybridization probe which lacked homology to Ad2 DNA (data not shown). Due to the cross-homology between Ad2 and Ad12 DNA, RNA sequences in double-infected cells could not be unequivocally differentiated.

Ad2- or Ad5-transformed hamster cell lines, which each carried different parts of the viral genome and jointly carried its left terminal segment (Fig. 1), were superinfected with Ad12. In Ad2- or Ad5-transformed cell lines selected for superinfection experiments, the late regions of the viral genome were deleted. Hence, late viral RNA in the Ad12-superinfected cells could not have been contributed by the Ad2 or Ad5 genome. At various times after superinfection, the total intranuclear DNA was extracted and analyzed after *Sma*I cleavage as described above. Furthermore, the cytoplasmic RNA or the total cellular RNA was extracted and analyzed by electrophoresis on agarose gels followed by Northern blotting and was then hybridized with the *Bam*HI-D fragment of Ad12 DNA (see below).

Patterns of viral DNA integration in the Ad2-transformed cell lines HE1, HE3, HE5, and HE7 and in the Ad5-transformed cell line BHK297-131. The patterns of viral DNA integration have been published previously for the Ad2-transformed cell lines (45), with the exception of cell line HE7, as well as for the Ad5-transformed hamster cell line BHK297-131 (47). In cell line HE5, the sites of linkage between viral and cellular DNA, the junction site at the internal viral deletion, and the unoccupied allelic site of insertion in hamster DNA have been cloned and sequenced (17, 18). The schemes presented in Fig. 1 show patterns of viral DNA persistence for all cell lines used in this study. The patterns of Ad2 DNA integration in cell line HE7 and of Ad5 DNA in cell line BHK297-131 were determined by using conventional Southern blotting and hybridization experiments (39). The cloned *Hin*dIII fragments of Ad2 DNA, in particular the terminal fragments *Hin*dIII-G and *Hin*dIII-K and the cloned *Hin*dIII fragments of Ad5 DNA (cf. above), were used as ^{32}P-labeled probes in these hybridization experiments. In some experiments, full-length Ad2 DNA was used as probe also. The data (T. Klimkait, thesis, University of Cologne, Federal Republic of Germany, 1984; J. L. Cook, T. Klimkait, A. M. Lewis, Jr., and W. Doerfler, manuscript in preparation; other data not shown) are schematically summarized in Fig. 1.

Cell line BHK297-131 proved to be the most useful in the analyses of late expression of Ad12 DNA in superinfection experiments because this cell line lacked the late regions of the Ad5 genome. There was some cross-homology between

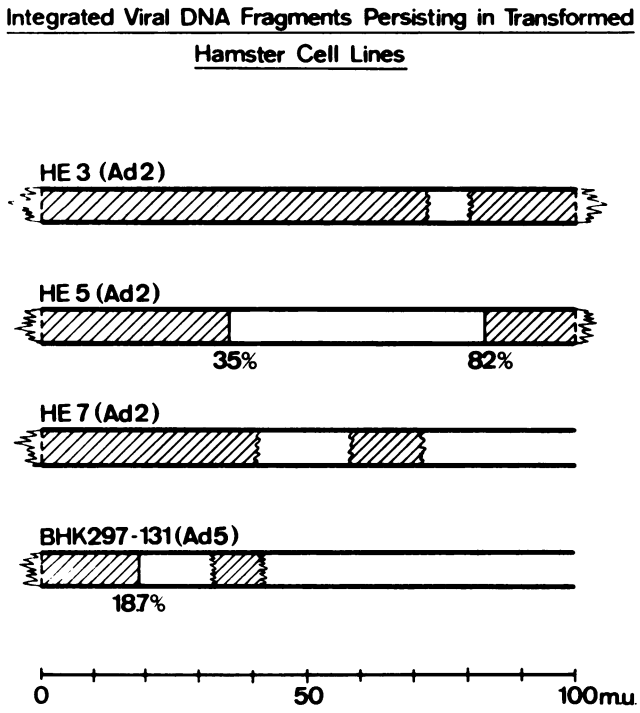

FIG. 1. Patterns of integration of Ad2 DNA in the Ad2-transformed cell lines HE3, HE5, and HE7 and of Ad5 DNA in the Ad5-transformed cell line BHK297-131. The data for cell lines HE3 and HE5 were taken from reference 45; the data for cell line BHK297-131 were taken, with modifications, from reference 47. The patterns of Ad2 DNA integration in cell line HE7 have been determined (Cook et al., in preparation) by conventional Southern blotting using clonal Ad2 DNA fragments as hybridization probes as described previously (39). Similarly, the cloned *Hin*dIII fragments of Ad5 DNA were used to determine the pattern of integration of Ad5 DNA in cell line BHK297-131. The hatched areas of the Ad2 or Ad5 genome have been shown to persist in cell lines as indicated. The jagged lines indicate continuing cellular DNA. The schemes do not represent the actual integration patterns of viral DNA, but designate viral DNA segments persisting in the cell lines as indicated. Except for cell line HE5, for which the bounds of the internal deletion have been located by sequence determination (17), all persistence patterns are approximate since they are based on Southern blot hybridization experiments.

Ad12 and Ad2 or Ad5 DNAs in the late regions of these viral genomes. In particular, when late Ad12 DNA expression was probed with the *Bam*HI-D fragment of Ad12 DNA (cf. Fig. 5), which comprised map units 34 to 47, it was important that the corresponding Ad2 or Ad5 DNA segments were absent from the Ad12-superinfected cell lines to rule out direct cross-hybridizations between the Ad2 or Ad5 DNAs and the Ad12 DNAs. The *Bam*HI-D and -I fragments of Ad12 DNA did not overlap with the early regions of the Ad12 genome (cf. map in Fig. 5). The cloned ^{32}P-labeled *Bam*HI-D or the *Bam*HI-E fragment of Ad12 DNA was hybridized to Southern blots of Ad2 DNA which had previously been cleaved with *Sma*I, *Sac*I, *Hin*dIII, or *Bgl*II. The data (not shown) demonstrated that the *Bam*HI-D fragment of Ad12 DNA had sequence homologies to the Ad2 genome in its corresponding segments. In contrast, the right terminal *Bam*HI-E fragment of Ad12 DNA exhibited no sequence homology to Ad2 DNA detectable by these methods. The *Bam*HI-E fragment of Ad12 DNA thus proved a very useful probe in our study. The *Bam*HI-D fragment, therefore, could be used as a probe for RNA from only those cell lines in which the internal parts of the Ad2 or Ad5 genomes had been deleted.

Expression of the left terminal sequences of Ad2 DNA or Ad5 DNA in cell lines HE1, HE3, HE5, HE7, and BHK297-131. To ascertain that all of the cell lines used here expressed all or part of the E1 region of the integrated Ad2 or Ad5 genome, cytoplasmic RNA was isolated from these cell lines, fractionated by electrophoresis on agarose-formamide gels, blotted onto nitrocellulose filters, and hybridized to the ^{32}P-labeled, cloned *Hin*dIII-G fragment (left 7.7%) of Ad2 DNA. The results (data not shown) clearly demonstrated that all of these cell lines expressed RNA of identical sizes with homology to the *Hin*dIII-G fragment of Ad2 DNA. It was also shown that the Ad2 fragment probe did not hybridize to RNA sequences isolated early (4.5 h) or late (48 h) after infection of human KB cells with Ad12.

The Ad12 genome can replicate in Ad2-infected BHK-21 cells. BHK-21 cells were infected with CsCl-purified Ad12 at a multiplicity of approximately 50 PFU per cell. At various times after infection, as indicated in Fig. 2a, the total intranuclear DNA was extracted and cut with *Sma*I, and the fragments were separated by electrophoresis on a 0.7% agarose gel. After blotting, the Ad12-specific DNA fragments were detected by hybridization to ^{32}P-labeled Ad12 DNA followed by autoradiography. As previously reported (10, 16, 46), there was no evidence for the replication of Ad12 DNA in BHK-21 cells. The amount of Ad12 DNA constantly decreased between 2 and 12 h after infection and appeared to be gradually lost from the nuclear DNA (Fig. 2a). Even when the autoradiogram was overexposed, evidence for Ad12 DNA replication could not be found (data not shown). Homologies of Ad12 DNA to BHK-21 DNA were not detected in this experiment. It should be noted that persistence and integration of Ad12 DNA in BHK-21 cells has previously been reported at much later times after infection than was the case in the present study (9, 11, 16). However, in these earlier experiments considerably higher multiplicities of infection were used. It has also been shown that Ad12 DNA cannot replicate in primary hamster embryo cells (U. Brüggemann and W. Doerfler, unpublished data).

BHK-21 cells were then simultaneously double infected with Ad2 and Ad12 as described below. At time intervals after infection, as indicated in Fig. 2b, the total intranuclear DNA was extracted, cut with *Sma*I, and analyzed as described above. In control experiments, BHK-21 cells were also infected with either Ad2 or Ad12 alone. Ad12 DNA by itself did not replicate in BHK-21 cells; Ad2 DNA did (Fig. 2b). By about 24 to 30 h after infection, Ad12 DNA replication was detectable in double-infected cells, as evidenced by the continuous increase in the intensities of the Ad12-specific signals (Fig. 2b). Ad2 DNA replication was also discernible by 12 h after infection. The data presented in Fig. 2b also shows a steady initial decrease of Ad12 DNA in double-infected cells between 2 and 12 h after infection. In the experiment shown in Fig. 2b, the entire Ad12 DNA molecule was used as hybridization probe. Therefore, Ad2-specific bands also lit up on the autoradiograms due to cross-hybridizations. There was no detectable homology between the right terminal *Bam*HI-E fragment of Ad12 DNA and Ad2 DNA. Hence, when the *Bam*HI-E fragment of Ad12 DNA was used as a hybridization probe, the increase in Ad12-specific signals was readily apparent at 23 and 47 h after infection (data not shown). We also demonstrated that the Ad12-specific band pattern was not due to incomplete cleavage on account of limiting enzyme concentrations,

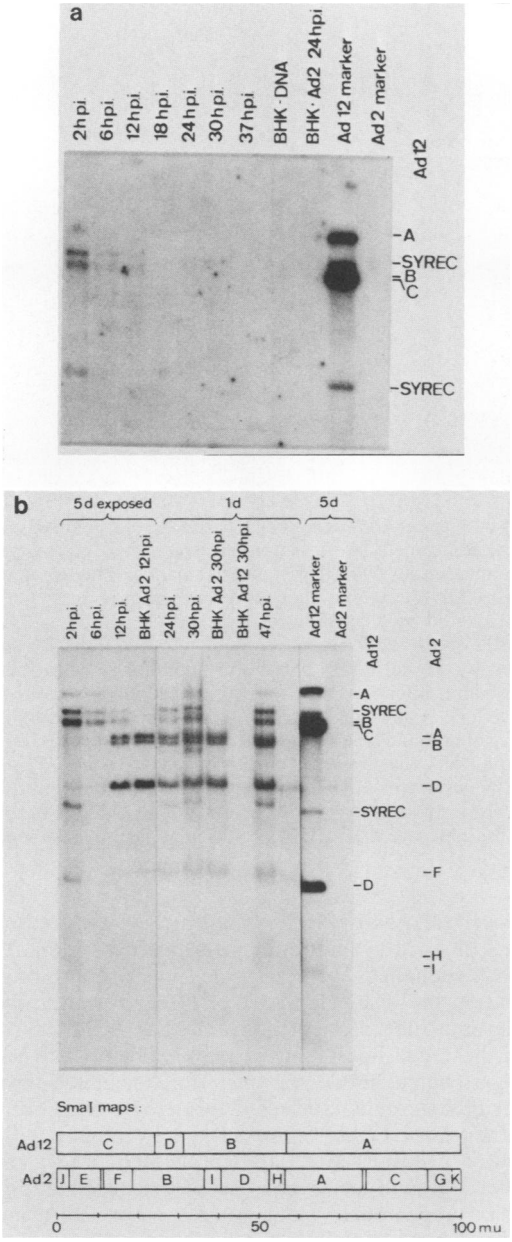

FIG. 2. Absence of Ad12 DNA replication in BHK-21 cells (a) and Ad12 replication in BHK-21 cells coinfected with Ad2 and Ad12 (b). (Panel a) BHK-21 cells were infected with Ad12 (50 PFU per cell), and the total intracellular DNA was extracted at the indicated time intervals and analyzed as described in the text. BHK-21 DNA, Ad12 DNA, Ad2 DNA, and DNA from BHK-21 cells 24 h after infection with Ad2 were also cleaved with *Sma*I and coelectrophoresed as internal controls. Ad12 DNA was ^{32}P labeled by nick translation and used as the hybridization probe. Cross-hybridizations between Ad2 and Ad12 DNAs were not apparent at the DNA concentrations and exposure times used in this experiment. The bands designated SYREC were due to a symmetric recombinant DNA between Ad12 DNA and KB cellular DNA (8) or to other recombinant DNAs present in many Ad12 DNA preparations. An autoradiogram is presented. (Panel b) BHK-21 cells were coinfected with Ad2 and Ad12 at multiplicities of 50 and 150 PFU per cell, respectively. At the indicated time intervals after infection, the total intranuclear DNA was extracted, cut with *Sma*I, electrophoresed on a 0.7% agarose gel, and blotted to nitrocellulose filter paper. The DNA was then hybridized to ^{32}P-labeled Ad12 DNA. Ad12 DNA, Ad2 DNA, and nuclear DNA from Ad2-infected BHK-21 cells isolated 12 or 30 h after infection or from Ad12-infected BHK-21 cells isolated 30 h after infection were coelectrophoresed as controls. Different parts of the blot were exposed for different times as indicated (1d, 1 day; 5d, 5 days). An autoradiogram was presented. The *Sma*I maps of Ad2 and Ad12 DNAs and a length scale in map units (m.u.) are also shown.

because 5 U of *Sma*I per μg of DNA yielded the same results as 1 U/μg. We concluded that Ad12 DNA could replicate in Ad2 and Ad12 double-infected BHK-21 cells by about 24 h after infection. The possibility could not be rigorously excluded that Ad12 DNA was capable of replicating due to recombination events with small segments of Ad2 DNA which were not detectable by restriction analysis.

Are Ad12 virions produced in Ad2 and Ad12 double-infected BHK-21 cells? It has been documented previously that Ad2 and Ad12 virions exhibit strikingly different buoyant densities by equilibrium centrifugation in CsCl density gradients: ρ = 1.334 g/cm^3 for Ad2 virions and ρ = 1.328 g/cm^3 for Ad12 virions (10). This difference was experimentally exploited to investigate the possible de novo synthesis of Ad12 virions in double-infected BHK-21 cells. At 2 h after double infection with Ad2 and Ad12, BHK-21 cells maintained in monolayers were exposed to 30 μCi of ^{32}P-labeled orthophosphate per ml of maintenance medium to label the newly synthesized viral DNA. The virions present were harvested 72 h after double infection and purified as described above. Viral DNA was then analyzed as described in the legend to Fig. 3.

Similar experiments were performed with human HeLa cells double infected with Ad2 and Ad12. Since HeLa cells are permissive for both viruses, this experiment served as a positive control. The results (Fig. 3a) demonstrated that both Ad2 (higher buoyant density) and Ad12 virions (lower buoyant density) had replicated. In contrast, in BHK-21 cells double infected with Ad5 and Ad12, there was no evidence for the presence of newly synthesized (i.e., ^{32}P-labeled) Ad12 DNA in the lower buoyant density stratum (Fig. 3b), even after much longer exposures than those shown here. Very similar results were obtained with BHK-21 cells double infected with Ad2 and Ad12. The autoradiogram in Fig. 3b demonstrated the distinct Ad5 DNA pattern (*Sma*I) with virions from the higher buoyant density stratum. The data thus failed to demonstrate replication of Ad12 virions. It remained unknown at what stage the assembly of newly synthesized Ad12 virions was blocked. The results presented in Fig. 3c show that parental, hence unlabeled, Ad12 DNA could still be recovered from the lower density stratum of the density gradients even 72 h after infection. These data were derived from the gel shown in Fig. 3b. In this experiment, the DNA from virions in different density strata was extracted and cut with *Sma*I, and the fragments were separated by electrophoresis. The gel was photographed using a UV illuminator (Fig. 3c). The gel was then dried, and newly synthesized DNA bands were visualized by autoradiography (Fig. 3b). It has been reported previously (11) that Ad12 virions remain intact for even longer periods after the inoculation of BHK-21 cells. The data presented here confirm this conclusion and also identify the buoyant density stratum of Ad12 virions. In this density stratum, newly synthesized Ad12 DNA was not detectable (Fig. 3b). We therefore conclude that Ad12 virions were not assembled de novo in BHK-21 cells double infected with Ad2 and Ad12. Ad12 DNA could replicate but was not packaged into virus particles.

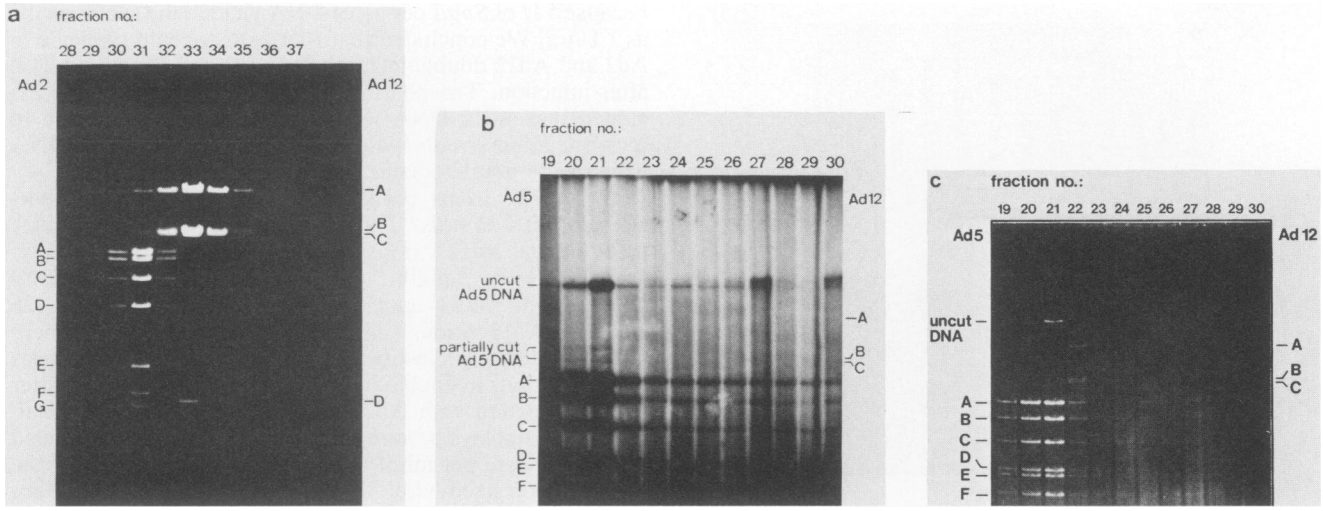

FIG. 3. Absence of replication of Ad12 virions in BHK-21 cells double infected with Ad2 (Ad5) and Ad12. (Panel a) As a positive control, HeLa cells were double infected with Ad2 and Ad12. At 72 h after infection, the total intracellular virus content was harvested and purified by equilibrium centrifugation in CsCl density gradients. Ad2 and Ad12 virions appeared as two clearly distinct bands. The gradient was collected in two drop fractions; from each fraction comprising the virion bands, viral DNA was extracted as described previously (43). The DNA from each fraction was then cleaved with *Sma*I, and the fragments were separated by electrophoresis on a 0.8% agarose gel. A UV photograph of the ethidium bromide-stained gel is presented. The Ad2 and Ad12 DNA peak fractions appear in fractions 31 and 33, respectively. The locations of the Ad2 and Ad12 *Sma*I fragments are designated by capital letters. Fraction numbers refer to the CsCl gradient. (Panel b) BHK-21 cells were double infected with Ad5 and Ad12, and at 2 h after infection 30 μCi of ^{32}P-labeled inorganic phosphate was added per ml of culture medium. At 72 h after infection, the total intracellular virus was harvested and purified by equilibrium centrifugation in CsCl density gradients as described in the text. A strong Ad5 virus band and a very weak Ad12 virus band were observed in the higher and lower density strata, respectively. Fractions were collected, and the DNA was extracted and analyzed as described for panel a. After electrophoresis, the agarose gel was dried and directly autoradiographed. Newly synthesized viral DNA was found only in the Ad5 virion band. The Ad12 virion band was devoid of newly synthesized DNA and therefore was due to parental virions. The positions of the Ad5 and Ad12 *Sma*I fragments are designated by capital letters. (Panel c) Before the gel was dried, it was ethidium bromide stained and photographed on a UV light transilluminator.

Ad12 DNA can replicate in Ad5-transformed hamster cells carrying the E1 region of Ad5 DNA. The Ad5-transformed hamster cell line BHK297-131 contained integrated in its genome the left 18.7% of Ad5 DNA including the E1 region and an additional internal segment of Ad5 DNA (comprising 32.4 to 41.4 map units) as indicated in Fig. 1. It was demonstrated that the left terminal DNA segment of Ad5 DNA was constitutively expressed as cytoplasmic RNA. The data presented in Fig. 4a demonstrate that by 25 h after the infection of BHK297-131 cells with Ad12 (100 PFU per cell), Ad12 DNA replication was evident from the steady increase of Ad12 DNA as determined by Southern blot analyses. The total intranuclear DNA of BHK297-131 cells infected with Ad12 was extracted and cut with *Sma*I, and Ad12 DNA sequences were visualized by hybridization to ^{32}P-labeled Ad12 DNA as the probe followed by autoradiography. It was concluded that the products of the E1 region or of the persisting internal segment of Ad5 DNA sufficed to elicit replication of Ad12 DNA in hamster cells. In this context it should be pointed out that the apparent complementation of Ad12 DNA replication by E1 gene products of Ad2 or Ad5 or of products of internal Ad5 DNA segments could also have been mediated indirectly via cellular gene products. Cellular genes essential for Ad12 DNA replication could have been induced or inhibitory cellular proteins could have been counteracted by the Ad2 or Ad5 gene products. In cell lines HE7 and BHK297-131 superinfected with Ad12, virion production could not be detected by the methods used here.

We also superinfected the Ad2-transformed hamster cell lines HE1 and HE7 with Ad12 at multiplicities of 100 and 150 PFU per cell, respectively. The nuclear DNA was extracted at various times after infection and cleaved with *Sma*I, and Ad12 DNA sequences were identified by Southern blotting, hybridization to ^{32}P-labeled Ad12 DNA, and autoradiography. The data (not shown) indicated that Ad12 DNA persisted in these cell lines, but the amount of Ad12 DNA did not seem to change between 2 and 71 h after infection, while the superinfected cells continued to grow. It was not clear yet whether Ad12 DNA replicated synchronously with cellular DNA or whether Ad12 DNA replicated and was simultaneously degraded at a rate such that replication and degradation appeared to balance each other. Both mechanisms would have explained the finding that the amount of Ad12 DNA in superinfected cells had remained approximately constant for at least 71 h after infection. It is also conceivable that Ad12 DNA replicated only in a limited number of superinfected cells.

Expression of late Ad12 genes in Ad2- and Ad5-transformed hamster cells. The Ad5-transformed hamster cell line BHK297-131 (Fig. 4b) or the Ad2-transformed hamster cell lines HE1, HE5, or HE7 (Fig. 5) were superinfected with Ad12 as described above. At the time intervals indicated (Fig. 4b and 5), RNA was isolated, electrophoretically fractionated, and transferred to nitrocellulose filters, and late Ad12-specific RNA sequences were identified by hybridization to the ^{32}P-labeled late *Bam*HI-D fragment of Ad12 DNA (Fig. 4b and 5). In control experiments, the late Ad12-specific RNA molecules were displayed on Northern blots of cytoplasmic RNA from Ad12-infected human KB cells as size markers (Fig. 4b and 5). In Ad2-infected human KB cells, RNA molecules with homologies to the Ad12 *Bam*HI-

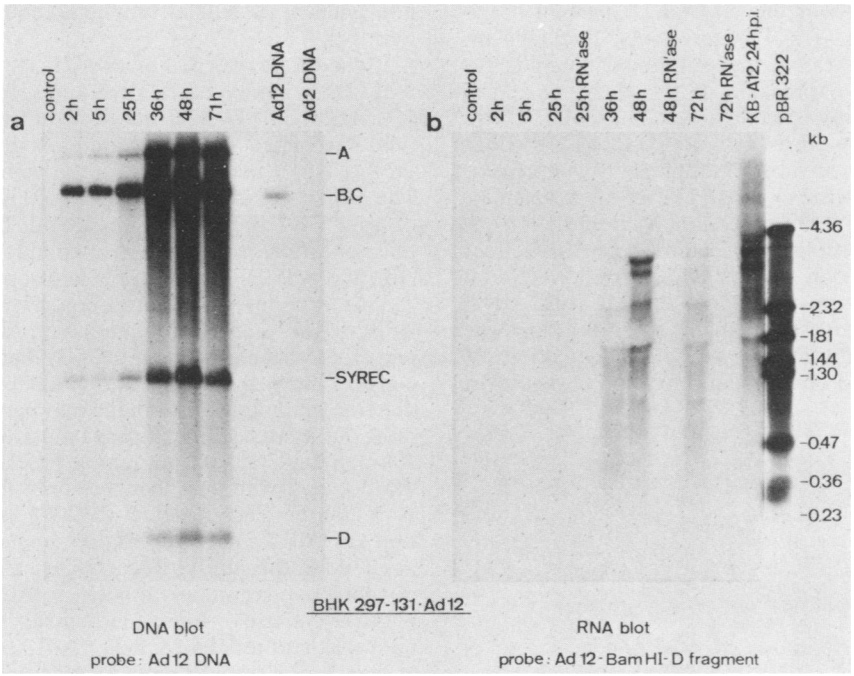

FIG. 4. (Panel a) Ad12 DNA replicates in BHK297-131 hamster cells. The Ad5-transformed hamster cell line BHK297-131 was superinfected with Ad12 at a multiplicity of 100 PFU per cell. At the indicated time intervals after superinfection, the nuclear DNA was extracted and cleaved with *Sma*I. The fragments were separated by electrophoresis on a 0.7% agarose gel and blotted to nitrocellulose filters, and Ad12-specific sequences were detected by hybridization to ^{32}P-labeled Ad12 DNA. The slot designated control contained BHK297-131 DNA from cells which were not superinfected. Ad2 and Ad12 virion DNAs cleaved with *Sma*I were coelectrophoresed to include marker fragments. (Panel b) Expression of late Ad12 functions after superinfection of BHK297-131 hamster cells with Ad12. Experimental conditions were similar to those described for panel a, except that the total cytoplasmic RNA was extracted, electrophoresed on a 1.0% agarose gel in the presence of 2.2 M formaldehyde (34), blotted to nitrocellulose filters, and hybridized to the ^{32}P-labeled *Bam*HI-D fragment of Ad12 DNA in pBR322 DNA. RNA from uninfected BHK297-131 cells was used as a negative control. In some experiments the RNA was destroyed by incubation with RNase as indicated. pBR322 DNA was cut with *Bgl*I, *Eco*RI, and *Taq*I, denatured, and coelectrophoresed as a size marker. Marker fragment sizes are indicated in kilobases (kb).

D fragment were not apparent (Fig. 4b and 5). In cell lines BHK297-131, HE1, HE5, and HE7, late Ad12 RNA synthesis began at 12 to 25 h after superinfection with Ad12 (Fig. 4b and 5). Most of the Ad12-specific RNA populations detected in the Ad12-superinfected cell lines corresponded in size to RNA size classes in human KB cells productively infected with Ad12 (Fig. 4b and 5). All Ad12-specific RNAs were fully sensitive to treatment with pancreatic ribonuclease (Fig. 4b). These RNA size classes were not found in transformed cell lines that had not been superinfected with Ad12 (Fig. 5). The amounts of late Ad12-specific RNAs synthesized in Ad12-superinfected Ad2- or Ad5-transformed cell lines were much lower than for those transcribed in human KB cells productively infected with Ad12. In some experiments with RNA from cell line BHK297-131, the ^{32}P-labeled *Bam*HI-I, -F, -C, -B, and -E fragments (map, Fig. 5) were used as hybridization probes (data not shown). Results similar to those shown in Fig. 4b and Fig. 5 were obtained with these probes.

It could also be demonstrated that in the superinfected cell lines, early Ad12 RNAs were produced (data not shown), as evidenced by RNA hybridizing on Northern blots to the early *Hin*dIII-F and *Bam*HI-E fragments of Ad12 DNA (map, Fig. 5). Similarly it was established that in the Ad12-superinfected cell lines, the early Ad2- or Ad5-specific RNAs continued to be transcribed (data not shown).

It was concluded that in Ad2- or Ad5-transformed hamster cells, the presence of the integrated E1 region and of internal segments of the Ad2 or Ad5 genome (32.4 to 41.4 map units) sufficed to elicit late Ad12 gene expression. The results of the Northern blot hybridization experiments did not yet establish whether all late Ad12 genes were expressed in the transformed hamster cell lines. However, it appeared from a comparison of Ad12 DNA transcription patterns in Ad12-superinfected transformed cells and productively infected human cells that Ad12 DNA expression was similar in both systems.

DISCUSSION

The block in DNA replication and in the expression of late genes of Ad12 DNA in hamster cells could be overcome, at least in part, by products of the left half of Ad2 or Ad5 DNA. Ad2 or Ad5 virions could replicate in hamster cells; thus, Ad2 or Ad5 DNA had to contain all of the functions required for the replication of these viruses in hamster cells. It was also apparent that Ad2 or Ad5 could utilize cellular functions involved in virus replication. It was odd that Ad12 DNA could not provide these functions or could not use cellular functions to promote its own replication. However, products of the left 18% and of internal segments of Ad5 DNA (32.4 to 41.4 map units) (map, Fig. 1), and of Ad2 DNA as well, were capable of complementing, by an as yet unknown *trans* effect, the deficiencies of Ad12 DNA in hamster cells.

trans-activating effects of the major E1A protein of Ad2 DNA on the expression of other early genes of this virus (4, 21, 26) and on the expression of some cellular genes (22) have been described. The exact biochemical nature of these

trans-activating functions of the major E1A protein, however, remains unknown. The E1A protein(s) might be involved in the modulation of a transcriptional complex required for the efficient expression of the early (and late) genes of Ad2 and possibly of cellular genes as well. These activities of the E1 proteins have not yet been directly demonstrated. It is conceivable, but presently unproven, that the E1 functions of Ad2 or of Ad5 DNA exert a similar *trans*-activating effect on the expression of late functions of Ad12 DNA and on its replication. This latter *trans* effect could be exerted directly on Ad12 functions or indirectly on cellular functions which, in turn, could activate Ad12 DNA replication and expression. Even if this interpretation was accepted, it would still not explain why the E1 functions of Ad12 should be incapable of effecting activation of their own late genes or of cellular functions required for Ad12 replication.

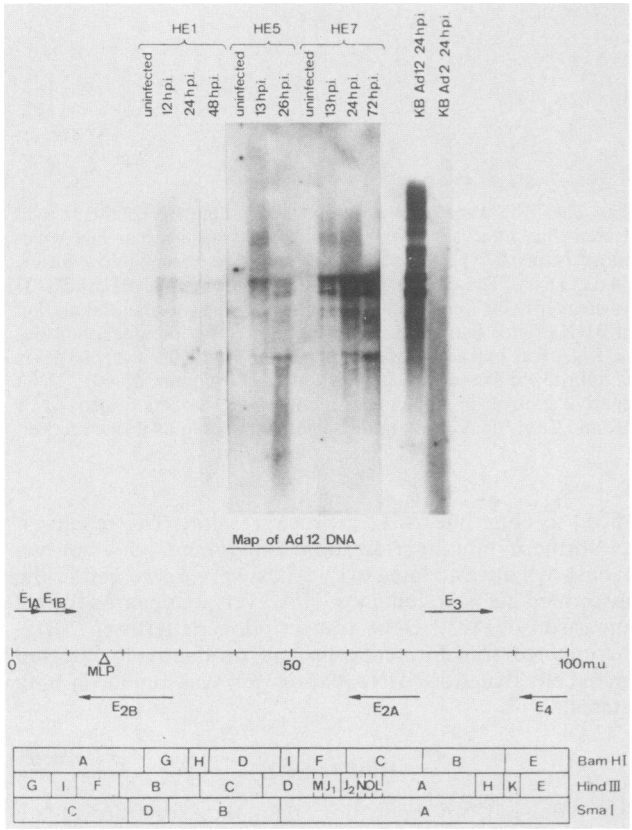

FIG. 5. Expression of late Ad12 functions in the Ad2-transformed hamster cell lines HE1, HE5, or HE7 upon superinfection with Ad12. Cell lines as indicated were superinfected with Ad12 at multiplicities of 100 to 150 PFU per cell. At the indicated time intervals, the total cytoplasmic RNA was extracted from the cells and analyzed as described in the legend to Fig. 4 (panel b) by using the ^{32}P-labeled *Bam*HI-D fragment of Ad12 DNA as a probe. RNAs from uninfected cells were used as negative controls. Cytoplasmic RNAs from human KB cells productively infected with Ad2 or Ad12, which were extracted 24 h after infection, were coelectrophoresed as positive controls and size markers. Also shown is a functional map of the Ad12 genome. The arrows indicate locations and directions of transcription of the early regions of Ad12 DNA (cf. references 23 and 28) designated E1A, E1B, E2A, E2B, E3, and E4. The *Bam*HI-D and -I fragments are diagnostic for late Ad12 genes; the *Hin*dIII-F fragment contains the major late promoter (MLP) but not the region encoding the VA RNAs. The Ad12 map was divided into 100 map units (m.u.).

It should be noted, however, that the early functions of Ad12 DNA can be efficiently expressed in hamster cells (14, 15, 23, 28, 34). Hence, the *trans*-activation by Ad2 or Ad5 functions appears to be required mainly for the expression of the late Ad12 functions and for the replication of Ad12 DNA. The possibility exists that expression and replication defects of Ad12 DNA in hamster cells are due to deficiencies in more than one function, hence the complementing functions of Ad2 and Ad5 DNA could be pleiotropic in this system.

Two experimental approaches relating the E1 region of Ad5 to Ad12 complementations are of interest in this context. It has been demonstrated (33) that in human HeLa cells coinfection with Ad12 can complement defects of Ad5 deletion mutants which are located between 1.3 and 3.7 map units of the Ad5 genome (class I mutants) or between 6.1 and 9.4 map units (class II mutants). In BHK-21 cells, however, Ad12 coinfection complements only the class I mutants of Ad5, not the class II defect (33). The effect of Ad5 coinfection on Ad12 DNA replication in hamster cells was not assessed in this study. Recently, it has been reported that a nondefective recombinant between Ad5 and the E1A region of Ad12 can grow slightly in hamster cells (37). These data indicate that the E1A region of Ad12 seems to be functional in hamster cells, as is already apparent from transcriptional analyses (28), but the data do not bear on the nature of the replication defect of Ad12 in hamster cells.

Clearly, it is necessary to determine which E1 function or which other functions are capable of complementing the defects in replication and late expression of the Ad12 genome in hamster cells. For this purpose, we have initiated experiments in which hamster cells are coinfected with deletion mutants of Ad5 (36) and with Ad12. Moreover, we plan to generate hamster cell lines carrying in an integrated form the E1A region of Ad2 DNA and to test these cell lines for their ability to complement Ad12 DNA replication.

Ad12 DNA does replicate in Ad2 and Ad12 double-infected hamster cells and in Ad2- or Ad5-transformed cell lines that carry in an integrated form the left part of viral DNA. In the Ad12-infected transformed cell lines, all or at least most of the Ad12-specific late functions, expressed as mRNA in Ad12 productively infected human cells, appear to be expressed in hamster cells as well. The biochemical basis for this complementation remains unknown. It has not yet been determined whether the Ad12-specific mRNAs are all translated into functional viral proteins. In fact, no evidence has been obtained as yet for the production of intact Ad12 virions in the hamster cell systems described above. The block in virion assembly could lie at several different stages, and we are currently trying to identify this block in virion assembly. Since only certain steps in Ad12 virus replication could be remedied by Ad2 or Ad5 functions, it is likely that Ad12 virus production is blocked at more than one single function.

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