Mechanism of Host Restriction of Adenovirus-associated Virus Replication in African Green Monkey Kidney Cells

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(Accepted 7 December 1978)

SUMMARY

Human adenovirus (Ad) serotypes provide an early factor(s) that is necessary for adenovirus-associated virus (AAV) multiplication in human cell lines. However, little, if any, AAV production occurs in primary African green monkey kidney (AGMK) cells co-infected with AAV and a helper human Ad (non-permissive infection), unless cells are additionally infected with SV40 (permissive infection). To determine the basis of the host restriction of AAV replication in AGMK cells, AAV DNA, RNA and protein synthesis were analysed under various conditions of infection. Hybridization reactions revealed no detectable AAV-specific DNA or RNA in infections with AAV alone or in combination with SV40. In co-infections with AAV and Ad5 or Ad7, the synthesis of both AAV- and Ad-specific DNA and RNA occurred without a significant rise in titre of either virus. During non-permissive infection, however, AAV DNA synthesis was abnormal in that an expected accumulation of single-stranded progeny molecules was not observed. Finally, although intact 20S AAV transcripts were present in the cytoplasm of AGMK cells during non-permissive infection (in amounts ranging from 50 to 80% of that found during permissive infection), AAV-specific polypeptides were not demonstrable by polyacrylamide gel electrophoresis. Taken together, these experiments indicate that the host restriction of AAV replication in AGMK cells is exerted at the level of translation of the single AAV messenger RNA. In addition, it appears that one or more of the AAV polypeptides specified by this message is required for the production of single-stranded AAV progeny DNA.

INTRODUCTION

The growth of adenovirus-associated virus (AAV), a defective parvovirus, is unconditionally dependent upon co-infection with a helper adenovirus (Ad) (Atchison et al. 1965). Experiments in which human cells were co-infected with AAV and DNA-minus temperature-sensitive (ts) mutants of Ad5 have indicated that the Ad helper effect is mediated by an early Ad gene function(s), possibly expressed at the onset of Ad DNA synthesis (Straus et al. 1976a). Since AAV multiplication proceeded when Ad DNA synthesis was blocked, AAV replication would also be expected to commence under conditions where Ad replication is restricted subsequent to initiation of Ad DNA synthesis. A restriction of this type occurs when primary African green monkey kidney (AGMK) cells are infected with human adenoviruses (O’Conor et al. 1963). Although T-antigen (Malmgren et al. 1966) and Ad DNA (Reich et al. 1966) and RNA (Reich et al. 1966; Baum et al. 1968) are synthesized, little
infectious virus is produced. This host restriction can be overcome by co-infection with SV40 (Rabson et al. 1964). It is therefore interesting to note that when AGMK cells were co-infected with a human Ad and AAV, AAV multiplication was not demonstrable, i.e. AAV structural protein was not detectable by fluorescent antibody staining (Blacklow et al. 1967). This suggests that, contrary to expectation, AAV replication did not commence. The present study was undertaken to characterize AAV macromolecular synthesis during Ad co-infection of AGMK cells. Our results indicate that (i) like Ad, AAV replication is restricted in primary AGMK cells, (ii) this restriction is exerted at the translational level and (iii) like Ad, additional infection with SV40 (i.e. triple infection) overcomes the host restriction, and effective multiplication of infectious AAV virions is promoted.

METHODS

Cells and viruses. Primary AGMK cells were grown to confluent monolayers (approx. $8 \times 10^4$ cells/cm$^2$) at 37°C in either 150 or 25 cm$^2$ plastic bottles containing Eagle’s medium supplemented with 5% foetal calf serum. Infections were carried out as indicated with two mean tissue culture infectious dose (TCID$_{50}$) units/cell of heated, CsCl-purified AAV type 2 (AAV; Straus et al. 1976a) and five TCID$_{50}$ units/cell of the wild type (WT) strain of Ad5, a DNA-minus $ts$ mutant of Ad5 ($ts_{125}$; Ensinger & Ginsberg, 1972) or the E46$^-$ strain of Ad7 (Rowe & Baum, 1965). Cultures inoculated with $ts_{125}$ were subsequently maintained at 39.5°C. Friedman et al. (1976) demonstrated that Ad multiplies more efficiently in AGMK cells pre-infected for over 16 h with SV40 than in simultaneously co-infected cells. Therefore, when required, cultures were pre-infected for 24 h with 10 p.f.u./cell of SV40. The indicated duration of infection was measured from the time of addition of AAV or Ad or both. For experiments that required virus titrations, parallel infections of bottles were carried out. Unadsorbed virus was removed after 1 h by a change of medium and pairs of bottles were harvested at 0, 43 and 88 h post infection (p.i.) for virus assays. The remaining bottles were used for labelling DNA and RNA as described below. AAV, Ad5 WT and Ad7 infectivities were assayed in primary human embryonic kidney (HEK) cells (Hoggan et al. 1966).

Labelling and analysis of nucleic acids. Except for the AAV RNA kinetic study, cultures were labelled for 1 h with 12.5 $\mu$Ci/ml of either $^3$H-thymidine or $^3$H-uridine (Schwarz-Mann Inc., Orangeburg, N.Y.) at 16 to 17 h p.i. Cells were harvested by scraping and centrifuging at 600g for 10 min at 4°C. DNA was extracted with phenol (Rose & Koczot, 1972) or by a modification of the Hirt method (Straus et al. 1976a). For the kinetics of RNA synthesis, cells were labelled for 2 h intervals with 75 $\mu$Ci/ml of $^3$H-uridine. Cells were harvested into tris-saline buffer (TSB; 140 mM-tris, pH 7.4, 140 mM-NaCl, 5 mM-KCl, 0.7 mM-Na$_2$HPO$_4$, 5 mM-dextrose), washed once with TSB and then resuspended in isotonic reticulocyte standard buffer (RSB; 20 mM-tris, pH 7.4, 150 mM-NaCl, 1.5 mM-MgCl$_2$) containing 0.5%, v/v, NP-40 (Shell Ltd). After gentle stirring at 4°C for 5 min, the lysate was centrifuged at 11000g for 5 min and the total cytoplasmic RNA purified for hybridization analysis by treatment with proteinase K (Merck, Darmstadt) and phenol (Bloom & Rose, 1978). DNA–RNA (Rose & Koczot, 1971) and DNA–DNA (Warnaar & Cohen, 1966) hybridizations were performed in duplicate on nitrocellulose filter membranes that contained saturating amounts of unlabelled AAV2, Ad7 or SV40 DNA that had been extracted from purified virions. In each experiment, non-specific binding values were determined from reactions with labelled DNA and RNA extracted from uninfected cells. These values have been subtracted and did not exceed 0.1% of added radioactivity for DNA–DNA reactions or
Host restriction of AAV replication

Table 1. Virus yields in AGMK cell cultures

<table>
<thead>
<tr>
<th>Virus infection</th>
<th>(TCID₅₀ units/ml)</th>
<th>AAV₂ titre (TCID₅₀ units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>43 h</td>
</tr>
<tr>
<td>Ad₅</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV₂</td>
<td>10⁴.₅</td>
<td>10⁵.₅</td>
</tr>
<tr>
<td>Ad₅</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV₂</td>
<td>10⁴.₀</td>
<td>10⁷.₀</td>
</tr>
<tr>
<td>SV₄₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad₇</td>
<td>10⁵.₀</td>
<td>10⁵.₀</td>
</tr>
<tr>
<td>AAV₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad₇</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV₂</td>
<td>10⁵.₀</td>
<td>10⁸.₀</td>
</tr>
<tr>
<td>SV₄₀</td>
<td></td>
<td></td>
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</tbody>
</table>

* Not done.

0-01 % of added radioactivity for DNA–RNA reactions. Velocity sedimentations of Hirt supernatants through 5 to 30 % neutral sucrose gradients were performed as before (Straus et al. 1976a).

For analysis of AAV mRNA, AGMK cells were co-infected with AAV and Ad₅ (with and without SV₄₀ pre-infection) and were labelled with 75 µCi/ml of ³H-uridine from 20 to 26 h p.i. Cells were then washed once with TSB and resuspended in 0.1 ml of RSB supplemented with 0.5 % NP-4₀ and 20 units/ml of heparin. After agitation on ice for 5 min the total cytoplasm was collected by centrifugation and treated with 500 µg/ml of proteinase K for 30 min at 30 °C. This digest was then layered on a 5 to 20 % (w/v) sucrose gradient containing 10 mM-tris (pH 7.4), 10 mM-EDTA, 50 mM-NaCl and 0.2 % SDS and centrifuged at 205600g in a SW41 rotor for 4 h at 20 °C. The fractionated gradient was first assayed for distribution of total radioactivity and then AAV-specific radioactivity was determined in selected fractions by nucleic acid hybridization.

Analysis of virus proteins. At 22 h p.i. AGMK cell monolayers infected with SV₄₀, Ad₅ or AAV in combination or alone were washed twice and recovered with Eagle’s medium reduced tenfold in L-methionine. ³⁵S-L-methionine (25 µCi/ml, Amersham/Searle) was added for a 2 h labelling period and samples then harvested and analysed by gradient polyacrylamide gel electrophoresis (Buller & Rose, 1978a).

RESULTS

Multiplication of AAV

AGMK cells were infected with Ad₅ or Ad₇ and AAV with or without pre-infection with SV₄₀. The infectious yields from these cultures (Table 1) reveal little, if any, multiplication of AAV and adenovirus in the absence of SV₄₀ (non-permissive infection), whereas pre-infection with SV₄₀ led to a 2 to 3 log increase in the infectious yields of AAV, Ad₅ and Ad₇. In control experiments (data not shown) AAV did not replicate in the absence of a helper adenovirus or in cultures co-infected only with SV₄₀. Thus, AAV multiplication in AGMK cells requires both adenovirus and SV₄₀ as helpers (permissive infection).

Virus nucleic acid synthesis

To determine whether or not the AAV genome is activated in the absence of SV₄₀ infection, AGMK cells were co-infected with AAV and Ad₇, with and without SV₄₀ pre-infection, and hybridization reactions were carried out with labelled DNA and RNA...
Table 2. Synthesis of virus-specific DNA and RNA in AGMK cells

<table>
<thead>
<tr>
<th>Virus infection</th>
<th>DNA-DNA hybridizations (\times)</th>
<th>DNA-RNA hybridizations†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA bound to</td>
<td>RNA bound to</td>
</tr>
<tr>
<td>Ad7</td>
<td>Ad7 DNA</td>
<td>AAV2 DNA†</td>
</tr>
<tr>
<td>AAV2</td>
<td>19387</td>
<td>11274</td>
</tr>
<tr>
<td>Ad7</td>
<td>13444</td>
<td>11131</td>
</tr>
</tbody>
</table>

* DNA–DNA hybridizations performed with 4 \(\mu\)g input DNA and 4 \(\mu\)g purified virus DNA bound to nitrocellulose filter membranes.
† DNA–RNA hybridizations performed with 8 \(\mu\)g input RNA and 4 \(\mu\)g purified virus DNA bound to nitrocellulose filter membranes.
†† In infections with AAV alone or AAV plus SV40, ct/min hybridizing with AAV/DNA at most were 50% greater than non-specific binding values of 100 ct/min for DNA–DNA hybridizations and 57 ct/min for DNA–RNA hybridizations.

extracted 17 h after Ad-AAV infection. The data (Table 2) demonstrate clearly that AAV DNA and RNA synthesis do not require SV40 infection. Also, in accordance with earlier observations, synthesis of Ad7 DNA (Reich et al. 1966) and RNA (Baum et al. 1968) was observed with and without SV40 pre-infection. If these reactions are taken to reflect relative quantitative responses, a significant suppression of Ad DNA synthesis (25 to 30%) was seen when cells were pre-infected with SV40. This contrasts with previous results showing either no effect of SV40 pre-infection on Ad DNA synthesis (Friedman et al. 1970) or an approx. twofold increase of Ad DNA synthesis in the presence of SV40 (Reich et al. 1966). The observed suppression of Ad DNA synthesis in our experiment probably relates to the induction of complete AAV replication, since a similar decrease in Ad5 DNA synthesis also was noted when KB cells were additionally infected with AAV (Straus et al. 1976a). SV40 pre-infection did not grossly alter Ad RNA or AAV DNA synthesis, but AAV RNA synthesis appeared to increase. Thus, although there is little or no production of infectious AAV when AGMK cells are co-infected with Ad and AAV, a relatively efficient synthesis of AAV DNA and RNA does occur. In this regard, at least the AGMK cell restriction on both AAV and human Ad multiplication is similar.

AAV DNA replicative intermediates

The intermediates of AAV DNA replication were analysed to determine whether AAV DNA synthesis proceeds normally in AGMK cells co-infected with AAV and Ad. The intermediates of AAV DNA replication in KB cells have been shown to include concatemers composed of covalently linked plus and minus strands (Straus et al. 1976b). In neutral sucrose gradients linear, unit length duplexes sediment at 15S, whereas longer concatemeric forms appear in a broad shoulder between 15S and 21S. Single-stranded plus and minus progeny molecules sediment at 20S. Neutral sucrose gradient profiles of Hirt extracts from Ad7- and Ad7-AAV-co-infected AGMK cells pulsed with \(^3\)H-thymidine 16 to 17 h p.i. are shown in Fig. 1 (a and b). Infection with Ad7 alone yielded a distinct 31S Ad DNA component, whereas co-infection with AAV additionally produced a typical pattern of AAV DNA replicating intermediates. To be sure that the Ad DNA component did not obscure any abnormality of AAV DNA synthesis, the same experiment was repeated with the Ad5 DNA-minus ts mutant, ts 125, as helper. Ts 125 is an effective AAV helper in KB cells at its restrictive temperature (Straus et al. 1976a). As is the case with either Ad7 or the Ad5 WT,
Host restriction of AAV replication

\[31S \quad 21S \quad 16S\]

\[0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]

Fig. 1. AAV DNA replicative intermediates. (a) Ad7-; (b) Ad7-AAV-; (c) \(ts\) 125- and (d) \(ts\) 125-AAV-infected cells were pulsed with \(12.5 \mu\text{Ci/ml of}^3\text{H-thymidine from 16 to 17 h p.i. Hirt extracts were analysed in 5 to 30\% neutral sucrose gradients. Ad2 (31S), SV40 Form I (21S) and SV40 Form II (16S) DNA were used as internal markers.}\)

\(ts\) 125 induces little, if any, infectious AAV production in primary AGMK cells, whereas additional SV40 infection results in normal AAV yields. As demonstrated in Fig. 1 (c and d), after a 1 h \(^3\text{H-thymidine pulse, little Ad DNA synthesis was apparent, but a normal AAV DNA intermediate pattern was obtained with the extract from co-infected cells. However, when the}^3\text{H-thymidine pulse was extended to 6 h, the expected appearance of 20S single-stranded progeny DNA (as observed in KB cells by Straus et al. 1976b) did not occur. Our inability to detect this 20S progeny DNA component strongly suggests that AAV DNA synthesis does not proceed normally in Ad-AAV-co-infected AGMK cells.}\)

AAV transcription

The AAV-specific RNA synthesized under non-permissive conditions (i.e. Ad-AAV co-infection; Table 2) was additionally analysed to determine whether it corresponded in size to the normal AAV mRNA. In KB cells Ad-AAV co-infection yields a single 20S AAV message (Carter et al. 1972; Carter & Rose, 1974) which accounts for all AAV-specified proteins (Buller & Rose, 1978a, b). To see if 20S AAV RNA was transcribed and transported into the cytoplasm of AGMK cells under non-permissive conditions, total cytoplasmic...
Fig. 2. Sedimentation profile of AAV-specific cytoplasmic RNA from permissive and non-permissive infections. Total cytoplasmic RNA from (a) permissive and (b) non-permissive infections was sedimented through 5 to 20% sucrose gradients. Selected fractions were then hybridized with 1 μg of AAV DNA. KB 28 and 18S ribosomal RNA were used as internal markers.

RNA labelled 20 to 26 h p.i. was sedimented in sucrose gradients and profiles of AAV-specific radioactivity were obtained by nucleic acid hybridization (Fig. 2). A major 20S AAV RNA component was synthesized in both the Ad-AAV-SV40 (Fig. 2a) and Ad-AAV (Fig. 2b) infections. The minor, faster sedimenting components seen in either the permissive or non-permissive infection have been observed before (Carter & Rose, 1974), but its basis is still uncertain. Nevertheless, RNA corresponding to normal-sized AAV mRNA was synthesized and transported to the cytoplasm of AGMK cells infected only with Ad-AAV, and the overall sedimentation pattern of AAV-specific RNA from these cells closely resembled that formed during permissive infection.
Host restriction of AAV replication

Fig. 3. Time course of the appearance of AAV-specific RNA in the cytoplasm of AGMK cells after permissive and non-permissive infection. At the indicated times after addition of Ad and AAV, AGMK cells in 25 cm² bottles were labelled with ³H-uridine for 2 h and samples of total cytoplasmic RNA then hybridized with 1 μg of AAV DNA: •—•, permissive infection; ○—○, non-permissive infection.

If ³H-uridine incorporation is taken as a measure of net synthesis in the previous experiment, the quantity of 20S AAV RNA produced in the non-permissive infection amounted to about half that produced in the presence of additional infection with SV40. To find out if this difference was due to a reduction in AAV RNA synthesis or an increased turnover rate in the non-permissive infection, the following experiments were carried out. First, we examined a time course of AAV RNA appearance in the cytoplasm during the virus growth cycle (Fig. 3). No major difference between permissive and non-permissive infections was found, except that less total ³H-uridine incorporation was seen in samples from the non-permissive infection. Secondly, we carried out an RNA pulse-chase experiment with similarly infected AGMK cells. RNA was labelled with ³H-uridine (75 μCi/ml) from 18 to 20 h p.i. and chased as described previously (Carter & Rose, 1974). At the end of a 6 h chase period, 81 and 97% of initial AAV-specific RNA radioactivity was recovered in permissive and non-permissive cell cytoplasmic samples, respectively. The results of the above experiments indicate that relative reductions of AAV-specific RNA observed in the absence of SV40 co-infection (ranging from 20 to 50% in five experiments) are due to reduced synthesis. Therefore, with the exception of a variable reduction in the rate of AAV RNA synthesis in non-permissive infections, there appears to be little difference between permissive and non-permissive infections in either the mode of accumulation or in the size of cytoplasmic AAV RNA species.

AAV protein synthesis

The previously observed absence of AAV-specific immunofluorescence following Ad-AAV infection of AGMK cells (Blacklow et al., 1967) did not rule out synthesis of an immunologically unreactive AAV protein. To determine whether or not a complete
Fig. 4. AAV-specific polypeptides synthesized during permissive and non-permissive infections. AGMK cell cultures infected with (a) SV40, (b) Ad-AAV, (c) Ad-AAV-SV40, (d) AAV-SV40, (e) AAV, (f) no virus, (g) Ad and (h) Ad-SV40 were labelled with 20 µCi/ml of 35S-L-methionine for 2 h at 22 h p.i. A constant quantity of radioactivity (100000 ct/min) from each sample was analysed in 6 to 17 % acrylamide gels. The polypeptide components of purified AAV-2 virions are displayed in track (i).

translational block exists during restrictive infection, AGMK cells infected with various combinations of Ad5, AAV and SV40 were pulsed for 2 h with 35S-L-methionine at 22 h p.i. and the labelled proteins were analysed in gradient polyacrylamide gels (Fig. 4). Track(c), Ad-AAV-SV40 infection, demonstrates synthesis of the four methionine-containing polypeptides (closed circles) identified previously in Ad-AAV infection of KB cells (Buller & Rose, 1978a). SV40 and Ad-specific polypeptides can be identified by comparisons with an SV40 (track a) and an Ad-SV40 infection (track h). The SV40 polypeptides in track (a) are indicated by open circles. Clearly, in the Ad-AAV infection normal AAV polypeptides could not be detected (compare track b with c) nor was any new AAV-specific polypeptide apparent. In addition, when the Ad-SV40 infection (track h) is compared with an infection of Ad alone (track g), certain Ad-specific polypeptides are deficient in the absence of SV40 enhancement. As previously reported by others, synthesis of the late hexon protein as well as the precursor to the major core protein (track h, first and third closed squares, respectively)
is greatly reduced (Baum et al. 1972; Klessig & Anderson, 1975) whereas quantities of the early 72K polypeptide are normal or slightly increased (track h, second closed square).

DISCUSSION

In this study we confirm the previously reported restriction of AAV multiplication in Ad-AAV-co-infected primary AGMK cells (Blacklow et al. 1967). The present work, however, indicates the specific biochemical level at which the host cell restriction operates. Under conditions of non-permissive infection, AAV DNA and RNA are produced but AAV mRNA-directed protein synthesis is not detected. The previous finding that AAV replication proceeded normally in human cell co-infections with AAV and Ad5 DNA-minus ts mutants provided strong evidence that the AAV multiplication requirement(s) is expressed early in adenovirus infection and possibly represented a function needed for initiation of both Ad and AAV DNA synthesis (Straus et al. 1976a). Our observation that production of AAV DNA and RNA occurred in Ad-AAV infections of AGMK cells is therefore consistent with a subsequent host-imposed restriction which very likely has some basic similarity with the AGMK restriction imposed on adenovirus replication. The apparent abnormality in AAV DNA synthesis (i.e. absence of single-strand progeny production) can be explained on the basis of the deficiency in synthesis of AAV structural protein. In the case of the non-defective parvovirus, H-1, Rhode (1976) found that two mutants, which were ts in the capsid protein, synthesized diminished single-stranded progeny DNA at the non-permissive temperature. Furthermore, recent studies in our laboratory have shown that when AAV protein synthesis is inhibited with cycloheximide, AAV DNA synthesis continues but single-stranded progeny DNA does not accumulate (E. Sebring, R. M. L. Buller & J. A. Rose, unpublished observations).

A deficiency of AAV protein synthesis is the obvious direct reason for the abortive AAV cycle in AGMK cells. Because approximations of the relative quantities and the turnover rate of 20S AAV RNA in permissive and non-permissive infections (Table 2 and Fig. 3) do not reveal differences which might reasonably account for the absence of AAV polypeptides in non-permissive infections (Fig. 4, track b), it appears that the underlying cause of impaired AAV protein synthesis resides in a faulty mRNA or in the translational machinery with which the AAV message must couple. Recent experiments that demonstrate comparable in vitro translation capabilities of AAV mRNA obtained from either permissive or non-permissive infections (R. M. L. Buller & J. A. Rose, unpublished observations) indicate that the 20S AAV RNA synthesized in non-permissive infection represents functional AAV mRNA. Host restriction in AGMK cells is thus exerted at the level of translation of the AAV message. Furthermore, it is very unlikely that this block is mediated by interferon, as measured by inhibition of VSV replication, since we were unable to detect any interferon production after infection of AGMK cells with Ad5, SV40 or AAV (R. M. L. Buller, H. Levy & J. A. Rose, unpublished observations).

The inhibition of multiplication of helper human Ad in AGMK cells may also involve a mechanism similar to that which restricts AAV. This implies that the observed host selectivity arises from differences in a specific translational control sequence(s) among certain virus mRNA species. Thus, messages coding for AAV structural protein as well as for several late Ad proteins would depend on an additional requirement to facilitate their translation. In the present case this requirement can be supplied directly or indirectly by the SV40 virus genome.

Technical help provided by Mrs D. Ortt was greatly appreciated.
REFERENCES


(Received 31 July 1978)