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Replication of Adeno-Associated Virus in Synchronized Cells without the Addition of a Helper Virus

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We investigated the helper-independent replication of adeno-associated virus (AAV) in cells synchronized by pretreatment with hydroxyurea, reversal of polyamine depletion, or physical mitotic detachment. In Chinese hamster cells (OD4 line) treated with hydroxyurea prior to infection, AAV underwent a complete cycle of replication. Transfection of such cells with plasmid-cloned AAV DNAs also gave rise to infectious viral progeny. Synchronization of OD4 cells by reversal of polyamine depletion or mitotic detachment led to independent AAV DNA synthesis (and infectious viral progeny in the case of the former procedure), but these procedures were not as effective as hydroxyurea pretreatment. Independent AAV DNA synthesis was also detected in some other cell lines of Chinese hamster, human, and monkey origin treated with hydroxyurea prior to infection. The results demonstrate that, in contrast to previous notions, the AAV infectious process is not absolutely dependent upon the addition of a coinfecting helper virus.

The parvoviruses contain a single-stranded DNA genome, of approximately 5,000 nucleotides, characterized by palindromic ends (for reviews, see references 2, 7, 8, 11, 18, 45, and K. I. Berns and M. A. Labow, J. Gen. Virol., in press). Traditionally, parvoviruses have been divided into two major groupings, depending upon their requirement for a helper virus. The autonomous group is generally capable of undergoing a complete infectious cycle in the absence of a helper virus, provided that viral DNA synthesis is initiated when the host cell is in the S phase of the cell cycle (18, 45). In contrast, the replication of the helper-dependent adeno-associated virus (AAV) (the name derives from its isolation as a contaminant of adenovirus stocks [24]) requires the presence of a coinfecting virus which can be a member of either the adenovirus or the herpesvirus family (2, 8). Very recently, coinfection with vaccinia virus has also been reported to aid the replication of AAV (41).

The mechanism by which adenovirus type 2 (Ad2) early-gene expressions assist the replication of AAV is unclear (8; Berns and Labow, in press). Furthermore, the taxonomic diversity of the DNA viruses which act as helpers raises the possibility that it is the helper virus-induced modulation of cellular functions which satisfies some of the special requirements for AAV replication. The first indication that AAV replication is not absolutely dependent upon coinfection with an added helper virus was provided by zur Hausen and colleagues, who discovered that simian virus 40 (SV40)-transformed Chinese hamster and human cells support AAV DNA and antigen synthesis after treatment with carcinogens or low concentrations of cycloheximide (20, 41, 42). Concomitant with the acquisition of the capacity to support AAV macromolecular synthesis, the replication of the endogenous SV40 genome was induced or enhanced. Previously, it had been demonstrated that chemical and physical carcinogens induce the replication of chromosomally integrated SV40 DNA and enhance the permissiveness of Chinese hamster cells to exogenous SV40 infection (28, 29). However, the replication of AAV DNA is not dependent upon the concomitant induction of endogenous SV40 DNA replication, since Chinese hamster cells transformed by replication-deficient (*ori*$^-$) SV40 mutant DNA will also support AAV DNA replication after treatment with cycloheximide or UV irradiation (B. Yakobson and E. Winocour, unpublished data).

Agents such as chemical carcinogens, UV irradiation, and cycloheximide, which transiently interfere directly or indirectly with cellular DNA synthesis, lead to gene amplification and chromosomal rearrangements after the recovery of the cell from the block (40). The acquisition of the capacity to support independent AAV replication might be associated with changes in cellular gene expression resulting from specific chromosomal rearrangements or amplification or both. However, DNA-damaging agents (and cycloheximide) also interfere with the normal progression of the cells through the cell cycle. Given the known link between the replication of the autonomous parvovirus group and the S phase of the host cell, it seemed feasible that independent AAV replication might also be associated with a specific phase of the cell cycle. In the present contribution, we show that in cell populations treated with hydroxyurea (by using conditions under which the majority of cells are arrested in the S phase prior to infection), a small proportion of cells will support complete AAV replication, giving rise to infectious virus progeny. The complete cycle of helper-independent AAV replication also ensues after transfection of hydroxyurea-pretreated cells with molecularly cloned AAV DNA. Furthermore, we show that cells synchronized by reversal of polyamine depletion or by physical mitotic detachment also acquire the ability to support independent AAV or AAV DNA replication. These observations reinforce the notion that AAV is much more similar to the autonomous parvovirus group than was previously considered. At the same time, the varied response of different cell lines, together with the relatively low proportion of synchronized cells converted to productive AAV infection, imply, as will be discussed below, that not all the intracellular requirements for independent AAV replication have been resolved.

MATERIALS AND METHODS

Plasmids and cell lines. The pSM620 (38, 39) and pAV2 (27) plasmid constructs containing the AAV type 2 genome were kindly provided by K. I. Berns and B. J. Carter, respectively. The Chinese hamster embryo line OD4, transformed

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by SV40 *ori*$^-$ DNA (29), was kindly provided by S. Lavi. CHO-K_1 cells (26) originated from the American Type Culture Collection, Rockville, Md., and the CHO 41-DD derivative, which carries chromosomally integrated and amplified SV40 *ori*$^-$ DNA, was a gift of H. L. Ozer. The SV40-transformed human newborn kidney line NB-E (30) was a gift of J. Rommelaere, and the 293 human embryonic kidney cells (transformed by Ad5 DNA; 15) were a gift of Y. Gluzman, who also provided the SV40 *ori*$^-$ DNA-transformed monkey cells of the COS7 line (14). Monkey CV1 and human HeLa cells originated from the American Type Culture Collection. Cell lines were grown at 37°C in Dulbecco modified Eagle medium (DME) with 10% fetal calf serum, except for the proline-auxotrophic CHO cells, which were cultured in 50% DME–50% Ham F12 medium supplemented with 10% fetal calf serum.

Viruses and antisera. Ad2 (from the American Type Culture Collection) was grown on HeLa cells and extracted and purified on CsCl density gradients as described previously (10). The purified virus, stored at −20°C in 50% glycerol, had a titer of 10^{10} PFU/ml by plaque assay on HeLa cells (52).

To prepare molecularly cloned AAV stocks (27, 38, 39), 10^7 HeLa cells were transfected with 1 μg of pSM620 DNA by using DEAE-dextran (33) as described elsewhere (13). After transfection, the cells were infected with Ad2 (20 PFU per cell) and incubated as a monolayer culture. At 40 to 48 h later, when Ad2-induced cytopathic effects were complete, the cells were lysed by freeze-thawing and the lysate was used to infect 30 9-cm plates of HeLa cells grown to 80% confluency. At 40 to 48 h later, the cultures were lysed by freeze-thawing and the pooled lysates were concentrated by centrifugation in the Spinco 35 rotor at 30,000 rpm for 3 h. Virus was extracted from the pellets by the deoxycholate-trypsin method (3, 4), and the AAV was separated from Ad2 by three consecutive cycles of equilibrium centrifugation in CsCl density gradients, as described previously (3). Finally, the AAV was dialyzed and heated at 56°C for 30 min to inactivate any residual Ad2 contamination. The titer of the purified AAV was 10^{11} infectious units/ml as measured by the dilution endpoint–quick-blot hybridization procedure or 3×10^{10} focus-forming units/ml as measured by the fluorescent-antibody staining method (see below).

Antiserum to AAV was prepared in a Hartley strain guinea pig inoculated three times with 10^{10} infectious units (30 μg of protein) of purified AAV in the presence of Freund complete adjuvant (first inoculation) and Freund incomplete adjuvant (second and third inoculations). The animal was bled twice, and the immunoglobulin G fraction was purified by ammonium sulfate precipitation and DEAE-52 ion exchange chromatography. The AAV antiserum displayed no detectable neutralizing activity against Ad2.

Cell cycle synchronization procedures and AAV infection. Prior to infection, cells were synchronized by hydroxyurea treatment (1, 37), reversal of polyamine depletion (19, 32), or mitotic detachment (1, 49). In the hydroxyurea pretreatment schedule, the cells were seeded (10^6 cells per 9-cm dish) in full medium supplemented with freshly prepared 1 mM hydroxyurea (Sigma Chemical Co., St. Louis, Mo.) and incubated for 36 to 40 h in the presence of the drug. The cells were then washed twice with medium, AAV at 1 or 20 infectious units/cell was adsorbed for 90 mins, the unadsorbed virus was removed, and the cells were again washed twice and refed with normal full medium. In the reversal-of-polyamine-depletion schedule, the cells were plated at the above density in full medium containing 5 mM α-difluoromethylornithine (DFMO; Merrell Dow Research Center, Cincinnati, Ohio), incubated for 96 h, infected with AAV as above, and refed with full medium supplemented with 10 μM putrescine (Sigma). To synchronize cells by mitotic detachment, subconfluent cultures in T75 flasks were refed with medium lacking serum, the flasks were tapped sharply (five times on each side), and the cells dislodged into the medium were collected. After addition of serum to 10%, the detached cells were replated at a density of 2×10^5 per 5-cm dish and incubated for 1 h. Unattached cells were removed by a medium change, and after a further incubation for 1 h, the attached cells were infected with AAV.

To determine the proportion of cells in the G1 and S phases of the cell cycle, samples of the synchronized cell populations were stained with propidium iodide (final concentration, 50 μg/ml in 0.1% Triton X-100) and analyzed by flow microfluorimetry (1) in a fluorescence-activated cell sorter (FACS II; Beckton-Dickinson and Co., Mountain View, Calif.).

AAV DNA synthesis. At different times after infection, extrachromosomal DNA was isolated from 10^6 cells, and the AAV replicative form (RF) DNAs were analyzed by Southern blot (48) hybridization with AAV [^{32}P]DNA. Briefly, low-molecular-weight DNA was isolated by the Hirt procedure (23) with the pronase digestion modification (34). The Hirt supernatant fraction was further deproteinized by two extractions with chloroform containing 2% isoamyl alcohol. The nucleic acids were collected by two successive precipitations with ethanol and suspended in TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). Prior to restriction enzyme digestion (performed under the conditions recommended by the manufacturers), RNA was degraded by RNase treatment. The DNAs were fractionated by electrophoresis on 1.4% agarose–Tris acetate gels (40 V, 16 to 18 h), transferred to nitrocellulose, and hybridized with AAV [^{32}P]DNA, as described previously (53). AAV [^{32}P]DNA was labeled by nick translation (36) of the pSM620 construct or the AAV DNA excised from pSM620 by *Sma*I digestion (39). For use as gel blot hybridization markers, single-stranded AAV DNA was isolated from purified virions as described previously (3), and the double-stranded RF DNAs were isolated from HeLa cells, 30 h after coinfection with Ad2 and AAV, by the procedure described above. Autoradiographic exposures are indicated in the figure legends.

AAV DNA synthesis was also monitored by the sodium iodide–quick-blot procedure of Bresser et al. (6). The AAV-infected cell pellets were lysed by proteinase K digestion (E. Merck AG, Darmstadt, Federal Republic of Germany), mixed with 0.81 volume of hot supersaturated sodium iodide (prepared by adding 3.5 ml of water to 12.5 g of sodium iodide and heating the mixture for 1 h at 90 to 95°C), and incubated at 90 to 95°C for 10 min. While still above 65°C, 100-μl samples (equivalent to an extract of 50,000 cells) were rapidly filtered through nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by using a slot template device. The device was assembled by clamping together the template (a piece of Perspex containing 24 slots of dimensions 10 by 2 by 10 mm), the nitrocellulose paper (presoaked in 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), a single sheet of 3MM paper (Whatman, Inc., Clifton, N.J.), a 5-mm-thick wad of adsorbent paper, and a plastic backing plate. The nitrocellulose filter was then briefly washed in 70% ethanol. Residual protein was acetylated by soaking the filter for 15 min in a solution prepared by adding 1.5 ml of triethanolamine (BDH, Poole, England) and 0.25 ml of acetic anhydride to 100 ml of water. For hybridization, the filter was incubated for 4 h at 42°C in a solution

containing 50% formamide, 5× SSC, 5× Denhardt buffer (12), 50 mM Na_3PO_4 (pH 7.0), and 100 μg of denatured salmon sperm DNA and then for 16 to 18 h at 42°C in the same solution supplemented with 400,000 cpm of AAV [^{32}P]DNA per ml. After hybridization, the filter was washed at room temperature with a solution containing 0.1% sodium dodecyl sulfate and 2× SSC (four 15-min washes) and then at 68°C with a solution containing 0.1% sodium dodecyl sulfate and 0.1× SSC (two 30-min washes). The hybridization results were scored by autoradiography and by excising the slot blots and measuring ^{32}P radioactivity in a scintillation counter.

Titration of AAV infectivity. The following method is based upon determination of the dilution endpoint for infectivity in Ad2-coinfected HeLa cells by the above quick-blot hybridization procedure of Bresser et al. (6). A 96-well microtitration plate was seeded with 50,000 HeLa cells per well. After 2 to 3 h of incubation for attachment, the cells in each well were inoculated with 100-μl portions containing 10^6 PFU of Ad2 (20 PFU per cell) and serial 10-fold dilutions of AAV in medium with 2% serum. At 28 h postinfection, 100 μl of 4 mM EDTA in TE buffer was added, and the cells were lysed by two cycles of freeze-thawing and proteinase K digestion (100 μg/ml at 37°C overnight). The lysates from each well were then mixed with 0.81 volume of hot supersaturated sodium iodide, and the DNA was immobilized on nitrocellulose (extract of 14,000 cells per slot) and hybridized (42°C in 50% formamide) as above. At the dilution endpoints, the results first scored by autoradiography were confirmed by excising the slot blots and measuring ^{32}P radioactivity in a scintillation counter. Control wells included in each microtitration plate contained HeLa cells inoculated with Ad2 alone, HeLa cells inoculated with AAV alone, and uninfected cells. The AAV infectious unit is defined as the highest dilution of the stock which generates a specific hybridization signal after passage in Ad2-coinfected HeLa cells.

AAV was also titrated by a fluorescent-focus assay (9). HeLa cells, grown on glass cover slips, were infected with Ad2 and serial dilutions of AAV, as above. At 30 h postinfection, the cells were washed, fixed for 30 min at room temperature in 3% paraformaldehyde–0.05% Triton X-100, incubated for 2 h at room temperature with anti-AAV guinea pig immunoglobulin G, and then stained by incubation for 1 h at room temperature with fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin G (Bio-Yeda, Rehovot, Israel). The cells were scored for nuclear fluorescence by using a Leitz microscope equipped with UV epifluorescent optics. Titers were calculated from the proportion of fluorescent nuclei, the number of cells, and the AAV dilution factor, as described elsewhere (9). The AAV titers determined by the fluorescent-focus assay were generally threefold lower than those determined by the dilution endpoint–quick-blot hybridization procedure.

Number of cells synthesizing AAV nucleic acids. The infected cells, trypsinized and suspended in 10 ml of phosphate-buffered saline at an appropriate concentration, were trapped on nitrocellulose by filtration under suction. The filters, cell side up, were placed for 1 min on top of Whatman 3MM paper saturated with 0.5 N NaOH containing 1.5 M NaCl. The filters were blotted dry, and the alkali denaturation step, with intermittent drying, was repeated twice more. Finally, the filters were neutralized by three 1-min contacts with Whatman 3MM paper saturated with 1 M Tris hydrochloride (pH 7.0) and 2× SSC, baked at 80°C for 2 h under vacuum, and hybridized at 68°C with AAV [^{32}P]DNA as described previously (53). The number of autoradiographic spots (see below) was directly proportional to the number of cells plated, indicating that each spot represents a cell synthesizing AAV nucleic acids.

DNA transfection. Subconfluent Chinese hamster OD4 cells (3×10^6 cells per 9-cm dish) were washed with DME alone and inoculated with 1 ml of solution containing DME, 50 mM Tris hydrochloride (pH 7.3), 500 μg of DEAE-dextran per ml, and 0.5 μg of the indicated AAV-containing plasmid DNAs per ml. After 20 min at room temperature (with frequent redistribution of the inoculum), the cultures were washed twice with DME supplemented with 10% fetal calf serum and refed with the same medium.

RESULTS

Helper-independent replication of AAV in hydroxyurea-pretreated Chinese hamster OD4 cells. The OD4 line of Chinese hamster embryonic cells, established by transformation with SV40 *ori*$^-$ mutant DNA incapable of replication (14, 29), was chosen for these experiments to avoid the possibility that the incoming AAV DNA might acquire an SV40 replication origin by recombination (16, 17). A second prerequisite was to confirm that the purified AAV stocks were free of infectious Ad2. Accordingly, HeLa and 293 cells were infected with dilutions of the purified AAV preparations and incubated for 8 days; no cytopathic effects

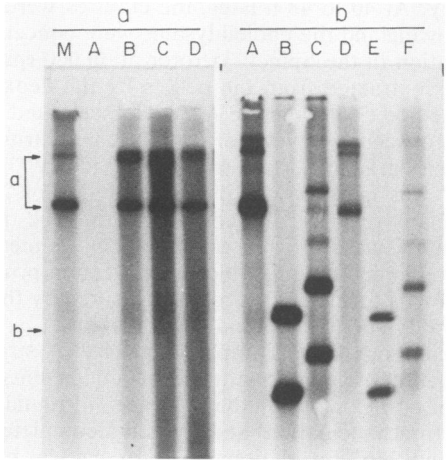

FIG. 1. Replication of AAV DNA in hydroxyurea-pretreated Chinese hamster OD4 cells. Cells grown for 36 to 40 h in the presence of 1 mM hydroxyurea were washed and infected with AAV (20 IU per cell; 10^6 cells per culture). After 1.5 h for virus adsorption, the inoculum was removed and the cells were washed and refed with medium. At the time intervals noted below, extrachromosomal DNA was isolated, electrophoresed (1/5 of the extract of 10^6 cells per slot), and blot-hybridized to AAV [^{32}P]DNA. (a) Time course. Lanes: A, 0.5 h; B, 4 h; C, 8 h; D, 22 h after virus adsorption; M, AAV DNA markers. Lane M shows the positions of single-stranded virion DNA (arrow b) and the double-stranded monomeric and multimeric RF DNAs (arrows a) isolated from AAV-Ad2 coinfected HeLa cells. (b) Restriction digests. Lanes A, B, and C show, respectively, AAV marker RF DNAs (isolated as above) undigested or digested with *Pst*I (only the 2,690- and 1,767-base-pair fragments are visible) or *Hin*dIII (2,793- and 1,882-base-pair fragments); lanes D, E, and F show, respectively, the undigested, *Pst*I digestion, and *Hin*dIII digestion patterns of DNAs isolated from hydroxyurea-pretreated OD4 cells at 10 h after AAV infection. Autoradiographic exposures were 16 h at room temperature (panel a) and 44 h at room temperature (panel b).

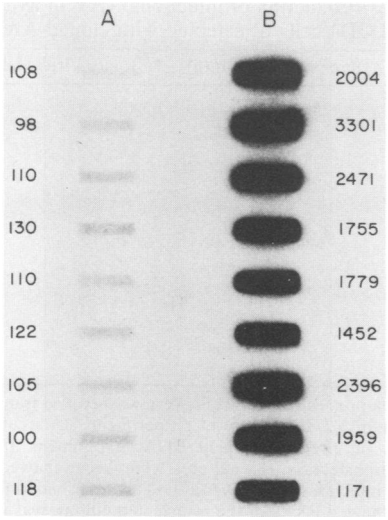

FIG. 2. AAV DNA replication in hydroxyurea-pretreated subclones of the OD4 line. Nine recloned populations of the parental OD4 line were pretreated with hydroxyurea and infected with AAV at 1 IU per cell. At 0.5 h (A) and 9 h (B) after virus adsorption, the DNA content of 50,000 cells was immobilized on nitrocellulose by the sodium iodide–quick-blot procedure described in Materials and Methods. After hybridization with AAV [^{32}P]DNA and autoradiography (20 h at −70°C in the presence of an intensifying screen), the individual slot blots were excised and their radioactivity was determined by scintillation counting (the numbers refer to ^{32}P cpm).

developed, and no Ad2 DNA was detected by quick-blot hybridization of the cell extracts with Ad2 [^{32}P]DNA.

To investigate the synthesis of AAV DNA in hydroxyurea-pretreated OD4 cells, extrachromosomal DNA was isolated from the infected cells and analyzed by blot hybridization with AAV [^{32}P]DNA. At the end of the 1.5-h period of virus adsorption (henceforth called postinfection), the only species of AAV DNA visible was the single-stranded DNA from the infecting virions (Fig. 1a, lane A). Starting at 4 h postinfection, double-stranded AAV monomeric and multimeric RF DNAs (8, 18) were detected (lane B); the formation of these DNA species was maximal at 8 h (lane C). Restriction digestion with *Pst*I (Fig. 1b, lane E) or *Hin*dIII (lane F) generated AAV-specific fragments which comigrated with those obtained by digestion of the AAV RF marker DNAs isolated from HeLa cells coinfected with Ad2 (Fig. 1b, lanes B and C). No AAV RF DNA formation was detected when the hydroxyurea pretreatment was omitted (not shown in Fig. 1, but see Fig. 5). Using the sodium iodide–quick-blot procedure which selectively immobilizes the DNA content of whole cells on nitrocellulose (6), we also examined the levels of AAV DNA synthesis in 20 single-cell clones derived from the parental OD4 population. The results with nine of these recloned populations are shown in Fig. 2. Without exception, all of the 20 cell clones examined supported the synthesis of AAV DNA after hydroxyurea pretreatment. Hence, AAV DNA replication is not confined to an OD4 subpopulation which is either genetically distinct or latently infected with a helper virus.

We next asked whether the synthesis of AAV DNA in hydroxyurea-pretreated OD4 cells leads to the production of infectious virions. To investigate this question, we made use of a microwell plate titration procedure (see Materials and Methods and Fig. 4) based upon coinfection of HeLa cells with a constant amount of Ad2 and serial dilutions of the freeze-thawed extracts of AAV-infected OD4 cell cultures. The production of infectious AAV virions was monitored in terms of the level of free virus released into the culture medium, total virus (free plus intracellular), and intracellular virus alone. From the results described in Table 1, it is evident that infectious AAV progeny are made in hydroxyurea-pretreated OD4 cells. The number of AAV infectious units recovered at 12 h postinfection (total virus and intracellular virus) was at least 4 orders of magnitude higher than the number recorded at time zero (immediately after removal of unadsorbed inoculum and cell washing). Free virus released into the culture medium accumulated at a slower rate and to a lower level; nevertheless, the titer at 24 h postinfection was at least 2 orders of magnitude higher than that at time zero. In the same experiment, the proportion of OD4 cells synthesizing AAV nucleic acids was determined by the single-cell hybridization assay illustrated in Fig. 3. The proportion of such cells reached a maximum of 3.2% at 12 h postinfection and then decreased (Table 1, % infected cells). The decrease in the proportion of infected cells with time (and the larger-than-expected parallel decrease in the levels of intracellular virus recorded) probably arose from cell lysis: hydroxyurea-pretreated OD4 cell cultures display cytopathic-like effects starting at approximately 24 h postinfection, and the selective killing of carcinogen-treated Chinese hamster cells by AAV has been noted by others (21). At 12 h postinfection, 3.2% of 10^6 cells yielded approximately 10^9 infectious units (IU), an average yield of 30,000 IU per cell (Table 1). This value for the yield per cell is probably an underestimate, since the recovery of intracellular virus from freeze-thawed cell suspensions is unlikely to be complete.

Production of infectious AAV in hydroxyurea-pretreated OD4 cells transfected with AAV plasmid DNAs. When bacterial plasmid constructs containing intact double-stranded AAV DNA are transfected into Ad2-infected HeLa or 293 cells, the AAV genome is efficiently rescued and a complete infectious cycle ensues (27, 38). Since the AAV terminal repeat sequences are absolutely required, as a *cis*-active element, for the initiation of DNA replication (39, 44), excision of the genome from the plasmid vector must be precise if it is to be biologically competent. Whether this excision activity is related to an Ad2 function or to a cellular function (or to both) is an open question (Berns and Labow, in press). It was therefore of interest to determine whether

TABLE 1. Growth of AAV on hydroxyurea-pretreated OD4 cells[a]

Time postinfection (h)	Free virus (IU/10^6 cells)	Total virus (IU/10^6 cells)	Intracellular virus (IU/10^6 cells)	% Infected cells
0	$<10^5$	$<10^5$	$<10^5$	0
4	$<10^5$	$<10^5$	$<10^5$	0.9
12	10^5	10^9	10^9	3.2
24	10^7	10^9	10^9	1.2
48	10^7	10^9	10^7	0.6

[a] Hydroxyurea-pretreated OD4 cells were washed and infected with purified AAV (1 IU per cell). At the time intervals noted, a sample of the culture medium was centrifuged at low speed and the supernatant was assayed for infectious AAV (free virus); any sedimented cells or cell debris were then added back to the culture, which, after being freeze-thawed six times, was assayed for infectious AAV (total virus). In parallel, cells infected as above were trypsinized, centrifuged, suspended in phosphate-buffered saline, and counted; samples of 1,000 cells each were plated on nitrocellulose filters and processed (see text and Fig. 3) for the number of cells synthesizing AAV nucleic acids (% Infected cells). Samples of 10^6 cells per ml were also freeze-thawed in phosphate-buffered saline and titrated for infectious AAV (intracellular virus). See text for the assay and definition of infectious units (IU).

transfection of hydroxyurea-pretreated OD4 cells with AAV plasmid DNAs might lead to the production of infectious progeny virus in the absence of Ad2 coinfection.

Pretreated OD4 cells were transfected with the following AAV-containing plasmid DNA preparations. The pAV2 construct, which contains the duplex AAV genome, inserted into a unique *Bgl*II plasmid DNA site via *Bgl*II linkers (27). This preparation was transfected both as a complete circular plasmid DNA and after in vitro cleavage with *Bgl*II, which excises the AAV genome in an intact form except for one-half of the *Bgl*II recognition sequence, which remains attached to the viral genome (27). The second plasmid construct was pSM620, which contains duplex AAV DNA inserted into a plasmid *Pst*I site by GC tailing (38). Because of the presence of three internal *Pst*I sites in the AAV genome (3), intact AAV DNA could not be obtained by in vitro enzymatic cleavage; hence the pSM620 DNA was transfected only in the circular plasmid DNA form. The yields of infectious AAV virions at different times after DNA transfection, measured both by the titration procedure described above and by fluorescent-antibody staining, are shown in Table 2. The highest yields were obtained by transfecting *Bgl*II-cut pAV2 DNA. Much lower yields of infectious AAV resulted from the transfection of uncleaved pAV2 DNA or pSM620 DNA. A higher level of AAV production after transfection with *Bgl*II-cleaved pAV2 DNA has also been observed in 293 cells coinfected with Ad2 (27). Nevertheless, the results with the uncleaved pAV2 and pSM620 DNA transfections show that hydroxyurea-pretreated OD4 cells contain an activity which, in the absence of Ad2, excises the AAV DNA insert in a form that gives rise to infectious virus.

TABLE 2. Production of infectious AAV in hydroxyurea-pretreated OD4 cells transfected with cloned AAV DNAs[a]

Expt	Time postinfection (h)	Virus yield (IU/10^6 cells)
1	1	0
	25	10^5
	48	10^7
	71	10^{7d}
	71[b]	0
	71[c]	0
2	1	0
	48	10^5
3	1	0
	48	10^4

[a] Hydroxyurea-pretreated OD4 cells were washed and transfected with 0.5 μg of *Bgl*II-cut pAV2 DNA per ml (experiment 1), circular pAV2 DNA (experiment 2), or circular pSM620 DNA (experiment 3). At the times indicated after transfection, the cultures were freeze-thawed six times, and their content of infectious AAV was titrated as described in the text (Fig. 4).

[b] The freeze-thawed lysate of the transfected culture was incubated for 30 min at room temperature with a 1:20 dilution of AAV antiserum before titration (Fig. 4).

[c] DNA was omitted from the transfection mixture.

[d] 4×10^6 focus-forming units per 10^6 cells as measured by fluorescent-antibody staining.

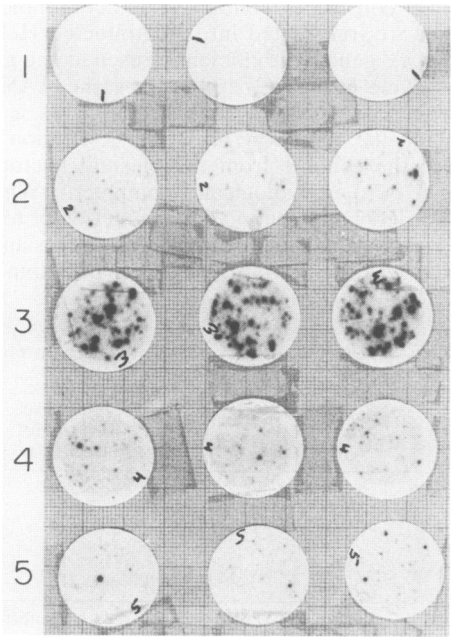

FIG. 3. Assay of the number of hydroxyurea-pretreated OD4 cells synthesizing AAV nucleic acids. Cultures of pretreated OD4 cells were infected with AAV at 1 IU per cell. At 0.5 h (row 1), 4 h (row 2), 12 h (row 3), 24 h (row 4), and 48 h (row 5), triplicate samples of 1,000 cells each were trapped on nitrocellulose filters and processed for hybridization with AAV [^{32}P]DNA as described in Materials and Methods. Autoradiographic exposure was 44 h at −70°C (with an intensifying screen). The photograph shows the autoradiogram superimposed upon the filters that were hybridized.

The experiment described in Table 2 was accompanied by several important controls. First, the infectivity of the yields of the transfected OD4 cells was neutralized by AAV antiserum (Table 2, footnote *c*; Fig. 4b, rows D to F) by using conditions under which the antiserum did not affect the replication of the helper Ad2 in the HeLa cell titration procedure (Fig. 4a, rows D to F). Second, the freeze-thawed lysates of the transfected OD4 cells, when passaged in HeLa cells (in the absence of added Ad2), gave rise to no detectable levels of Ad2 DNA synthesis as judged by sensitive hybridization of the HeLa cell extracts with Ad2 [^{32}P]DNA; thus, chance contamination of the OD4 lysates with Ad2 is excluded. Third, passage of the freeze-thawed lysates of the transfected OD4 cells onto a fresh culture of hydroxyurea-pretreated OD4 cells (under virion infection conditions) gave rise, at 24 h postinfection, to a 10-fold increase in the number of AAV infectious units, all of which were neutralized by AAV antiserum. These results provide strong supporting evidence that a complete AAV infectious cycle can occur in hydroxyurea-pretreated OD4 cells in the absence of a helper virus.

AAV replication in cell lines of other origin. The helper-independent synthesis of AAV DNA in hydroxyurea-pretreated cells is not confined solely to the OD4 line of Chinese hamster embryonic origin (Fig. 5). Human cells transformed by SV40 (NB-E line) or Ad5 DNA (293 line) supported the synthesis of AAV RF DNA (Fig. 5a, lanes A to F); no such synthesis was detected in the absence of the hydroxyurea pretreatment (Fig. 5a, lanes G and H). Human NB-E cells also gave rise to infectious AAV progeny; 10^8 IU/10^6 cells at 10 h postinfection compared with $<10^4$ IU/10^6 cells at 0.5 h postinfection. The ability of NB-E cells to support AAV DNA and protein synthesis after postinfection treatment with a carcinogen has been reported previously (41). With cells of monkey origin, hydroxyurea-pretreated cells of the SV40 *ori*$^-$ DNA-transformed COS7 line supported AAV RF DNA synthesis (Fig. 5b, lanes A to C), in contrast to the parental CV1 line (Fig. 5b, lanes E to G).

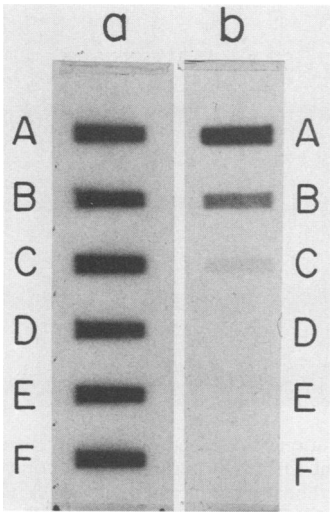

FIG. 4. Titration of AAV infectious units by dilution endpoint–quick-blot hybridization. HeLa cells growing in a 96-well microtitration plate (50,000 cells per well) were infected with 100-μl samples of medium containing 10^6 PFU of Ad2 (all wells) and a 10^{-3} (rows A and D), 10^{-4} (rows B and E), or 10^{-5} (rows C and F) dilution of the 48-h freeze-thawed lysate of hydroxyurea-pretreated OD4 cells transfected with pAV2-*Bgl*II DNA (Table 2). Prior to infection, inocula D, E, and F were incubated for 30 min at room temperature with a 1:20 dilution of AAV antiserum. At 28 h after infection, the DNA content of 14,000 cells was immobilized on nitrocellulose by the sodium iodide–quick-blot procedure (see Materials and Methods). Blot "a" was hybridized with Ad2 [^{32}P]DNA; blot b was hybridized with AAV [^{32}P]DNA. The slot blots hybridized with Ad2 [^{32}P]DNA were excised, and their radioactivity was measured by scintillation counting; all contained 26,000 ± 3,000 cpm of ^{32}P. Blot a was autoradiographed for 1 h at room temperature; blot b was autoradiographed for 17 h at −70°C (with an intensifying screen).

However, the synthesis of AAV RF DNA was not confined to cells bearing a known chromosomally integrated viral genome; for example, the spontaneously transformed CHO line of Chinese hamster ovary origin responded just as well as its 41-DD derivative, which carries integrated SV40 *ori*$^-$ DNA (Fig. 5c). The number of cells synthesizing AAV nucleic acids varied among the different cell lines. For example, under the same conditions of hydroxyurea pretreatment and multiplicity of AAV infection, only 0.3% of CHO cells were positive, compared with 3% of OD4 cells (data not shown). Possible reasons for the varied responses of different cell lines are discussed below.

Helper-independent AAV replication and the cell cycle. As noted above, when hydroxyurea treatment was omitted prior to infection, AAV RF DNA synthesis was not detected. Hydroxyurea, a drug which inhibits ribonucleotide reductase (50), is widely used in cell cycle synchronization procedures. The arrest of the cycle occurs at the G1/S boundary or in the S phase, depending upon the concentration of the drug, the time of exposure, and the type of cells (1). To determine the effect of hydroxyurea on the cell cycle distribution of OD4 cells, the flow microfluorimetry (FACS) analyses shown in Fig. 6 was carried out. When OD4 cells were plated and grown in the presence of 1 mM hydroxyurea for 36 h (the standard pretreatment schedule prior to AAV infection), approximately 90% of the cells were found to be arrested in the S phase of the cell cycle (Fig. 6a). After removal of hydroxyurea and reincubation of the cells in normal medium, the majority of OD4 cells remained in the S phase for at least another 24 h (Fig. 6b and c). The retention of Chinese hamster cells in the S phase after prolonged exposure to hydroxyurea has also been observed by others (S. Lavi and Y. Berko, personal communication). These data, taken together with the time course data in Tables 1 and 2, suggest that the entire AAV infectious cycle occurred in cells arrested in the S phase of the cell cycle (but see Discussion). Since hydroxyurea is also known to be a DNA-damaging agent which induces chromosomal rearrangements in animal cells (22, 40, 50), the question arose of whether the efficacy of the hydroxyurea pretreatment in promoting helper-independent replication of AAV is due solely to its cell cycle-synchronizing properties. To answer this question, we used two other synchronizing procedures which are considered to be nongenotoxic: reversal of polyamine depletion and mitotic detachment.

Depletion of the intracellular polyamine content by growing cells in the presence of 5 mM DFMO, an inhibitor of

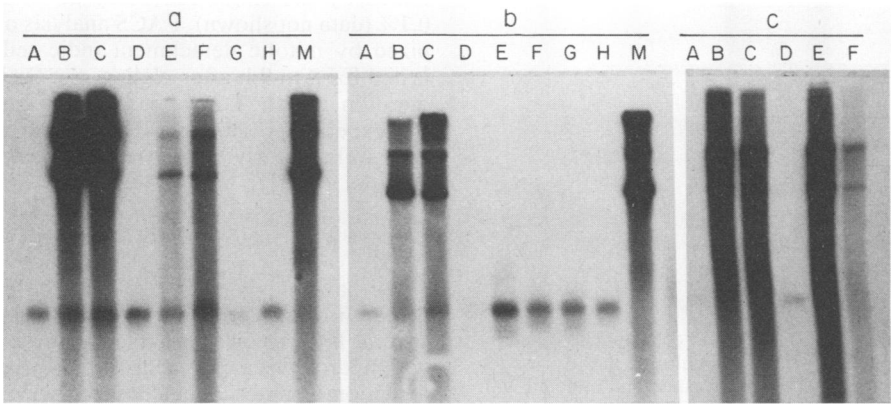

FIG. 5. Replication of AAV DNA in cell lines of human (a), monkey (b), and Chinese hamster (c) origin. Cultures of 10^6 hydroxyurea-pretreated or untreated cells were infected with AAV (20 IU per cell), and at the times noted below, AAV DNA replication was assayed as for Fig. 1. (a) NB-E cells at 0.5 h (lane A), 10 h (lane B), and 24 h (lane C); 293 cells at 0.5 h (lane D), 10 h (lane E), and 24 h (lane F); NB-E (lane G) or 293 (lane H) cells without hydroxyurea pretreatment, extracted at 24 h postinfection; AAV RF DNA markers (lane M). (b) COS7 cells at 0.5 h (lane A), 7 h (lane B), 24 h (lane C), and 24 h without hydroxyurea pretreatment (lane D); CV1 cells at 0.5 h (lane E), 7 h (lane F), 24 h (lane G), and 24 h without hydroxyurea pretreatment (lane H); AAV RF DNA markers (lane M). (c) CHO cells at 0.5 h (lane A), 8 h (lane B), and 24 h (lane C); CHO 41-DD cells at 0.5 h (lane D), 8 h (lane E), and 24 h (lane F). Autoradiographic exposures were 17 h (panel a), 24 h (panel b), and 8 h at −70°C with an intensifying screen (panel c). The sources of the cell lines are given in Materials and Methods.

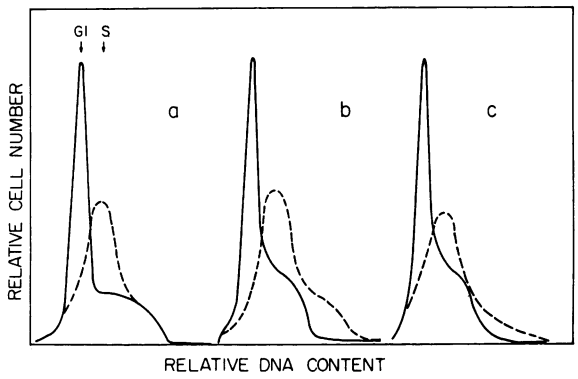

FIG. 6. Cell cycle analyses of hydroxyurea-treated OD4 cells. OD4 cells were plated and grown in the presence of 1 mM hydroxyurea for 36 h; the drug was then removed, and the cells were washed and incubated in normal medium. The broken line shows the relative proportions of the hydroxyurea-treated cells in the G1 and S phases of the cell cycle, as determined by FACS analysis, immediately after removal of the drug (a) or after 12 h (b) or 24 h (c) of incubation in normal medium. The solid line shows the FACS analyses of a population of OD4 cells that were plated and grown to 80% confluency in the absence of hydroxyurea. A total of 10,000 to 20,000 cells were analyzed in each case.

ornithine decarboxylase (35), arrests cells in the G1 phase or at the G1/S boundary of the cycle; the arrest is readily reversable by removal of the drug and reincubation of the cells in medium containing 10 μM putrescine (32, 35). OD4 cells synchronized by the DFMO-putrescine method supported the synthesis of AAV RF DNA (Fig. 7). Furthermore, infectious AAV progeny was produced: approximately 10^6 IU/10^6 cells at 24 h postinfection compared with 10^4 IU/10^6 cells at 0.5 h postinfection. Compared with the hydroxyurea pretreatment schedule, the peak of AAV RF DNA synthesis occurred at a later time (cf. Fig. 1a and Fig. 7), and the yields of AAV infectious units were about 10-fold lower. FACS analysis of OD4 cells grown for 96 h in medium containing 5 mM DFMO confirmed that the cells were arrested in the G1 or G1/S boundary phase. Upon reversal with putrescine, the majority of cells moved into the S phase within 4 h; by 10 h, a substantial fraction had progressed into the G2 phase; and by 24 h, the flow microfluorimetry pattern was indistinguishable from that of randomly cycling cells. In contrast to the situation with the hydroxyurea treatment, we observed no cytotoxicity with the DFMO-putrescine schedule, and the cells progressed normally through the cycle.

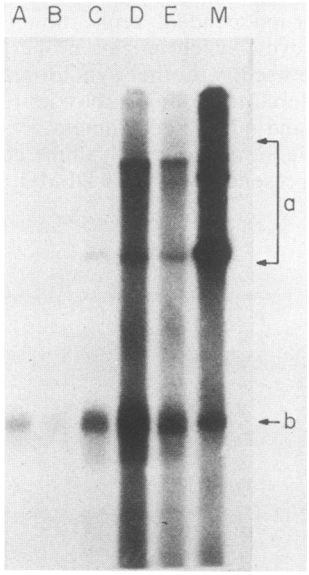

FIG. 7. Replication of AAV DNA in OD4 cells synchronized by reversal of polyamine depletion. OD4 cells, cultured in the presence of 5 mM DFMO for 96 h (see text), were washed and infected with AAV at 20 IU per cell. After virus adsorption, the cells were refed with medium containing 10 μM putrescine. At 4 h (lane A), 8 h (lane B), 12 h (lane C), 24 h (lane D), and 32 h (lane E) postinfection, AAV DNA replication was assayed as for Fig. 1. Lane M shows AAV marker RF DNAs (arrows a) and single-stranded virion DNA (arrow b). Autoradiographic exposure was 27 h at −70°C (with an intensifying screen).

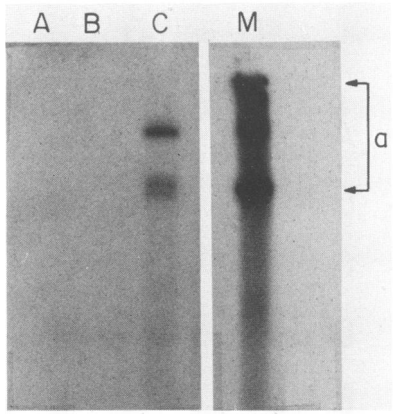

FIG. 8. Replication of AAV DNA in OD4 cells synchronized by mitotic detachment. OD4 cells harvested by mitotic detachment (see Materials and Methods) were infected with AAV (20 IU per cell) 2 h after plating. At 0.5 h (lane A), 10 h (lane B), or 24 h (lane C) postinfection, AAV DNA replication was assayed as for Fig. 1 (DNA extract of 10^5 cells per slot). Lane M shows AAV RF DNA markers (arrows a). Autoradiographic exposure was 17 h at −70°C (with an intensifying screen).

Synchronization of OD4 cells by physical mitotic detachment (a procedure which avoids the use of all drugs) (1, 49) also led to the synthesis of AAV RF DNA (Fig. 8). The percentage of cells synthesizing AAV nucleic acids, determined by the single-cell assay at 36 h postinfection, was 0.1% (data not shown). FACS analysis of OD4 cells synchronized by mitotic detachment indicated that the G1 phase lasted for 8 to 9 h after plating, the S phase was of 3 to 4 h duration, and the G2/M phase was of 2 h duration. Hence, in the experiment shown in Fig. 8, the mitotically detached cells were in early G1 phase at the time of AAV infection.

DISCUSSION

We have shown that AAV, in the absence of an added helper virus, can replicate its DNA in established cell lines of several host species, provided that the cells are pretreated with hydroxyurea or synchronized by reversal of polyamine depletion or mitotic detachment. AAV DNA synthesis culminated in the production of infectious progeny virus in Chinese hamster OD4 cells and in human NB-E cells, and a complete infectious cycle ensued after transfection of hydroxyurea-pretreated OD4 cells with plasmid-cloned AAV DNAs. Thus, in contrast to older conceptions (2, 8, 11), the completion of the AAV infectious cycle is not

absolutely dependent upon coinfection with a helper virus. Indeed, the present results underscore the increasing awareness (Berns and Labow, in press) that the *Parvovirus* (autonomous) and *Dependovirus* (helper-dependent) genera of the *Parvoviridae* family (46) share many more common features than previously recognized and should, perhaps, be viewed as a single genus.

It has been known for many years that the replication of the autonomous parvoviruses is optimal in rapidly growing cells, and a link between the onset of viral DNA replication and the S phase of the host cell cycle has been demonstrated (18, 45). Under the conditions of hydroxyurea treatment used in the present study, at least 90% of the OD4 cells were found to be arested in the S phase prior to infection. Of the total treated cell population, about 3% replicated AAV. Because the proportion of cells converted to AAV production was small and because it was difficult to demonstrate that all of the cells were in the S phase prior to infection, our results do not establish that independent AAV replication is confined to S-phase cells. Nevertheless, in view of the known effects of hydroxyurea on the cell cycle (1, 22, 40), we think it highly likely that AAV replication, like that of the autonomous parvoviruses, is strongly dependent upon events that occur in the S phase of the cell cycle. It is probably not fortuitous that the conditions of the hydroxyurea pretreatment which we find optimal for independent AAV replication are essentially identical to those used to synchronize cells for the replication of the autonomous bovine parvovirus (37). Like the situation with bovine parvovirus (37), cellular DNA polymerase α (25) is involved in the independent replication of AAV DNA, as judged by sensitivity to aphidicolon (B. Yakobson and E. Winocour, manuscript in preparation). There are also striking similarities in the time course of the infectious cycle. In hydroxyurea-pretreated Chinese hamster OD4 or human NB-E cells, AAV DNA synthesis occurs as early as 4 h after the end of the 90-min virus adsorption period and reaches a maximum level at 8 to 12 h, when infectious progeny virus can be detected. The replication of various autonomous parvovirus DNAs, in synchronized cells, starts within a similar time interval after infection (18, 51); and in synchronized HeLa cells, LuIII progeny virus begins to accumulate at about 10 h after infection (47).

Hydroxyurea (like cycloheximide, UV irradiation, and chemical carcinogens) is one of a variety of agents that transiently interfere with cellular DNA synthesis such that upon reversal of the block, overreplication of the cellular DNA (gene amplification) and chromosomal rearrangements can occur (22, 40). The question therefore arose of whether the hydroxyurea pretreatment, which we used in the present experiments to arrest the cells in the S phase prior to AAV infection, might promote AAV replication as a side effect of its ability to induce chromosomal rearrangements or changes in cellular gene expression or both. To help clarify this issue, we made use of other cell synchronization procedures. Independent replication of AAV DNA and the production of infectious progeny occurred in OD4 cells synchronized by putrescine reversal of DFMO-induced polyamine depletion. In contrast to the hydroxyurea pretreatment (which prolongs the S phase and is accompanied by cytotoxicity involving approximately 50% of the treated cells), the DFMO-putrescine method results in no discernable levels of cytotoxicity and allows the cells to progress through the entire cycle. Reversal of DFMO-induced polyamine starvation, however, has been reported to affect cellular differentiation, as judged by the conversion of mouse fibroblasts into adipocytes (5); therefore, indirect effects of the DFMO-putrescine cell synchronization schedule cannot be rigorously excluded. To some extent, synchronization of cells by physical mitotic detachment circumvented the problem of drug side effects in preparing the cell for helper-independent AAV replication. On the other hand, the efficacy of the mitotic detachment procedure was much lower than that of the hydroxyurea method; even though the efficiency of synchronization was high, the number of mitotically detached OD4 cells supporting AAV DNA synthesis was 30-fold lower than the number of hydroxyurea-pretreated cells supporting AAV DNA synthesis. Thus, although cell synchronization is required, other factors seem to contribute to the number of cells which acquire the ability to replicate AAV. Perhaps the efficacy of the hydroxyurea pretreatment is linked to the extensive prolongation of the S phase, which allows the accumulation of cells in a state transiently permissive for AAV.

It should be stressed that the number of hydroxyurea-pretreated OD4 cells rendered permissive for AAV DNA synthesis (approximately 3%) is at least 10-fold lower than the number supporting AAV replication after coinfection with Ad2, as judged by comparing the intensities of Southern blots of viral DNA extracted from cells (data not shown). Ad2 coinfection is still, by far, the most efficient method of replicating AAV. The precise nature of the Ad2 helper functions is unclear, in particular the question of whether the helper activity represents a direct exploitation by AAV of an Ad2-encoded product or an indirect consequence of the Ad2 infection of the host cell (8; Berns and Labow, in press). The present experiments do not address the question of why Ad2 coinfection is so much more efficient in supporting AAV replication; however, it may be relevant, in this context, that Ad2 infection enhances the expression of some S-phase-dependent cellular genes (31).

Several of the cell lines that supported AAV replication after hydroxyurea pretreatment (Chinese hamster OD4, human NB-E, monkey COS7) were originally established by SV40 transformation; and the observation that monkey CV1 cells did not support AAV DNA synthesis, in contrast to the SV40-transformed COS7 derivative, might suggest a role for the integrated SV40 genome in the acquisition of AAV permissiveness. However, the analysis of other cell lines showed that the presence of integrated SV40 DNA is not absolutely required; nor is its presence by itself sufficient for the acquisition of permissiveness. Thus, Chinese hamster ovary cells (CHO-K_1 line) and Ad5-transformed human newborn kidney cells (293 line), neither of which harbors integrated SV40 DNA, supported the synthesis of AAV DNA after hydroxyurea pretreatment, albeit at a lower level than the SV40-transformed OD4 and NB-3 cells. Furthermore, a line of rat embryonic cells transformed by SV40 *ori*$^-$ DNA (the same mutant DNA used to transform OD4 and COS7 cells) has so far failed to support the synthesis of AAV DNA after hydroxyurea pretreatment, even though these cells will replicate AAV after coinfection with Ad2 (Yakobson and Winocour, unpublished data). It seems, therefore, that the ability of the cell to support independent AAV replication depends upon the interplay of additional factors which have yet to be resolved. The results obtained so far suggest that at least some of those factors are linked to S-phase gene expression. The level of synthesis of such factors may well be enhanced in transformed cells, since the transformed phenotype is associated with the activation of certain cellular genes (43). It will now be of interest and importance to analyze in detail the intracellular milieu which

favors AAV replication. Perhaps this analysis will also provide insight into the mechanism by which some parvoviruses limit the growth and spread of tumor cells (11, 45).

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