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Simian Virus 40 DNA Replication, Transcription, and Antigen Induction During Infection with Two Adenovirus 2-SV40 Hybrids That Contain the Entire SV40 Genome

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The Ad2^{++}HEY hybrid virus population produces simian virus 40 (SV40) efficiently during lytic infection, whereas Ad2^{++}LEY does not, although both hybrids contain a complete SV40 genome. In this report, we demonstrate the synthesis of nonhybrid SV40 DNA in Ad2^{++}HEY-infected cells but not in Ad2^{++}LEY-infected cells. Hybridization-competition experiments indicate that both early and late SV40 RNA is transcribed efficiently in Ad2^{++}HEY-infected Vero cells, but only early SV40 RNA is transcribed efficiently in Ad2^{++}LEY-infected cells. Ad2^{++}HEY induces SV40 U, T, and V antigens during lytic infection of African green monkey kidney cells, whereas Ad2^{++}LEY induces only SV40 U and T antigens. These variations in the behavior of Ad2^{++}HEY and Ad2^{++}LEY regarding expression of SV40 functions probably reflect differences in the rate of SV40 excision from the hybrid genomes.

Two stable variants have been isolated from the adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid population Ad2^{++} (21). These hybrid-virus populations were first distinguished by the amount of SV40 that they produced during lytic infection of African green monkey kidney (AGMK) cells. One population, designated Ad2^{++}HEY (high-efficiency yielder), contains adeno-encapsidated particles (neutralized by Ad2 antiserum) that produce SV40 in concentrations of 10^6 or 10^7 PFU/ml during low-multiplicity infection of AGMK cells. The other population, designated Ad2^{++}LEY (low-efficiency yielder), contains Ad-encapsidated particles that produce 1,000- to 10,000-fold lower concentrations of SV40 during low-multiplicity infection than do Ad2^{++}HEY hybrid virions. Combined with studies on the progeny of Ad2-$^{++}$HEY and Ad2^{++}LEY plaques isolated from AGMK cells (21), these data indicate that the hybrid particles in both variants must contain at least one infectious SV40 genome. Although both hybrid populations induce early SV40 T antigen, only Ad2^{++}HEY induces detectable late SV40 (V) antigen (21). Both the Ad2-$^{++}$HEY and the Ad2^{++}LEY hybrid virions are defective and require co-infection with nonhybrid Ad2 for replication.

Since the SV40 genome in the hybrid particles apparently enhances Ad2 replication in AGMK cells, 90 to 98% of the particles in the populations are nonhybrid Ad2 (21). Ad2-SV40 hybrid particles that give rise to SV40 progeny comprise 2 to 10% of each population; very small amounts (0.01 to 0.001%) of nonhybrid SV40 are also present. The Ad2 and SV40 DNA in the hybrid genomes of both populations are covalently linked, since they cannot be separated by centrifugation in alkaline CsCl or alkaline sucrose gradients (4). Hybrid virions in the Ad2^{++}LEY population have the same buoyant density as Ad2, whereas hybrid virions in the Ad2^{++}HEY population are 4 mg/ml lighter (34) and the DNA from Ad2^{++}HEY hybrid virions is 3 mg/ml lighter than nonhybrid Ad2 DNA (4).

Kelly et al. (10) have recently studied the structure of the hybrid genomes in the Ad2-$^{++}$HEY and Ad2^{++}LEY populations by electron microscope examination of heteroduplexes. The results of these studies indicate that Ad2-$^{++}$HEY hybrids contain a large tandem repetition of SV40 DNA, such that an intact SV40 genome is more likely to be excised from Ad2-$^{++}$HEY hybrid molecules than from Ad2^{++}LEY hybrid molecules. Thus, the higher probability of excision of complete SV40 molecules could account for the higher efficiency of SV40 production in cells infected with Ad2^{++}HEY. However, the observations on the hybrid genome structure do not exclude the possibility that

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differences in SV40 yielding are associated with biochemical lesions at the level of SV40 DNA replication, transcription, or translation. In the present study, cells infected with Ad2++HEY or Ad2++LEY have been examined to determine the extent of nonhybrid SV40 DNA replication and the pattern of SV40 transcription. We have also tested both hybrid populations for their ability to induce SV40 U and T antigens.

MATERIALS AND METHODS

Tissue culture. AGMK and human embryonic kidney cells, obtained commercially, were maintained on Eagle minimal essential medium containing penicillin (250 U/ml), streptomycin (250 μg/ml), and 2 mM glutamine (EMEM) plus 10% fetal bovine serum (EMEM-10).

Continuous cultures of the Vero and BSC-1 lines of AGMK cells were maintained on EMEM-10 between passages 147 and 172. All cell lines were repeatedly negative for mycoplasma by anaerobic culture on Hayflick media (6).

Viruses. For these studies, pools of the Ad2++HEY population (representing the fourth and fifth monkey cell passages of the progeny of plaque 1208) were prepared in either primary AGMK or Vero cells. Ad2++LEY pools, representing the fourth monkey cell passage of the progeny of plaque 1567, were prepared in Vero cells. Cultures were infected with >10 PFU/cell. The size of the inoculum was estimated from the concentration of nonhybrid Ad2 virions in the population. The inocula for Ad2++HEY pools were pretreated with 125 U of SV40 rabbit antiserum per ml prior to infecting the cultures. Earlier studies (21) demonstrated that this SV40 antibody concentration reduced the concentration of nonhybrid SV40 in the Ad2++HEY pools to less than 10^3 PFU/ml. These studies also showed that the concentration of nonhybrid SV40 in Ad2++LEY pools remained below 10^3 PFU/ml without pretreating the inoculum with SV40 rabbit antiserum. The concentrations of nonhybrid Ad2 in Ad2++HEY and Ad2++LEY pools ranged between $10^{7.9}$ and $10^{9.3}$ PFU/ml, whereas the concentration of hybrid particles in the pools that registered as plaques on lawns of nonhybrid Ad2 in AGMK cells varied between $10^{7.1}$ and $10^{8.1}$ PFU/ml. The concentrations of hybrid particles in the two Ad2++HEY pools that registered as SV40 plaques on AGMK cells without Ad2 lawns were $10^{6.9}$ and $10^{7.1}$ PFU/ml. The techniques used to assay the various components of the Ad2++HEY and Ad2++LEY populations have been described (21).

SV40 strain 777 has been maintained in our laboratory by serial, low-multiplicity passage in monolayers of BSC-1 cells (27). A single SV40 pool (titer $10^{8.5}$ PFU/ml) was used as the inoculum for the SV40 stocks used to obtain SV40 DNA and to produce SV40-specific RNA. These stocks were produced in 32-oz (ca. 960 ml) bottle cultures of Vero cells with an inoculum of $<10^{-5}$ PFU/cell and containing $10^{8.4}$ and $10^{8.7}$ PFU of SV40 per ml as determined by plaque assay in AGMK cells.

Ad2 (strain Ad.6) has been maintained by serial passage in human embryonic kidney cells. The pool (titer $10^{9.3}$ PFU/ml) of Ad2 used in these studies was prepared in human embryonic kidney cells infected with an inoculum of 10 or more PFU/cell. The isolation, propagation, and characterization of Ad2+ND$_4$ have been described (7, 18, 20).

All virus pools were demonstrated to be free of mycoplasma by anaerobic cultures on Hayflick agar (6). Complement fixation testing for adeno-associated viruses types 1 through 4, reovirus, and SV5 were performed on each Ad2 and hybrid virus pool, and, with the exception of the Ad2++LEY population that was persistently contaminated with AAV-4, all such tests were negative.

Selective extraction of viral DNA, velocity sedimentation in alkaline sucrose, and hybridization of gradient fractions. Parallel cultures of confluent primary AGMK cells in 100-mm petri dishes (1×10^7 to 1.2×10^7 cells per dish) were infected with Ad2++HEY or Ad2++LEY virus at a multiplicity of 10 nonhybrid Ad2 PFU/cell, SV40 at a multiplicity of 10 PFU/cell, or Ad2 (10 PFU/cell) plus SV40 (1 PFU/cell); each virus inoculum was in 4 ml of EMEM supplemented with 2% agammaglobulinemic calf serum (EMEM-2). The Ad2++HEY and Ad2++LEY infections were carried out in the presence of 125 U of SV40 rabbit antiserum per ml. After a 2-h adsorption, cultures were refed with 10 ml of EMEM-2 containing 1 μCi of [2-^{14}C]thymidine (20 to 30 mCi/mmol, New England Nuclear) per ml and, in the case of Ad2++HEY and Ad2++LEY, 125 U of SV40 rabbit antiserum per ml. Thirty-six hours after infection, the medium was discarded and the cell sheets were washed twice with iced Tris-buffered saline. Viral DNA was then extracted by a modification of the method of Hirt (8). A 2-ml amount of 0.6% sodium dodecyl sulfate-0.01 M EDTA (pH 7.5) was added to each dish, and after 20 min at room temperature the viscous lysates were gently scraped into flasks. Nuclease-free Pronase (2 mg/ml) was added to each flask, and the lysates were incubated at 37 C for 1 h. After precipitation of cellular DNA with 5 M NaCl and centrifugation in a Spinco L265B at $17{,}000 \times g$ for 45 min at 4 C, the supernatant was extracted three times with an equal volume of Tris (1 M)-saturated redistilled phenol. Viral DNA was precipitated with iced ethanol, pelleted for 1 h in a Sorvall RC-2B at 15,000 rpm at 4 C, and dissolved in $0.1 \times$ SSC (SSC = 0.15 M NaCl, 0.01 M sodium citrate, pH 6.9).

DNA in the Hirt supernatants was analyzed by velocity sedimentation on 5 to 20% alkaline sucrose gradients. The DNA solutions (0.1 ml) were layered onto 4.5-ml gradients (0.2 M NaOH, 0.7 M NaCl, 0.15% Sarkosyl) and centrifuged 2 h at 40,000 rpm at 5 C in a Spinco SW50.1 rotor. After centrifugation, 0.2-ml fractions were collected from the bottom of the tube by using a constant-volume displacement device, and samples of each fraction were neutralized and counted in Aquasol (New England Nuclear) in a Packard Tri-Carb scintillation spectrometer. Approximately equal amounts of DNA from each fraction of

the Ad2++HEY and Ad2++LEY alkaline sucrose gradients were diluted in cold 6× SSC and collected by gravity on nitrocellulose filters. Filters were challenged with saturating amounts of ^{3}H-labeled SV40 complementary RNA synthesized in vitro (19), and processed as described for in vivo RNA-DNA hybrids.

Radiolabeling, virus purification, and extraction of SV40 DNA. Radioactive SV40 was prepared, as described previously (27), with 0.5 μCi of [2-^{14}C]thymidine (New England Nuclear, 20 to 30 mCi/mmol) per ml of medium. Labeled and unlabeled SV40 virus was purified from cells and medium by a modification of the technique of Burnett et al. which has been reported in detail (27).

Viral DNA was extracted from purified virus by Pronase (B grade, nuclease-free, Calbiochem)-sodium dodecyl sulfate digestion followed by phenol extraction and dialysis (11). Viral DNA was stored at −30 C in 0.1× SSC. This DNA is free of detectable host cell (monkey) nucleotide sequences as determined by sensitive hybridization assays (19).

Preparation of early and late SV40 RNA and hybrid virus RNA. Early and late SV40-specific RNA was prepared in roller bottle cultures of Vero cells as described previously (18, 27). Early SV40 RNA was prepared in the presence of cytosine arabinoside. Radiolabeling of SV40 RNA was performed by refeeding cultures 4 h postinfection with 15 ml of EMEM-2 and 20 mCi each of [5-^{3}H]uridine, [2,8-^{3}H]adenosine, and [5-^{3}H]cytidine (greater than 20, 20 to 30, and 15 to 30 Ci/mmol, respectively, New England Nuclear) per ml.

Hybrid virus-specific RNA was prepared by infecting 32-oz bottle cultures of Vero cells with 10 PFU of hybrid virus per cell (estimated from the concentration of nonhybrid Ad2) in 10 ml of EMEM-2. After a 3- to 4-h adsorption period, an additional 20 ml of EMEM-2 was added to each culture. Cell cultures were harvested by scraping 24 or 48 h after infection. Tritiated hybrid virus-specific RNA was prepared by refeeding the cultures 4 h postinfection with 15 ml of EMEM-2 medium containing concentrations of the three radioisotopes identical to those employed for SV40-specific RNA.

Extraction of nucleic acids from infected and uninfected cells. DNA from *Escherichia coli* was extracted by the method of Marmur (24). RNA from infected and uninfected Vero cells was extracted by a hot phenol-sodium dodecyl sulfate procedure (17) and stored at −30 C in 2× SSC plus 0.05% sodium dodecyl sulfate. The concentrations of DNA solutions were determined by a modified diphenylamine reaction (3) with calf thymus DNA (Calbiochem) as a standard. RNA concentrations were determined by an orcinol reaction (2) with yeast tRNA (Calbiochem) as a standard.

RNA-DNA hybridization and hybridization competition. The procedure for hybridization of [^{3}H]RNA with single-stranded DNA immobilized on nitrocellulose membrane filters (Millipore Corp., HAWP) has been described (17, 18). Hybridization reactions were performed at 60 C with 13-mm filters in a 0.25-ml volume of 2× SSC plus 0.05% sodium dodecyl sulfate. Unless otherwise stated, an incubation period of 20 h was employed. All RNA-DNA hybrids were washed at 60 C, treated with pancreatic RNase (XII-A, Sigma Chemical Co.; 20 μg/ml) and RNase T_1 (B grade, Calbiochem; 10 U/ml) for 1 h at room temperature, and washed again before scintillation counting.

Hybridization-competition experiments were performed by preincubating the DNA-containing filters with increasing amounts of unlabeled competitor RNA for 12 h at 60 C and then adding a saturating amount (separately determined with the same batch of DNA filters and [^{3}H]RNA) of radioactive RNA. Incubation was then continued for an additional 14 h, after which the filters were washed, RNase treated, and washed again before scintillation counting. The extent of nonspecific competition by cellular RNA was determined by mixing unlabeled RNA from uninfected Vero cells directly with the unlabeled competitor RNA (from virus-infected Vero cells) in varying ratios so that the same total amount of RNA was present at each point in the competition experiment. Selected competition experiments were performed by treating the initial unlabeled RNA-SV40 DNA hybrid with RNase, extensive washing, and retreatment with RNase after the second stage of hybridization (23).

The SV40 DNA on the filters was ^{14}C labeled to permit an accurate determination of the amount of DNA still present at the end of the final hybridization and washing procedures. Filters were counted for sufficient time to achieve a counting accuracy of ±5% or better for both ^{3}H and ^{14}C. The ^{3}H counts per minute bound to SV40 DNA filters were corrected for ^{14}C counted in the ^{3}H channel and for nonspecific binding to *E. coli* DNA. The net virus-specific ^{3}H counts per minute were then normalized to the stated amount of SV40 DNA per filter on the basis of the ^{14}C counts per minute.

Antigens, immune sera, and serologic tests. Preparation of SV40 T^+U^+ and T^+U^- hamster antiserum and complement fixation testing of virus-infected cells have been described in detail (22). SV40-neutralizing antiserum (rabbit antiserum) was obtained from rabbits immunized intravenously at biweekly intervals with 10^8 to 10^9 PFU of SV40 strain 777 and exsanguinated at 5 to 6 days after the seventh injection. One unit of SV40 rabbit antiserum is the minimum amount of antibody that inhibits the development of cytopathic effects in AGMK cells infected with 10^7 to 10^8 PFU of SV40 in 5 days in a graded response assay.

RESULTS

Forms of SV40 DNA synthesized in cells infected by Ad2++HEY or Ad2++LEY. The observed differences in the structure of Ad2++HEY and Ad2++LEY (10) suggest that excision of intact SV40 genomes could occur with a greater probability from Ad2++HEY than Ad2++LEY and result in a more extensive replication of nonhybrid SV40 DNA. We there-

fore examined the various forms of SV40 DNA synthesized in cells infected by Ad2++HEY or Ad2++LEY. AGMK cells were infected for 36 h with each of these viruses, as well as with wild-type SV40 or with a mixed infection of Ad2 plus SV40. Radiolabeled viral DNA was selectively extracted and analyzed by velocity sedimentation in alkaline sucrose gradients. It can be seen in Fig. 1A that wild-type SV40 infection induces the synthesis of ^{14}C-labeled DNA that sediments as two peaks at approximately 53*S* and at 16- to 18*S*; the major peak corresponds to denatured form I SV40 DNA (denatured superhelices) and the smaller peak corresponds to forms II (single-stranded circular) and III (single-stranded linear) SV40 DNA. As anticipated, infection with 10 PFU of Ad2 per cell plus 1 PFU of SV40 per cell induces the synthesis of viral DNA that sediments as only one peak (34*S*), since in this mixed infection only Ad2 DNA replicates significantly (5). Ad2++LEY also induces the synthesis of viral DNA that sediments as only one peak, corresponding to Ad2 DNA. We were able to detect the peak corresponding to Ad2 DNA in the 36-h Ad2++HEY infection, but no peak corresponding to a nonhybrid form of SV40 DNA despite the production of SV40 virus in Ad2++HEY infection. Attempts to demonstrate form I SV40 by isopycnic banding in ethidium bromide-cesium chloride were also unsuccessful (data not shown). It is not clear why the Ad2++HEY DNA appears to sediment more slowly than Ad2 DNA (Fig. 1A), since about 90% of the DNA should consist of nonhybrid Ad2 DNA. Since a marker DNA was not included in each gradient, this difference may be due to an artifact. However, the result is reproducible and may reflect the fact that hybrid molecules are up to 20% smaller than nonhybrid (4, 10) and a mixture of the two may appear to move more slowly than nonhybrid Ad2 DNA.

To further examine the forms of viral DNA synthesized in Ad2++HEY and Ad2++LEY infection, we collected single-stranded ^{14}C-labeled DNA from the gradient fractions (Fig. 1A) on nitrocellulose filters and challenged these filters with saturating amounts of in vitro-made complementary SV40 RNA labeled with tritium. The results of hybridization are expressed as the ratio ^{3}H/^{14}C (Fig. 1B), and the assumption is made that if all of the SV40 DNA synthesized in the hybrid virus infections is replicated only as a part of the hybrid genome (i.e., remains covalently linked to Ad2 DNA) then the ^{3}H/^{14}C ratio will remain constant in all fractions of the gradient. It can be seen that, whereas the ratio does remain nearly constant for the Ad2++LEY infection, two distinct peaks of nonhybrid SV40 DNA are synthesized in the case of Ad2++HEY, one sedimenting at 53*S* and one at 16- to 18*S*. These data suggest that, by the more sensitive technique of hybridizing gradient fractions with very high specific activity complementary RNA, nonhybrid forms of SV40 DNA can be demonstrated in the case of

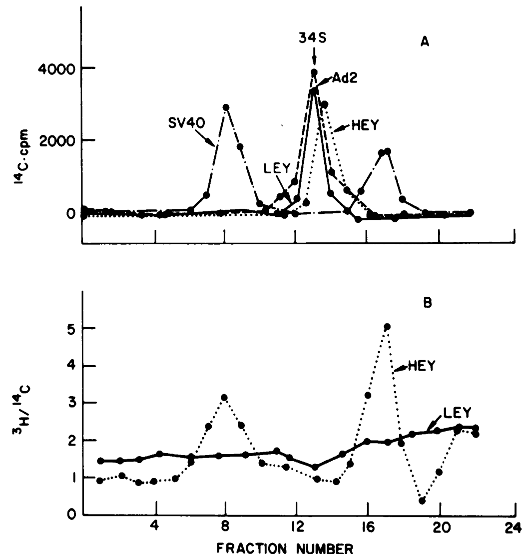

FIG. 1. *Velocity sedimentation of Hirt supernatant DNA molecules in alkaline sucrose, and hybridization of gradient fractions. (A) Initial centrifugation of ^{14}C-labeled DNA selectively extracted from AGMK cells infected for 36 h with various viruses: (-.-) SV40 alone; (- - -) SV40 plus Ad2; (·····) HEY; and (——) LEY. The 34S Ad2 marker was established in separate, identical gradients in which we employed a mixture of purified ^{14}C-labeled Ad2 DNA and ^{3}H-labeled SV40 DNA (53S and 16- to 18S). (B) Hybridization of ^{14}C-labeled Hirt supernatant DNAs, fractionated on alkaline sucrose gradients (Fig. 1A), with ^{3}H-labeled complementary SV40 RNA. The RNA was synthesized in vitro and had an estimated specific activity of 1.1 × 10^7 counts/min per µg. Each hybridization mixture contained a DNA filter from the gradient fraction and 100,000 counts of ^{3}H-labeled SV40 complementary RNA per ml in a total volume of 0.25 ml of 2× SSC. In addition, each vial contained an E. coli DNA filter (0.1 µg per filter) as a blank. The data shown are based on the average counts per minute for duplicate viral DNA filters after subtraction of the average counts per minute (85) bound to E. coli DNA and correction for spill-over between channels. The statistical significance of these data can be estimated from the average ^{3}H and ^{14}C counts per min (6,148 and 1,216, respectively) observed for filters representing the major peak of the Ad2++HEY gradient. (·····) HEY; (——) LEY.*

Ad2++HEY but not Ad2++LEY. Although the newly synthesized SV40 DNA appears to consist of more 16- to 18*S* material than of 53*S* material, it is likely that rapid reannealing of form I DNA precludes efficient hybridization with complementary SV40 RNA.

Comparison of the SV40-specific RNAs induced by Ad2++HEY and Ad2++LEY with SV40-specific RNA induced by Ad2+ND$_4$ and wild-type SV40. In lytic SV40 infection, early SV40 RNA is transcribed before and after viral DNA replication begins, so that late RNA is a mixture of early and late species (1, 18, 26, 27, 33). The early template has been reported to comprise about one-third of the (−) strand of the SV40 genome and the late template comprises two-thirds of the (+) strand (14, 30). To determine the extent of transcription of the SV40 genome in the Ad2++HEY and Ad2++-LEY hybrids during lytic infection, RNA was extracted from Vero cells acutely infected with Ad2++HEY, Ad2++LEY, or wild-type SV40, and employed in hybridization-competition reactions. The SV40 transcription product of each hybrid could then be compared with the other and with the early and late RNA species associated with wild-type SV40 infection.

The results of competition against [^{3}H]RNA extracted from cells infected for 48 h with wild-type SV40 (late SV40 RNA) are shown in Fig. 2. As expected, unlabeled late SV40 RNA competed efficiently with the late [^{3}H]RNA for SV40 DNA binding sites, and early SV40 RNA (made in the presence of cytosine arabinoside) competed with about one-third of the late RNA. The unlabeled RNA from a 48-h infection with Ad2++HEY was also an efficient competitor of ^{3}H-labeled late SV40 RNA, indicating that this hybrid virus induces all of the SV40 RNA sequences induced by SV40 itself. However, unlabeled RNA from a 48-h Ad2++LEY infection was not an efficient competitor of late SV40 RNA (Fig. 2); whereas one component of the 48-h Ad2++LEY competition curve suggests that a small percentage of the SV40 RNA sequences is present in an abundance equal to that in late SV40 infection, the majority of SV40 sequences is present in much lower concentration than in the wild-type infection. It was possible that these less abundant sequences were transcribed only after replication of infectious SV40 DNA began and that RNA harvested at an earlier point in the Ad2++LEY infectious cycle might be more representative of transcription from the hybrid viral genomes. Therefore, competition against ^{3}H-labeled late SV40 RNA was also carried out with unlabeled RNA harvested after 24-h Ad2++HEY and Ad2++LEY infections. Whereas the SV40-specific RNA sequences in a 24-h infection with Ad2++HEY are present in very low concentration as compared with the 48-h Ad2++HEY infection, the 24-h Ad2++HEY RNA contains more sequences complimentary to the entire SV40 genome than does early SV40 RNA (Fig. 2). On the other hand, unlabeled RNA from a 24-h infection with Ad2++LEY reaches a competition plateau such that this RNA appears to contain only about 20% of the RNA sequences present in late lytic SV40 RNA. Thus, it seems likely that at a point during Ad2++LEY infection which is well advanced in terms of Ad2 cytopathic effect, and which should therefore be representative of transcription from the entire Ad2 portion of the hybrid genome, transcription of the SV40 moiety within the hybrid DNA represents only a small fraction of the SV40 genome.

To determine whether the small fraction of

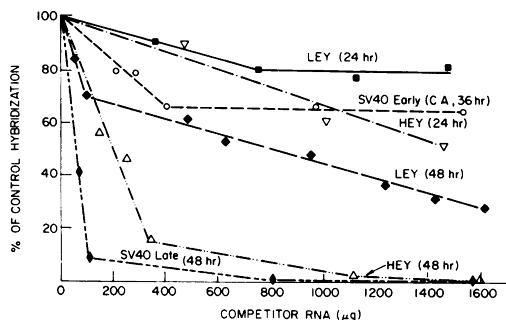

FIG. 2. *Hybridization-competition of unlabeled hybrid virus-specific RNAs or SV40 early RNA with ^{3}H-labeled SV40 late RNA. Hybridization-competition experiments were performed with a saturating amount of [^{3}H]RNA and 0.1 μg of ^{14}C-labeled SV40 DNA filters as described in the text. Radioactive RNA was added to reaction vials after filters had been preincubated with unlabeled RNA; hybrids were washed and treated with RNase after the second stage of hybridization. Nonspecific competition was internally controlled by mixing unlabeled, uninfected Vero cell RNA with unlabeled competitor RNA in varying ratios, so that a constant total amount of unlabeled RNA was present at each point in the competition experiment. The control [^{3}H]RNA counts per minute bound (only unlabeled uninfected Vero RNA present) are the ^{3}H counts per minute bound to the SV40 DNA filter minus the counts per minute bound to an appropriate E. coli DNA (blank) filter. The average of duplicate determinations is shown, and the counts per minute bound at other points in the competition curves are plotted as percentage of this control value. Input of ^{3}H-labeled SV40 late RNA = 93 μg; 86 × 10^3 counts/min per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 10. Control [^{3}H]RNA counts per minute bound = 214.*

competition demonstrated with 24-h $Ad2^{++}$-LEY RNA could be ascribed to early sequences or to true late sequences, we compared the 24-h $Ad2^{++}$LEY RNA with that induced in an infection with $Ad2^{+}ND_4$. This nondefective Ad2-SV40 hybrid virus appears to contain all or most of the early region of the SV40 genome (as well as a small fraction of the SV40 late region), i.e., 0.11 to 0.59 SV40 map units (9, 15, 16, 18, 25, 29); although the topography of the early and late templates within this early region has not been firmly established (15, 29, 31), $Ad2^{+}ND_4$-specific RNA is known to represent most, if not all, of the early SV40 template and a small amount of SV40 (−) strand anti-late template (13). [^{3}H]$Ad2^{+}ND_4$ RNA was chosen for this experiment because its early SV40 sequences are present in much greater abundance than in a wild-type early SV40 infection, thus making the competition experiment technically more feasible (18). It can be seen in Fig. 3, that whereas early SV40 RNA competes with 92% of the ^{3}H-labeled SV40-specific RNA sequences induced by $Ad2^{+}ND_4$, 24-h $Ad2^{++}$LEY RNA reaches a plateau at approximately 70% competition, suggesting that SV40 sequences transcribed from the $Ad2^{++}$LEY hybrid genome correspond to about two-thirds of the early SV40 region; these data do not exclude the possibility that $Ad2^{++}$LEY RNA also contains some or all of the anti-late SV40 sequences induced by $Ad2^{+}ND_4$.

To determine whether the SV40-specific RNA induced by $Ad2^{++}$LEY in the first 24 h of infection might contain additional sequences not present in an $Ad2^{+}ND_4$ infection, e.g., late sequences transcribed from the SV40 (+) strand, we employed unlabeled RNA from cells infected with $Ad2^{+}ND_4$ against ^{3}H-labeled $Ad2^{++}$LEY RNA from a 24-h infection. The $Ad2^{+}ND_4$ RNA competed efficiently with the $Ad2^{++}$LEY RNA (Fig. 4), indicating that $Ad2^{++}$LEY induces no SV40 RNA sequences not induced by $Ad2^{++}ND_4$.

All of these data indicate that, whereas Ad2-$^{++}$HEY virus induces the synthesis of SV40-specific sequences complementary to the entire SV40 genome almost as efficiently as does wild-type SV40 itself, the $Ad2^{++}$LEY virus is very inefficient in the first 24 h of infection, inducing transcripts that appear to be representative of only a portion of the early region of the SV40 genome. Certain of the competition-hybridization experiments were repeated by treating the initial labeled RNA-SV40 DNA hybrid with RNase and by extensive washing, followed by another RNase-washing treatment after the second stage of hybridization. The results obtained (data not shown) did not differ from those observed with the one-step RNase treatment, unlike the variable results obtained by Lucas and Ginsberg with Ad2 RNA-DNA hybrids (23). Although the competition-hybrid-

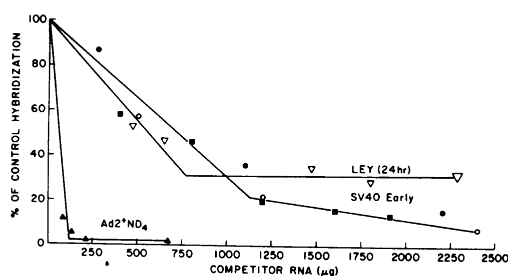

FIG. 3. *Hybridization-competition of unlabeled SV40 early RNA or LEY RNA (24-h infection) with $Ad2^{+}ND_4$ [^{3}H]RNA. Hybridization-competition experiments were performed with a saturating amount of [^{3}H]RNA and 0.1 μg of ^{14}C-labeled SV40 DNA filters. All conditions as indicated in the legend to Fig. 2. Input of $Ad2^{+}ND_4$ [^{3}H]RNA = 73 μg; 63 × 10^3 counts/min per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 10. Control [^{3}H]RNA counts per minute bound (only uninfected, unlabeled Vero cell RNA present) = 229. Three different batches of unlabeled SV40 early RNA were employed, as indicated by the different symbols (■, ○, and ●).*

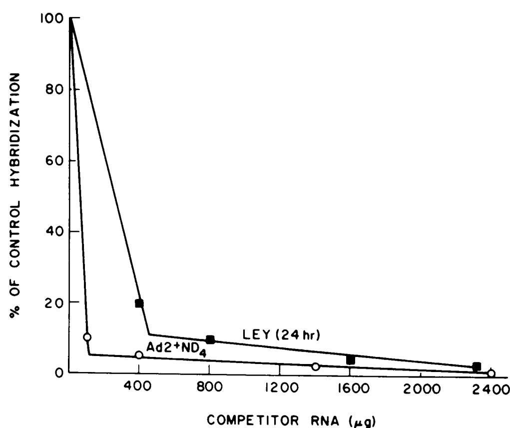

FIG. 4. *Hybridization-competition of unlabeled $Ad2^{+}ND_4$ RNA with LEY [^{3}H]RNA (24-h infection). Hybridization-competition experiments were performed with a saturating amount of [^{3}H]RNA and 0.1 μg of ^{14}C-labeled SV40 DNA filters. All conditions as indicated in the legend to Fig. 2. Input of LEY [^{3}H]RNA = 273 μg: 70 × 10^3 counts/min per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 23. Control [^{3}H]RNA counts per minute bound (only uninfected, unlabeled Vero cell RNA present) = 77.*

ization experiments reported here do not measure directly the strand orientation and extent of transcription, the results are consistent with previous data from competition-hybridization experiments involving Ad2^{+}ND$_{4}$ RNA and early or late SV40 RNA (18) and with hydroxyapatite chromatography experiments involving separated strands and fragments of SV40 DNA hybridized to Ad2^{+}ND$_{4}$-specific RNA (13).

Induction of SV40-specific antigens by the Ad2^{++}HEY and Ad2^{++}LEY hybrid populations. Since the SV40-specific RNA present in cells lytically infected by Ad2^{++}LEY appeared to represent less of the early SV40 region than SV40-specific RNA induced by Ad2^{+}ND$_{4}$, it was of interest to compare the pattern of early SV40 antigen induction in AGMK cells infected by Ad2^{++}HEY and Ad2^{++}LEY. A previous report (21) demonstrated that both early SV40 T and late SV40 V antigens were present in AGMK cells infected with Ad2^{++}HEY, whereas only SV40 T antigen was detected in Ad2^{++}LEY-infected cells. SV40 U antigen induction by these hybrids has not been studied previously. To determine whether an interruption of SV40 U antigen synthesis was associated with the inefficient yielding of SV40 in Ad2^{++}LEY infected cells, the ability of both hybrid populations to induce SV40 U and T antigens was compared.

Both hybrid populations induced serologically detectable quantities of heat-labile SV40 T and heat-stable SV40 U antigens during lytic infection of AGMK cells (Table 1). To determine whether SV40 T and U antigens are induced by input hybrid DNA or newly synthesized nonhybrid SV40 DNA, it was necessary to compare Ad2^{++}HEY antigens prepared with and without cytosine arabinoside. In Ad2^{++}HEY-infected cells, detectable levels of SV40 T and U antigen were produced in the presence of 20 μg of cytosine arabinoside per ml. Similar concentrations of cytosine arabinoside have been shown to inhibit SV40 DNA synthesis by 99% in Vero cells and produce a greater than 100-fold reduction in the number of AGMK cells containing SV40 V antigen detectable by immunofluorescence (27). Thus, SV40 U and T antigen induction by Ad2^{++}HEY would appear to be associated with the input hybrid genome. These experiments do not rule out, however, the induction of SV40 T and U antigens from excised (but unreplicated) SV40 genomes. It should be noted that Ad2^{++}HEY failed to induce SV40 V antigen detectable by complement fixation in this experiment. However, low titers (2 to 4) of SV40 V antigen have been detected by complement fixation in other Ad2^{++}HEY cell packs, and 1 to 10% of Ad2^{++}HEY-infected cells contain SV40 V antigen by immunofluorescence (21).

DISCUSSION

The Ad2^{++}HEY and Ad2^{++}LEY virus populations are of interest because they might serve as useful models for analyzing the properties of integrated SV40 DNA. In another report (10), we have described anatomical features of the Ad2^{++}HEY and Ad2^{++}LEY hybrid genomes that appear to explain why Ad2^{++}HEY-infected cells yield SV40 much more abundantly than

TABLE 1. *Induction of SV40 antigens by the Ad2^{++} HEY and Ad2^{++} LEY populations*[a]

Antigen	Heat treatment (50 C, 30 min)	Antigen titer by CF, hamster sera				
		SV40 $T^{+}U^{+}$	SV40 $T^{+}U^{-}$	Ad2 T	SV40 V	Ad2 V
Ad2^{++}HEY	−	≥64	≥64	≥64	0	≥64
	+	32	0			
Ad2^{++}HEY + CA	−	32	32	≥64	0	4
	+	8	0			
Ad2^{++}LEY	−	32	16	≥64	0	≥64
	+	8	0		0	
SV40	−	32	32	0	64	
	+	8	0		64	
Ad2^{+}ND$_{1}$	−	32	0		0	128
	+	32	0		0	
Ad2 + CA	−	0	0	≥64	0	0

[a] Ad2^{++}HEY, Ad2^{++}LEY, and SV40 cell pack antigens were prepared in AGMK cells. Ad2^{+}ND$_{1}$ and Ad2 cell pack antigens were prepared in human embryonic kidney cells. Where indicated, Ad2^{++}HEY and Ad2 cell pack antigen were prepared in the presence of 20 μg of cytosine arabinoside per ml. Complement fixation (CF) titer is expressed as the reciprocal of the highest antigen dilution giving 3+ fixation (0 = <2).

cells infected with Ad2^{++}LEY. The majority of hybrid virions in the Ad2^{++}HEY population contain SV40 DNA segments that are either 1.43 or 2.39 SV40 units in length, whereas the majority of Ad2^{++}LEY hybrid virions contain an SV40 DNA segment that is 1.05 SV40 units in length. The extra SV40 DNA in each of these segments is arranged in a tandem repetition. The large tandem repetition of SV40 DNA in the Ad2^{++}HEY genome creates many possibilities for excision of an intact SV40 genome from the hybrid molecule, whereas few such possibilities exist with the very small tandem repetition in the Ad2^{++}LEY genome. With the increased probability of excision of a unit length of SV40 DNA, replication of SV40 progeny in cells singly infected with hybrid particles should be greatly increased. The results of the present experiments appear to confirm this expectation, since there was no detectable synthesis of nonhybrid SV40 DNA (forms I and II) during Ad2^{++}LEY infection. However, such molecules were detected in the DNA of Ad2^{++}HEY-infected cells by hybridization with SV40 complementary RNA. These findings also tend to exclude the possibility that the difference in SV40 yielding by Ad2^{++}HEY and Ad2^{++}LEY is due to a defect in a post-DNA replication event such as viral coat protein synthesis or encapsidation. It is possible that SV40 DNA is excised with equal efficiency from both hybrid genomes but replicates more efficiently in the case of Ad2^{++}HEY. However, selective suppression of free SV40 DNA replication in the case of Ad2^{++}LEY is unlikely, since inhibition of SV40 replication in a mixed Ad2-SV40 infection is an Ad2 function (5) and both the Ad2^{++}HEY and Ad2^{++}LEY hybrid populations consist primarily of nonhybrid Ad2 virions (21). Thus, one would expect this late Ad2 function to be expressed in cells infected with either hybrid.

In contrast to the 10^6 to 10^7 PFU of SV40 virions per ml produced by Ad2^{++}HEY hybrid particles during low-multiplicity infection, the yield of nonhybrid SV40 virions in high-multiplicity infection (without SV40 antiserum) is about 10^4 PFU/ml (21). Therefore, it is not surprising that alkaline gradients (Fig. 1A) failed to demonstrate nonhybrid SV40 DNA synthesis in this high-multiplicity Ad2^{++}HEY infection until sensitive hybridization techniques were employed. Because the amount of nonhybrid SV40 DNA detectable in Ad2^{++}HEY infection is small, one cannot exclude the possibility that some component of nonhybrid SV40 DNA synthesis originates from input SV40 that was not neutralized by SV40 rabbit antiserum.

The failure of Ad2^{++}LEY to induce efficiently the synthesis of late SV40 RNA transcripts, despite the presence in the hybrid particle of the entire SV40 genome, is similar to two other situations in which the expression of integrated SV40 DNA has been studied. In SV40-transformed cells, viral-specific RNA is transcribed primarily (and in certain lines exclusively) from the (−) strand of SV40 DNA and, therefore, represents only early or anti-late SV40 templates even though late templates (+ strand) may be present (12, 30). This same pattern of transcription has been found in cells acutely infected with the nondefective Ad2-SV40 hybrid viruses Ad2$^+$ND$_1$ − Ad2$^+$ND$_5$ (13), where the SV40-specific transcripts are complementary only to the (−) strand of SV40 DNA despite the presence of late (+ strand) templates within the SV40 moiety of these hybrids (28, 29). Thus, it seems likely that, unless SV40 DNA is in a nonintegrated form (as in Ad2^{++}HEY-infected cells after excision), expression of the late genes (+ strand) is limited or absent. Additional information on this point might be obtained by determining the sedimentation value of SV40 RNA in Ad2^{++}HEY-infected cells and by determining whether the SV40 RNA is covalently linked to Ad2 RNA.

Although the Ad2^{++}LEY hybrid fails to induce all of the sequences comprising early SV40 RNA, it induces SV40 T and U antigens in AGMK cells at levels comparable to those in Ad2^{++}HEY infection. Thus, these missing sequences do not appear to be associated with the induction of SV40 T and U antigens. They could, however, be associated with the induction of other early SV40 antigens such as transplantation antigen or with some unrecognized SV40 function. Preliminary studies have demonstrated that Ad2^{++}LEY fails to induce detectable SV40 transplantation antigen in a hamster assay (A. M. Lewis, Jr., unpublished data). This hybrid does induce SV40 transplantation antigen in a mouse assay, whereas the Ad2^{++}HEY hybrid induces SV40 transplantation antigen in both hamster and mouse (S. S. Tevethia and A. M. Lewis, Jr., unpublished data). Moreover, although the competition-hybridization experiments suggest that 24-h Ad2^{++}LEY RNA does not contain a complete compliment of early SV40 RNA sequences, this apparent lack may be quantitative. The overriding fact is that Ad2^{++}LEY does yield complete SV40 virions, albeit in low number, indicating that all RNA needed for virus maturation is synthesized. The apparent failure of early RNA expression is, therefore, unlikely to ac-

count for the truncated appearance of late functions.

To summarize, permissive infection with Ad2++HEY gives rise to early and late SV40 antigens, early and late SV40 RNA, and nonhybrid SV40 progeny. In contrast, cells permissively infected with Ad2++LEY contain only early SV40 antigens, primarily early RNA, and only very small quantities of nonhybrid SV40 virions. These findings are consistent with the following concepts. (i) Transcription and translation of the entire SV40 genome requires the replication of nonintegrated SV40 DNA (12, 13, 30). (ii) Replication of nonintegrated SV40 DNA will not occur in Ad2++LEY or Ad2++HEY infection in the absence of SV40 excision from the hybrid genome. (iii) Excision will be infrequent unless a sufficiently large segment of SV40 DNA is present in a tandem repetition within the hybrid (10). Certain aspects of transformation by SV40 resemble lytic infection by Ad2++LEY rather than Ad2++HEY, in that transformed cells contain only integrated SV40 genomes, only early SV40 antigens, and primarily early RNA (1, 12, 26, 30, 32, 33). Given these comparisons, further studies on lytic infection by the Ad2++HEY and Ad2++LEY hybrids may help to further detail the manner in which integration and excision of viral genomes influence the ultimate course of viral replication in cells infected with SV40.

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