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Adeno-Associated Virus Rep Protein Synthesis during Productive Infection

BRUNHILD E. REDEMANN, ELLA MENDELSON, AND BARRIE J. CARTER*

Laboratory of Molecular and Cellular Biology, Building 8, Room 304, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, Maryland 20892

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Adeno-associated virus (AAV) Rep proteins mediate viral DNA replication and can regulate expression from AAV genes. We studied the kinetics of synthesis of the four Rep proteins, Rep78, Rep68, Rep52, and Rep40, during infection of human 293 or KB cells with AAV and helper adenovirus by in vivo labeling with [^{35}S]methionine, immunoprecipitation, and immunoblotting analyses. Rep78 and Rep52 were readily detected concomitantly with detection of viral monomer duplex DNA replicating about 10 to 12 h after infection, and Rep68 and Rep40 were detected 2 h later. Rep78 and Rep52 were more abundant than Rep68 and Rep40 owing to a higher synthesis rate throughout the infectious cycle. In some experiments, very low levels of Rep78 could be detected as early as 4 h after infection. The synthesis rates of Rep proteins were maximal between 14 and 24 h and then decreased later after infection. Isotopic pulse-chase experiments showed that each of the Rep proteins was synthesized independently and was stable for at least 15 h. A slower-migrating, modified form of Rep78 was identified late after infection. AAV capsid protein synthesis was detected at 10 to 12 h after infection and also exhibited synthesis kinetics similar to those of the Rep proteins. AAV DNA replication showed at least two clearly defined stages. Bulk duplex replicating DNA accumulation began around 10 to 12 h and reached a maximum level at about 20 h when Rep and capsid protein synthesis was maximal. Progeny single-stranded DNA accumulation began about 12 to 13 h, but most of this DNA accumulated after 24 h when Rep and capsid protein synthesis had decreased.

The human defective parvovirus adeno-associated virus (AAV) has a single-stranded linear DNA genome of 4.7 kilobases (kb) with inverted terminal repeats of 145 bases at each end (5, 6, 8, 43). Both plus and minus strands can be encapsidated and are capable of initiating genome replication (4, 5, 8, 21, 40). Generally, AAV depends on coinfection with a helper adenovirus or herpesvirus to replicate efficiently (1, 5, 9, 11, 23, 38). In the absence of helper virus coinfection, the AAV genome integrates into the host chromosomes and establishes a latent infection (7, 14, 20, 28). Recent studies showed that AAV can also replicate in the absence of helper virus (41, 52), although at much lower efficiency, in synchronized cells or in cells treated with carcinogens.

Infection of cells with AAV results in conversion of the parental single-stranded (SS) DNA to a parental duplex replicative-form (RF) molecule (11, 29, 38) and then in synthesis of progeny RF monomers, dimers, and larger duplex concatemers (5, 21, 37, 44). The SS progeny DNA is displaced from the double-stranded RFs and associates rapidly with preformed empty capsids (36). Subsequent maturation of stable virus particles takes several hours (36). Biochemical studies (37) and genetic analyses (22, 45) showed that SS DNA does not accumulate if capsid protein synthesis is prevented.

The AAV genome contains two major open reading frames (ORF): ORF-1 in the left half and ORF-2 in the right half of the viral genome (12, 43). Three transcription promoters are used to generate three families of mRNA (12, 19, 30–32) having overlapping sequences and common 3′ termini but exhibiting alternate splicing. Thus, three virus capsid proteins, VP1, VP2, and VP3, are produced from ORF-2 (2, 3, 22, 24, 31, 39, 45, 49), and four noncapsid Rep proteins (Rep78, Rep68, Rep52, Rep40) are produced from ORF-1 (34, 45, 50). Genetic analysis of recombinant AAV vectors having mutations in ORF-1 (22, 42, 45) revealed that this region is essential for viral DNA replication. Additional studies (22, 26, 27, 33a, 46–48) showed that the Rep proteins, encoded by ORF-1, also have two more functions of negative and positive regulation of gene expression.

In the present study, we examined the synthesis of Rep proteins during infection of human KB or 293 cells and correlated it with viral DNA and capsid protein synthesis. In the earliest stage, up to 12 h after infection, duplex RF DNA was first formed and two Rep proteins, Rep78 and Rep52, were synthesized at low levels. Then, between 14 and 24 h, the bulk of all four Rep proteins and the capsid proteins were synthesized and the pool of RF DNA synthesis reached a maximum. Later (after 24 h), AAV protein synthesis was decreased but the bulk of SS DNA accumulated and assembled into mature virus particles. This suggests at least two discrete stages in the AAV growth cycle.

MATERIALS AND METHODS

Cells and viruses. All experiments were performed in human KB cells or in human 293 cells, an established line of adenovirus-transformed human embryonal kidney cells (18). Cells were grown in monolayers at 37°C in 5% CO_2 in Eagle minimal essential medium supplemented with antibiotics, glutamine, and 10% fetal calf serum.

AAV type 2 (AAV2) and human adenovirus type 5 were grown and assayed as described previously (10).

Antibodies. For AAV Rep protein analysis, an antibody raised in rabbits against a truncated Rep protein expressed in *Escherichia coli* (50) was used in immunoblotting experiments and the immunoglobulin G (IgG) fraction of antibody raised in rabbits against the synthetic oligopeptide S18K (34) was used in immunoprecipitations. An anticapsid antibody

* Corresponding author.

against AAV2 that was raised in guinea pigs (a gift from D. Hoggan) was used for immunoprecipitation of capsid proteins, and an anticapsid antibody against AAV2 that was elicited in rabbits (10) was used for immunoblotting.

Infection of cells. One day prior to infection, KB cells or 293 cells were plated at a density of 3×10^5 or 5×10^5 cells, respectively, per 35-mm dish. At the time of infection, cells were 60 to 70% confluent and had approximately doubled in number. Medium was removed, and 2 ml of fresh medium was added. Cells were then coinfected with adenovirus type 5 and AAV2 at multiplicities of 20 infectious units per cell for each virus. After 1 to 2 h of incubation at 37°C in 5% CO_2, the inoculum was removed and fresh medium was added.

Radiolabeling proteins in vivo. To label proteins in KB or 293 cells growing in 35-mm dishes, we replaced the culture medium with 1 ml of fresh methionine-deficient Dulbecco minimum essential medium supplemented with 10% dialyzed calf serum and antibiotics, and cells were incubated for 30 min. [^{35}S]methionine (200 to 360 μCi) was added, and incubation continued for the required labeling period. The [^{35}S]methionine-containing medium was then removed, and the cells were either harvested immediately or chased by the addition of fresh medium containing a 100-fold excess of unlabeled methionine. Cells were harvested by scraping, washed twice with ice-cold phosphate-buffered saline (PBS) in 1.5-ml microcentrifuge tubes, collected by low-speed centrifugation, and stored frozen at −20°C.

Immunoprecipitation. Immunoprecipitation was done in 1.5-ml microcentrifuge tubes essentially as described previously (17). Frozen pellets of 6×10^5 KB cells or 1×10^6 293 cells were thawed quickly by vortexing into 360 μl of lysis buffer (10 mM Tris hydrochloride [pH 8], 10 mM disodium EDTA, 150 mM NaCl, 1% Nonidet P-40, 2% aprotinin, 2 mM phenylmethylsulfonyl fluoride). A 90-μl portion of 10% sodium dodecyl sulfate (SDS) (wt/vol) was added to a final concentration of 2% SDS. The lysate was incubated for 10 min at 37°C, then for 20 min at 60°C, and finally for 10 min at 70°C with occasional vortexing. After cooling to room temperature, 20 μl of the IgG fraction of rabbit preimmune serum was added and incubated in an end-over-end mixer for 20 to 30 min at room temperature. Meanwhile, fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.) were washed in lysis buffer containing 1% ovalbumin and resuspended at 10% (wt/vol) concentration in lysis buffer.

A 200-μl portion of 10% Pansorbin in lysis buffer was then added to the antibody-extract mixture and incubated for 20 min at room temperature in an end-over-end mixer. Nonspecific immune complexes bound to Pansorbin were collected by centrifugation at 12,000 × *g* at room temperature. The supernatant was transferred to a new microcentrifuge tube, and this clearance step was repeated twice with 100 μl of prewashed Pansorbin to remove any remaining nonspecific immune complexes.

The supernatant was finally diluted to 3.6 ml with lysis buffer (final concentration of SDS was 0.25%). A 1-ml sample was incubated with 15 μl of anti-Rep IgG or antiserum for 20 h at 4°C. Prewashed Pansorbin (10%) (150 μl) was then added, and incubation continued at 4°C for 40 min. Specific immune complexes bound to Pansorbin were collected by centrifugation in an Eppendorf microcentrifuge at 4,000 × *g* and 4°C for 3 min. The supernatant was removed and reprecipitated with anticapsid antiserum and Pansorbin.

Pellets of anti-Rep and anticapsid precipitations were washed three times with 1 ml of lysis buffer at 4°C, and the final pellets were suspended in 100 μl of sample buffer (50 mM Tris hydrochloride [pH 7.5], 10 mM disodium EDTA, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1% sodium deoxycholate, 0.2% SDS, 150 mM NaCl). The sample was then mixed with 0.2 volumes of 5× loading buffer (1× loading buffer contains 0.5% SDS, 10% glycerol, 25 mM Tris hydrochloride [pH 7.5], 0.2% bromophenol blue, and 1% 2-mercaptoethanol) and boiled for 5 min and centrifuged for 2 min at 12,000 × *g*. The supernatant (80 to 100 μl) was loaded on an SDS-12% polyacrylamide gel with a 3% stacking gel and electrophoresed at 40 to 60 V for 16 h (34). The gel was stained with Coomassie blue and destained in 30% methanol–6% acetic acid; then it was incubated with 10% glycerol for 60 min and with Enlightning (Dupont, NEN Research Products, Boston, Mass.) for 30 to 60 min, dried, and autoradiographed at −70°C.

Cell extracts for immunoblotting. Cells were harvested by scraping and washed with ice-cold PBS and then lysed in cold lysis buffer (50 mM Tris hydrochloride [pH 8], 1 mM disodium EDTA, 150 mM NaCl, 1% sodium deoxycholate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5% SDS) and passed three times through a 21-gauge needle to shear the cellular DNA.

Immunoblotting. Experiments were performed as described previously (34). Briefly, cell extracts were boiled in loading buffer (see above) and centrifuged at 12,000 × *g*, and the supernatants were electrophoresed on a 12% polyacrylamide-SDS gel. After electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) at 1,000 mA for 1.5 h in transfer buffer (25 mM Tris, 190 mM glycine [pH 8.3], 20% methanol). Membranes were soaked in PBS-Tween 20 (PBS containing 0.5% Tween 20) for 1.5 h and then incubated with rabbit anti-Rep antibody diluted in 3% bovine serum albumin–0.2% α-casein in PBS-Tween 20 for 2.5 h at 37°C. After two 15-min washes with PBS-Tween 20, the membrane was incubated with ^{125}I-labeled goat anti-rabbit IgG (10^5 cpm/ml; Dupont, NEN Research Products) for 1 h at 37°C and then washed twice for 15 min with PBS-Tween 20, dried, and autoradiographed. Some blots were further reacted with rabbit anticapsid antibody and developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, Calif.) to show the capsid proteins.

Extraction of viral DNA. Viral DNA was extracted by a modified Hirt procedure (13). Medium was removed from infected cell monolayers, and 800 μl of lysis buffer (10 mM Tris [pH 8], 1 mM EDTA, 1% SDS) containing 200 μg of proteinase K per ml was added to each 35-mm dish. After 2 h of incubation at 37°C, the lysed cells were put on ice and 200 μl of 5 M NaCl was added slowly. Cellular DNA was precipitated at 4°C overnight and collected by centrifugation at 12,000 × *g* for 20 min at 4°C. A 800-μl sample of the supernatant was removed, extracted with chloroform, precipitated with ethanol–0.3 M sodium acetate, washed once with 70% ethanol, dried, and suspended in 50 μl of TE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA). Samples of 15 μl were used for each analysis and were digested with pancreatic RNase (20 μg/ml at room temperature for 15 min) immediately prior to electrophoresis.

Analysis of viral DNA by Southern blotting. Viral DNA was separated on a 0.8% agarose gel and transferred to nitrocellulose following denaturation in 0.5 M NaOH–1.5 M NaCl and neutralization in 1 M ammonium acetate in the gel. The filter was baked for 2 h at 80°C and hybridized to a random-primed, ^{32}P-labeled AAV DNA at 68°C for 16 to 22 h in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus

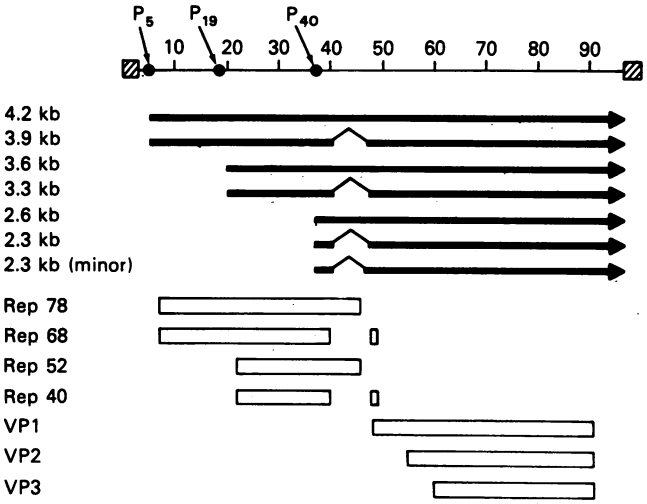

FIG. 1. Diagram of AAV2 DNA, mRNA, and proteins. The 4.7-kb viral genome is shown at the top on a scale of 100 map units. The viral DNA terminal palindromes and inverted repeat sequences are shown as hatched boxes. The three viral transcription promoters p_5, p_{19}, and p_{40} are indicated by solid circles. Viral mRNA species are shown below the genome as solid arrows. The arrowheads indicate the common polyadenylation site, and the caret indicates the intron. The size of each mRNA in kilobases is shown at the left. The intron of the minor 2.3-kb mRNA is shorter by 27 nucleotides than the major intron (49). Viral proteins are illustrated below the mRNA as open boxes. The diagram depicts only the viral proteins identified so far, and the name of each protein is shown at the left side.

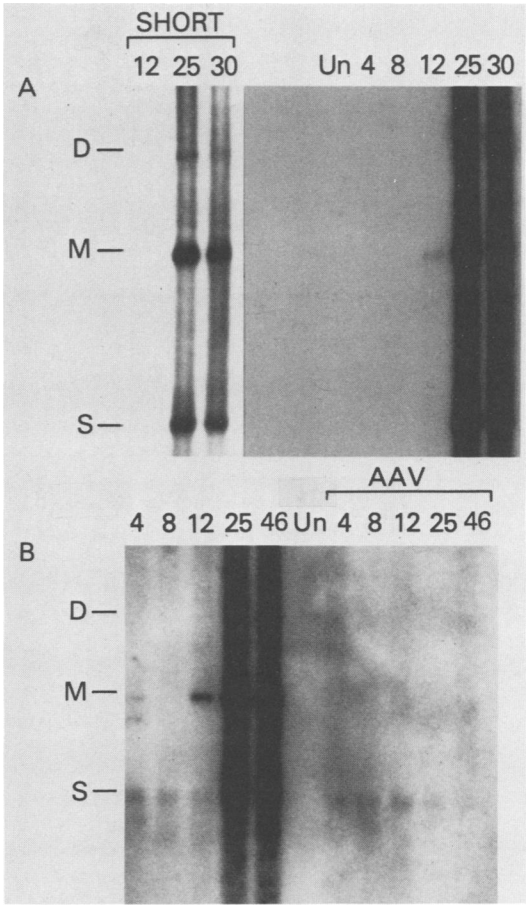

FIG. 2. AAV DNA synthesis in 293 cells (A) or KB cells (B) coinfected with AAV and adenovirus. Viral DNA was extracted at various times after infection and analyzed by Southern blot hybridization with ^{32}P-labeled AAV DNA as the probe. The number above each lane indicates the time (hours after infection) of isolation of each sample. Un, Uninfected cells; AAV, infection with AAV alone; SHORT, autoradiographic film exposure of 2 h; otherwise, films were exposed for 20 h. AAV DNA species are indicated: D, dimer RF; M, monomer RF; S, single strands.

0.015 M sodium citrate), 0.5% SDS, 5× Denhardt solution, 200 μg of salmon sperm DNA per ml, 10 mM EDTA, and 10 mM sodium PP_i. The filter was then washed in 0.2× SSC at 68°C for 15 min, dried at room temperature, and autoradiographed at −70°C. In both the DNA and protein analysis, the relative intensities of various bands in autoradiograms was estimated with a Bio-Rad videodensitometer (model 360) and software supplied by the manufacturer.

RESULTS

Accumulation of AAV proteins and AAV DNA in infected cells. At least four AAV Rep proteins (34, 50) and three AAV capsid proteins (3, 23, 39) are detected in cells infected with AAV. The regions of the AAV genome coding for these proteins are shown in Fig. 1. For the Rep proteins we retained the designations Rep78, Rep68, Rep52, and Rep40 since these were used in previous studies (33, 50) and were apparent molecular weights.

In a previous study (34) in human 293 cells coinfected with AAV and adenovirus, the two major Rep proteins (Rep78 and Rep52) were detected by immunoblotting analysis earlier after infection than the minor proteins (Rep68 and Rep40). In the present study, we first examined the steady-state levels of AAV DNA or AAV proteins in 293 cells at various times after infection with AAV and adenovirus. Viral DNA was isolated from infected cells and examined by Southern blot analysis (Fig. 2A), and protein extracts from the same cells were analyzed by Western blot (immunoblot) analysis (Fig. 3A). In the Western blot (Fig. 3A), Rep78, Rep68, and Rep52 were first detected at 12 h after infection, while Rep40 was first detected only at 25 h. The amount of all four Rep proteins was much higher at 25 h and remained at the same level at 30 h. At 12 h, Rep78 was the most abundant species, but by 25 or 30 h, Rep52 and Rep78 were present in equal amounts and were more abundant than Rep68 and Rep40. The capsid proteins VP1, VP2, and VP3 were readily detected in the same experiment, but not until 25 h after infection (Fig. 3C).

In the Southern blot analysis (Fig. 2A), AAV DNA synthesis was first detected at 12 h as a band of approximately 4.7 kb which corresponds to the monomer RF. At 25 and 30 h, the amount of monomer RF DNA in the cells was greatly increased and double-stranded dimer RF and progeny SS DNA molecules were also present. The experiment in Fig. 2A and Fig. 3A suggests that in 293 cells coinfected with AAV and adenovirus, accumulation of Rep proteins and AAV DNA synthesis followed similar kinetics. They were first detected concomitantly at 12 h after infection and reached maximum levels by 25 to 30 h after infection.

293 cells are adenovirus-transformed human cells constitutively expressing the adenovirus early genes E1a and E1b (18). These adenovirus regulatory genes are required for AAV replication (11), and their constitutive accumulation

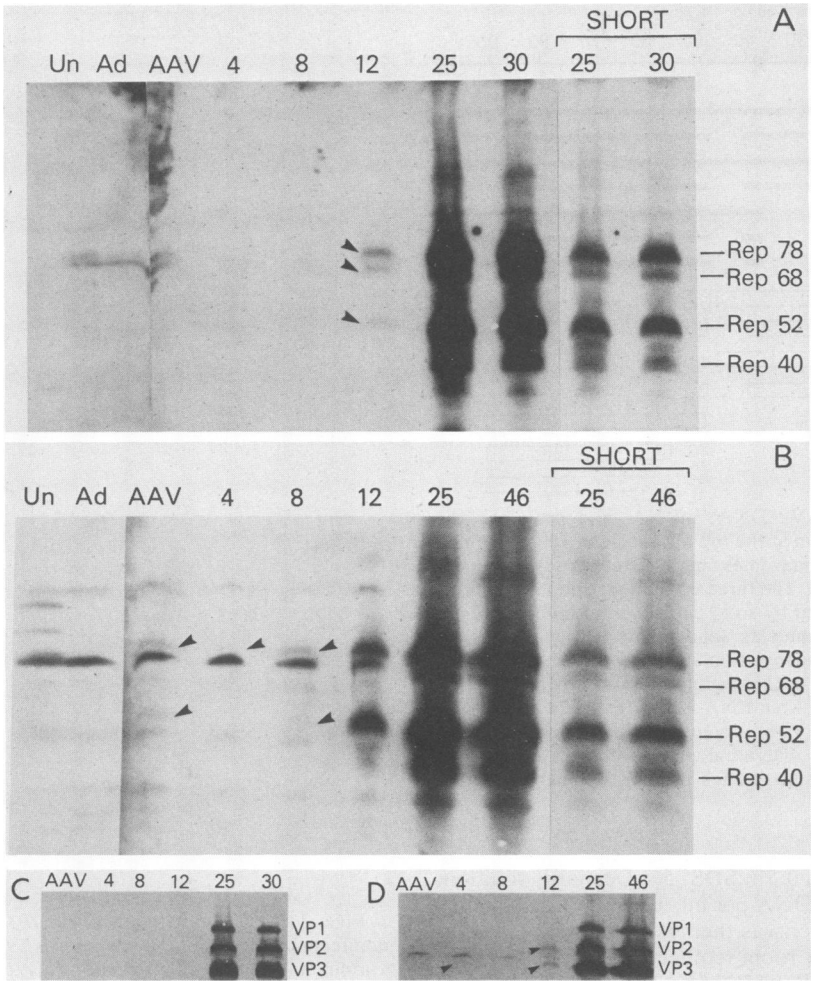

FIG. 3. AAV protein accumulation in 293 cells (A) or KB cells (B) infected with AAV2 and adenovirus. Cell extracts were made at various times after infection as indicated (in hours) above the lanes showing infection with AAV and adenovirus. Un, Uninfected cells; AAV, AAV2-infected cells; Ad, adenovirus type 5-infected cells. SHORT indicates 24-h autoradiographic film exposure; otherwise, blots were exposed for 1 week. The locations of Rep proteins are indicated at the right of each panel and marked by arrowheads in some slots. Panels C and D show reaction of the same blots as in panels A and B, respectively, with anti-AAV capsid antibody followed by reaction with horseradish peroxidase-conjugated second antibody (see Materials and Methods). The locations of VP1, VP2, and VP3 are indicated at the right of each inset, and VP2 and VP3 detected early (4 and 12 h) are marked by arrowheads.

could have affected the expression of AAV Rep proteins. We therefore performed similar experiments in human KB cells, which do not contain any adenovirus genes. Analysis of viral DNA and protein synthesis in infected KB cells is shown in Fig. 2B and Fig. 3B and D. In the immunoblot analysis (Fig. 3B), Rep78 was first detected at 4 h after infection (as shown by inspection of the original autoradiogram, although it does not reproduce well in the photograph), and a small increase in its level was observed at 8 h when Rep52 also appeared. At 12 h, Rep78 and Rep52 were detected in similar amounts, while Rep68 and Rep40 were first detected only at 25 h. High levels of all Rep proteins were observed at 25 h and remained the same at 46 h. Rep78 and Rep52 were more abundant than Rep68 and Rep40. Low levels of Rep78 and Rep52 were also observed in KB cells 25 h after infection with AAV alone (Fig. 3B).

The analysis of AAV DNA in KB cells (Fig. 2B) showed SS DNA and some monomer RF DNA at 4 h after infection. The SS DNA came from the infecting viral particles, since they were also observed in cells infected with AAV alone (Fig. 2B). Although monomer RF was barely detected at 4 h and was not readily detected at 8 h, it clearly increased at 12 h when dimer RF molecules were also detected. We conclude that RF DNA probably was synthesized as early as 4 h but only at the limit of detection of the assay. At 25 and 46 h, all three major forms of AAV DNA (monomer RF, dimer RF, and progeny SS DNA) had accumulated to high levels. In cells infected with AAV alone, only parental SS DNA was clearly detected up to 46 h after infection (Fig. 2B). Comparison of the protein (Fig. 3B) with the DNA (Fig. 2B) analyses showed that Rep78 and monomer RF DNA were first detected concomitantly at 4 h after infection. Increases in DNA replication and accumulation of Rep78 and Rep52 were observed at 12 h, and a large increase in viral DNA at 25 h correlated with a large increase in the amount of Rep78 and Rep52 and the appearance of Rep68 and Rep40. The capsid protein VP3 (Fig. 2D) was also detected at a very low level at 4 h but not at 8 h, and its amount increased at 12 h when some VP2 was also detected. This suggests that de novo synthesis of VP3 started only between 8 and 12 h

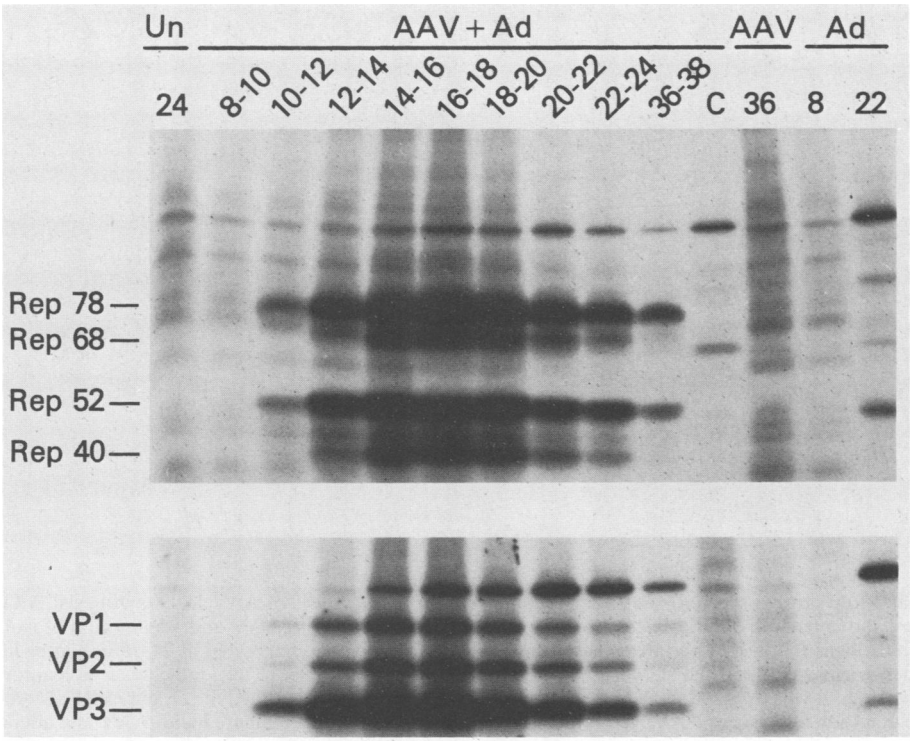

FIG. 4. Kinetics of AAV Rep and capsid protein synthesis after infection of 293 cells with AAV2 and adenovirus. Cells were labeled in vivo with [^{35}S]methionine for 2-h pulses at various times after infection and harvested immediately after the pulse. Rep or capsid proteins were immunoprecipitated, electrophoresed, and autoradiographed. The top panel shows the autoradiogram of proteins precipitated with anti-Rep antiserum, and the bottom panel shows the proteins precipitated with anticapsid antibody. The numbers above the lanes indicate the time (hours after infection) of each pulse label. C, Control precipitation with preimmune serum of extracts 22 h after infection with AAV and adenovirus; Un, uninfected cells; Ad, adenovirus-infected cells; AAV, AAV2-infected cells; AAV + Ad, cells coinfected with AAV2 and adenovirus. The locations of each Rep and capsid protein are indicated at the left.

postinfection and that the VP3 observed at 4 h may have come from the infecting parental virus particles. All three capsid proteins, VP1, VP2, and VP3, were found in large amounts at 25 and 46 h postinfection.

The main difference found between 293 and KB cells was the time at which RF monomer and Rep proteins were first detected. In KB cells, Rep78 was detected at a very low level as early as 4 h (Fig. 3B) compared with the initial detection at 12 h in 293 cells (Fig. 3A), but no significant increase was observed in KB cells until 12 h. In other experiments with KB cells, we did not detect Rep proteins or viral RF DNA before 12 h after infection (data not shown), which suggests that the levels earlier than 12 h are close to the limits of detection in our experiments. The monomer RF DNA found before 12 h could be the product of limited replication or of reannealing of parental plus and minus single strands. This duplex DNA is presumably the template for transcription of the Rep gene. In both cell lines, Rep78 appears to be the predominant species when replication begins at about 12 h after infection. By 25 h, both cell lines were similar in that accumulation of SS DNA was detected and all the Rep and capsid proteins were present at high levels. The molar ratios among the viral proteins and among the three major viral DNA forms were also similar in KB and 293 cells. Thus, the data shown in Fig. 2 and 3 suggest that, in general, AAV infection of 293 cells is similar to infection of KB cells. In both cell lines, Rep and capsid proteins seem to accumulate mainly between 12 and 25 h after infection and then remain at the same level. However, the immunoblot analysis measured only steady-state levels and not the rate of synthesis or stability of the Rep proteins. To analyze this, we performed in vivo labeling and immunoprecipitation experiments.

Rate of AAV protein synthesis. We measured the rate of synthesis of AAV proteins during infection by in vivo labeling with [^{35}S]methionine for 2 h at different times after infection. Cell extracts were prepared immediately after labeling and first immunoprecipitated with anti-Rep antibody. The supernatants from these reactions were then used to immunoprecipitate capsid proteins. The result of one such experiment in 293 cells is shown in Fig. 4.

Synthesis of Rep proteins (Fig. 4) was first detected at 10 to 12 h after infection, but only Rep78 and Rep52 were detected, and Rep78 was slightly more abundant. By 12 to 14 h, Rep68 and Rep40 could also be labeled. The rate of synthesis of all four Rep proteins increased to a maximum level that was constant between 14 and 20 h and then decreased gradually. Between 24 and 36 h, all four Rep proteins were still synthesized but at much reduced rates. Synthesis of cellular proteins, as indicated by the general background in each lane (Fig. 4), was also inhibited after 20 h, and a similar inhibition (Fig. 4) was also observed at 22 h postinfection in cells infected with adenovirus alone, but not at 36 h postinfection in cells infected with AAV alone.

Synthesis of capsid proteins (Fig. 4) followed kinetics similar to those of Rep protein. All three capsid proteins, VP1, VP2, and VP3, were first detected after the 10- to 12-h labeling period, and synthesis of each reached a maximum rate between 14 and 18 h and then decreased gradually. Interestingly, the relative rates of synthesis of each Rep

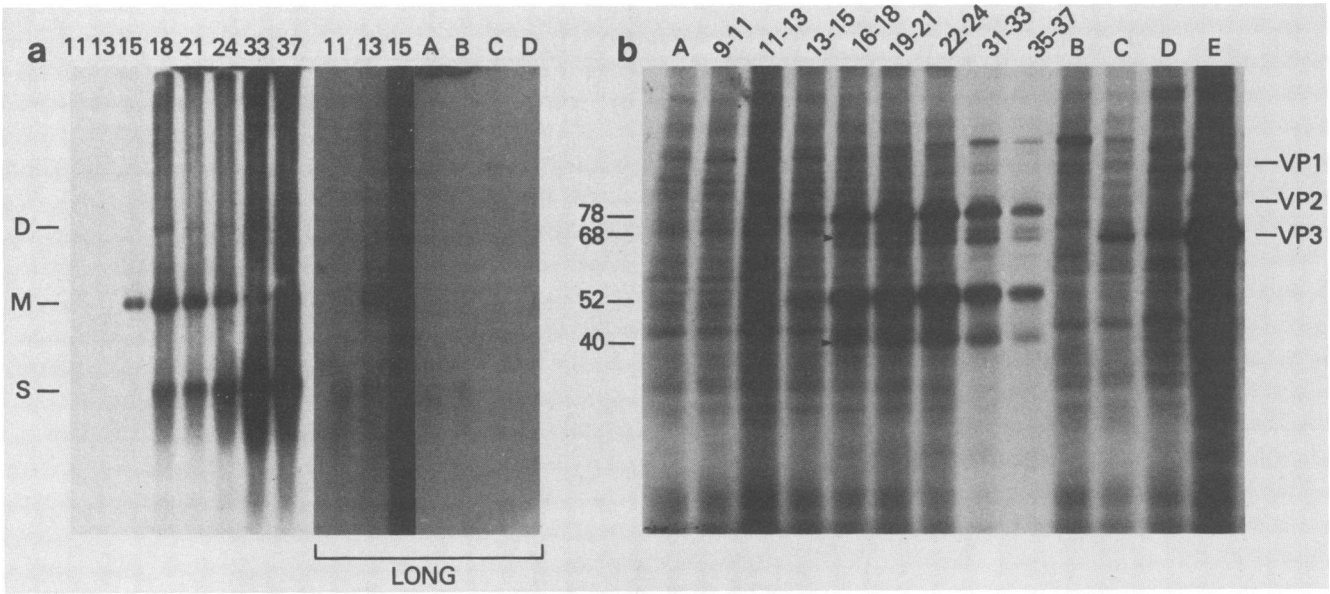

FIG. 5. AAV DNA and Rep protein synthesis in KB cells. (a) Southern blot analysis of AAV DNA synthesis. AAV [^{32}P]DNA was used as a probe. The numbers above each line indicate the time (hours after coinfection with AAV and adenovirus) of isolation of DNA. Lanes A, B, C, and D show controls as follows: A and B, cells infected with AAV alone and harvested at 24 or 33 h after infection, respectively; C, cells infected with adenovirus and harvested at 24 h after infection; D, uninfected cells. LONG indicates a 48-h autoradiographic exposure of the X-ray film; otherwise, film was exposed for 3 h. AAV DNA species are indicated at the left: D, dimer RF; M, monomer RF; S, single strands. (b) Immunoprecipitation analysis of AAV Rep proteins. KB cell cultures were coinfected with AAV and adenovirus in parallel with those used in panel a but were labeled with [^{35}S]methionine for 2-h pulses for the periods (hours after infection) indicated above each lane. Protein extracts were then immunoprecipitated with anti-Rep antibody. Lanes A, B, C, D, and E are controls: A, uninfected cells labeled for 2 h in parallel with the 9- to 11-h pulse of infected cells; B, cells labeled at 22 to 24 h after infection with adenovirus alone and immunoprecipitated with anti-Rep antibody; C, cells labeled at 22 to 24 h after infection with AAV alone and immunoprecipitated with preimmune serum; D, cells labeled at 22 to 24 h after infection with AAV and adenovirus and immunoprecipitated with preimmune serum; E, same cells as lane D, but immunoprecipitated with anticapsid antiserum. The controls show nonspecific precipitation of several cellular and viral proteins, including VP3. The location of each AAV capsid protein is shown to the right, and the Rep proteins are indicated to the left of panel b. Rep68 and Rep40 are also indicated by arrowheads in one of the lanes.

protein remained constant. Similarly, the relative rates of synthesis of the three capsid proteins remained constant. This indicates the tight regulation of synthesis of all the viral proteins. Similar experiments in KB cells (data not shown) gave kinetics similar to those seen in 293 cells (Fig. 4) for both Rep and capsid protein synthesis.

Kinetics of Rep protein synthesis and viral DNA replication. To compare Rep protein synthesis and AAV DNA replication, we infected KB cells with AAV and adenovirus and labeled them with [^{35}S]methionine for 2-h intervals. Protein extracts made at the end of each labeling period were examined by immunoprecipitation with an anti-Rep antibody (Fig. 5B), and AAV DNA was examined by Southern blot hybridization (Fig. 5A). The relative band intensities in the autoradiograms were estimated by densitometry (see Materials and Methods).

In the Southern blot (Fig. 5A), SS DNA from the infecting virions was readily detected at 11 and 13 h after infection, whereas duplex monomer RF was barely detected at 11 h but clearly increased at 13 h. Duplex dimer RF was also first detected at 13 h. Thereafter, both monomer and dimer RF DNA increased rapidly to a maximum at about 18 h. For example, between 15 and 18 h, the amount of both species increased about 6- to 10-fold. After 18 to 21 h, the level of both RF species was fairly constant. Because of the presence of parental SS DNA even at 11 h, it is difficult to measure the initial onset of net SS DNA accumulation, but this probably began about 13 to 15 h. Following this, there was a relatively slow accumulation of SS DNA up to 24 h, but thereafter a large increase occurred. Thus, from 18 to 24 h the amount of SS DNA increased by less than 2-fold, whereas between 24 and 33 h there was an increase of at least 10-fold.

These results suggest at least two clearly defined stages. First, between 12 to 20 h, bulk RF DNA synthesis began and reached a maximum level. Although SS DNA accumulation also began in this period, most (i.e., at least 80%) of the SS DNA accumulated after 24 h when the RF pool was constant. It is noteworthy that in this latter stage there was also a general increase in the background hybridization that likely represents branched molecules from which single strands are being displaced. Such replicative intermediate (RI) molecules of AAV have been described previously (44) and are expected to be a heterogeneous population with various electrophoretic mobilities as compared with the more discrete fully duplex RF or SS DNA species.

Consistent with the previous experiment (Fig. 4), synthesis of Rep78 and Rep52 (Fig. 5B) was first detected at, or soon after, the onset of RF DNA synthesis. Also, the synthesis of Rep68 and Rep40 was again detected about 2 h after Rep78 and Rep52 (Fig. 5B). In addition, Fig. 5 also shows that the synthesis rate of each Rep protein species was constant and maximal between 16 and 24 h and lower at later (31 to 37 h) or earlier (13 to 15 h) times (Fig. 5). The synthesis rates at 13 to 15 h and at 31 to 33 h were 50% of the maximum and decreased to 20% at 35 to 37 h postinfection

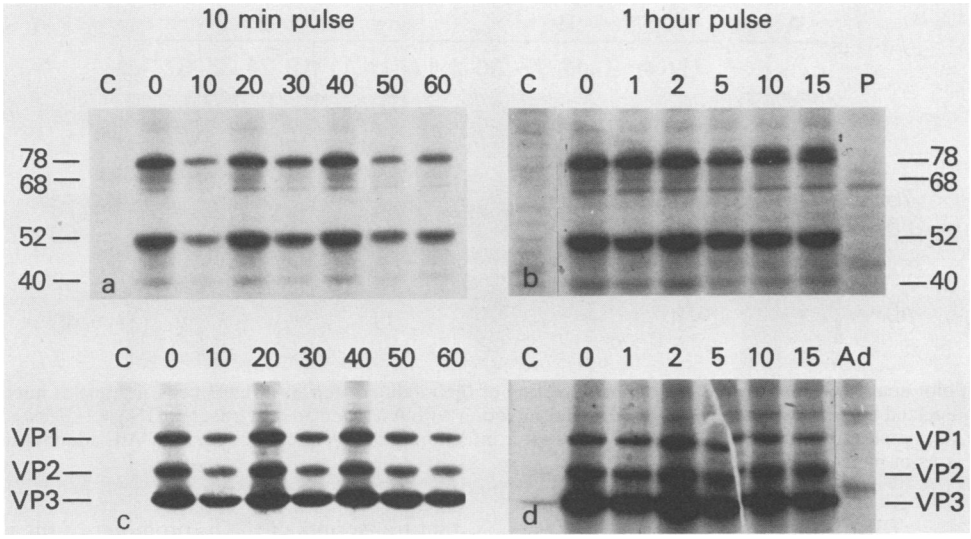

FIG. 6. Isotopic pulse-chase analysis of AAV Rep and capsid protein synthesis in KB cells infected with AAV and adenovirus. Beginning 18 h after infection, cells were labeled with a 10-min or 1-h pulse of [^{35}S]methionine. Immunoprecipitation of AAV Rep proteins (a) or capsid protein (c) following a 10-min pulse and at 10-min chase intervals is shown on the left. Immunoprecipitation of Rep proteins (b) or capsid protein (c) following a 1-h pulse and after a chase of 0, 1, 2, 5, 10, or 15 h is shown on the right. Lanes: C, control uninfected cells; P, immunoprecipitation with preimmune serum of extracts from cells infected with AAV and adenovirus and harvested at the end of the pulse; Ad, extracts from cells pulse-labeled for 1 h at 18 h after infection with adenovirus alone and immunoprecipitated with anticapsid antiserum. The locations of AAV Rep proteins and capsid proteins are indicated at the side of each panel.

(Fig. 5). However, the synthesis rate of each Rep protein species relative to the overall Rep protein synthesis was constant between 15 and 37 h after infection (Fig. 5). This is consistent with the constant ratios of total amounts of the four Rep proteins observed in immunoblots at 25 and 46 h after infection of KB cells (Fig. 3B).

The experiments in Fig. 4 and 5 show that the period of maximal Rep and capsid protein synthesis (about 15 to 24 h) correlated with a low level of viral SS DNA accumulation. The large increase in SS DNA accumulation replication after 24 h (about 80% of total) corresponded to a decrease in Rep and capsid protein synthesis. However, there was no obvious correlation between changes in the relative abundance of RF and SS viral DNA and changes in the relative abundance or synthesis of any particular Rep protein between 16 and 37 h postinfection. Prior to this, increasing Rep protein and capsid protein synthesis rates corresponded to increasing RF DNA accumulation.

Pulse-chase labeling of AAV proteins. AAV capsid proteins are very stable, at least after assembly into virion particles (36), but there was no information about the stability of Rep proteins during the replication cycle. The experiments described above showed continued accumulation of Rep proteins during infection (Fig. 3) even at later times, when their rate of synthesis had decreased (Fig. 4 and 5). This suggested that the Rep proteins were relatively stable. Genetic analysis (34, 50; Mendelson et al., in press) indicated that each of the four Rep proteins identified so far (Rep78, Rep68, Rep52, and Rep40) is synthesized from its own mRNA (see Fig. 7). To examine these points further, we used an isotopic pulse-chase labeling and immunoprecipitation assay of AAV proteins (Fig. 6). KB cells were labeled at 17 h after infection with [^{35}S]methionine either for 10 min and chased for up to 1 h (Fig. 6a and c) or for 1 h and chased for 1, 2, 5, 10, and 15 h (Fig. 6b and d). Rep and capsid proteins were immunoprecipitated from cell extracts and electrophoresed.

For the capsid proteins, the short pulse (Fig. 6c) showed that proteins labeled for 10 min were stable for at least 60 min. The relative amounts of VP1, VP2, and VP3 observed immediately at the end of the pulse were similar to those observed in virions (39) and remained the same throughout the chase. Similarly, capsid proteins labeled for 1 h and chased for 15 h were very stable. For the Rep proteins, after a 10-min pulse (Fig. 6a), all four Rep proteins were present and remained stable for 60 min. After 1 h of labeling, Rep52 and Rep40 were stable and unchanged for up to 15 h. Rep78 remained stable for 2 h and then began to decrease, while a new, slower-migrating species appeared and accumulated. Rep68 was not detected very clearly after a 10-h chase. This experiment suggests that after its synthesis, Rep78 was gradually modified to a more slowly migrating species. The modified Rep protein was not detected in the previous immunoprecipitation experiments (Fig. 4 and 5) because the length of the labeling pulse was only 2 h. In similar pulse-chase experiments in 293 cells infected with AAV and adenovirus, similar results were obtained for both capsid and Rep proteins, again suggesting independent synthesis of each protein and stability for up to 15 h after synthesis.

Further analysis showed that the modified Rep78 was also detected by immunoblotting (Fig. 7). As shown for infected 293 cells, this component was more readily detected if the nuclear extracts were treated with DNase I (Fig. 7A). We then examined the appearance of the modified Rep78 during productive infection of 293 and KB cells (Fig. 7B and C) by immunoblotting of DNase I-digested extracts. In 293 cells, this component was detected at 25 h after infection, and in KB cells, it was detected at 33 h (Fig. 7D). In both cell lines, the modified Rep78 appeared when the rate of Rep protein synthesis was low and accumulation of viral SS DNA was high (Fig. 2, 3, and 4). Also, its appearance at relatively late times is consistent with the pulse-chase analysis.

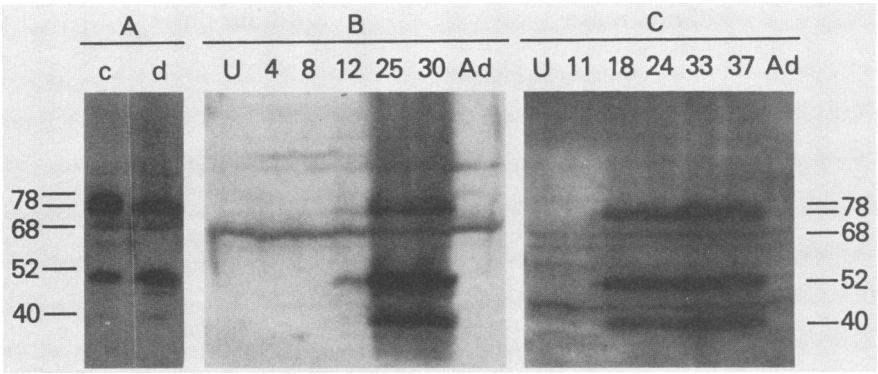

FIG. 7. Western blot analysis of cell extracts showing detection of the modified Rep78. (A) 293 cells. Lanes: c, nuclear extract digested with DNase I; d, undigested nuclear extract isolated at 30 h after infection with AAV and adenovirus. (B) DNase I-digested whole cell extracts of 293 cells. Lanes: U, uninfected; 4, 8, 12, 25, and 30, hours after infection with AAV and adenovirus; Ad, adenovirus-infected cells. (C) KB cells. Analysis is as in panel B.

DISCUSSION

The experiments reported here analyzed the kinetics of synthesis and accumulation of AAV proteins and DNA during productive infection of human cells in the presence of adenovirus. The data suggest that the AAV growth cycle can be conveniently divided into three stages. In the first 10 to 12 h after infection, only very small amounts of duplex AAV DNA were sometimes detected in addition to infecting parental single strands. Two Rep gene products, Rep78 and Rep52, were also detected at very low levels in this period. The second stage occurred from 12 to 24 h when the RF DNA pool was established and most of the Rep and capsid protein was synthesized. In the final stage, after 24 h, production of viral proteins was inhibited and RF DNA was relatively constant, but there was extensive accumulation of most of the progeny SS DNA which apparently was packaged into preformed capsids to produce mature virions (36). We did not notice any significant differences between 293 and KB cells in the overall AAV growth cycle. Thus, the adenovirus E1a and E1b genes expressed in 293 cells are insufficient for helper-mediated AAV DNA replication, consistent with evidence that one important adenovirus gene for AAV DNA replication is E4 (13, 23), which is not expressed in either KB or 293 cells.

In the first stage, the immunoblotting analysis detected the AAV proteins Rep78 and Rep52 apparently before the beginning of bulk (progeny) RF DNA synthesis. They were presumably expressed from the earliest duplex (parental) RF DNA formed, but several caveats must be mentioned. We have reported previously (34) that synthesis of AAV Rep proteins occurs in the absence of helper virus or bulk AAV DNA replication. Also, our experiments may not clearly identify the formation of parental RF, and the requirement for generation of these remains unclear. It is not known whether this normally requires a helper virus function, although in certain cases it clearly does not (52). Also, it is possible to detect duplex AAV DNA after infection in the absence of helper virus and in the presence of DNA replication inhibitors (33). This duplex DNA, which might represent annealing of infecting single strands or rapid integration of AAV single strands into cellular chromosomes, may account for the earliest detected Rep78 and Rep52. More interestingly, in this early phase, Rep78 was more abundant than Rep52, while later on, both were present in similar amounts. Synthesis of capsid proteins was not detected in this earliest phase. This is comparable to a recent report (15) that transcripts of the p_4 promoter of the parvovirus minute virus of mice (MVM), which express the viral nonstructural proteins NS-1 and NS-2, were detected before the p_{38} promoter transcripts, which express the virus structural proteins. Also, NS-1 appears to regulate this process (51). In addition, synthesis of the nonstructural proteins of porcine parvovirus was seen before detection of the structural proteins during infection in cell culture (35).

The appearance of AAV Rep proteins at or before the beginning of progeny RF synthesis is consistent with genetic evidence that this requires Rep protein coded by p_5 transcripts (22, 45). The idea (25) that Rep proteins may mediate inhibition of AAV DNA replication early in infection until permissive conditions can be established is neither refuted nor supported by our results. Progeny RF DNA synthesis begins around 10 to 12 h after infection. One function apparently required for this is strand nicking in or near the terminal repeat sequence within the hairpin loop of the RF to allow replication of the genome termini (5, 44). This putative activity is an essential part of all the models suggested so far for parvovirus DNA replication (5, 16). In MVM, it may be mediated by the NS-1 protein (17). In AAV, it may also be mediated by one or more of the Rep proteins.

Between 12 and 24 h, replication of RF DNA increased to reach a relatively constant level by about 18 to 21 h, but only small amounts of progeny SS DNA accumulated. Viral protein synthesis was extensive during this time. Rep protein synthesis reached maximal rates which remained constant for 6 to 8 h in parallel with the establishment of the RF DNA pool. Capsid protein synthesis probably started shortly after the beginning of Rep protein synthesis and followed very similar kinetics. Thus, the maximum rates of synthesis of both Rep and capsid proteins occurred several hours before the RF pools reached maximum levels and well in advance of bulk SS DNA accumulation. Testing the stability of Rep and capsid proteins by pulse-chase labeling experiments indicated that they were stable for at least 15 h, thus allowing their accumulation.

Similar high stability and kinetics of synthesis were also reported for MVM proteins (16). In highly synchronized A9 cells infected with MVM, synthesis of NS-1, VP1, and VP2 was first detected within 2 h from the beginning of the cellular S phase and reached maximal levels for all the proteins within the next 6 h, while duplex viral DNA synthesis was low. Synthesis of all the major viral proteins of the parvovirus H-1 (16) also begins and reaches maximum

rates before bulk viral DNA synthesis. Thus, establishing peak synthesis of both nonstructural and structural proteins before the maximum rate of DNA synthesis may be a general characteristic of parvoviruses.

We observed constant ratios between the relative rates of synthesis of all AAV proteins. The relative amounts of the four mRNA species (32) coding for the Rep proteins were similar to the relative amounts of the Rep proteins which we observed. Thus, the unspliced 4.2- and 3.6-kb mRNAs coding for Rep78 and Rep52, respectively, were more abundant than the 3.9- and 3.3-kb spliced messages coding for Rep68 and Rep40, respectively, much like their protein products. Our pulse-chase experiments are also consistent with independent synthesis of the four Rep proteins. This suggests that the main regulation of Rep protein expression is at the level of RNA transcription and splicing. The capsid proteins have a more complicated regulation since the most abundant protein, VP3, and the much less abundant protein VP2 are both synthesized from the same 2.3-kb mRNA. A weak ACG translation initiation codon is used for VP2, whereas VP3 is initiated at a strong AUG codon (3). VP1 is synthesized from a different AUG codon contained in a minor 2.3-kb mRNA species (Fig. 1) which is spliced differently from the major 2.3-kb mRNA (2, 49). For the capsid proteins, the correlation between their relative rates of synthesis and their relative proportions in mature virions is consistent with their independent synthesis. Thus, capsid protein synthesis is regulated mainly by alternate splicing and alternate translation initiation codons.

The final period of the AAV growth cycle beginning at about 24 h was characterized by decreased AAV protein synthesis and increased accumulation of AAV SS DNA, while the pool of duplex RF was constant. Studies with AAV (22, 37, 45) and MVM (16) showed that accumulation of progeny single strands depends on capsid proteins synthesis and is probably coupled to association of SS DNA with preformed capsids (36, 37). Recently, Cotmore and Tattersall (17) reported that most of the newly synthesized MVM DNA is covalently associated with NS-1 at the 5′ termini, which suggests that NS-1 plays a role in directing the synthesis of SS DNA and packaging into MVM virions.

In the late stage of AAV infection, we observed accumulation of a modified form of Rep78 having a slightly lower electrophoretic mobility. The nature of the modification is unknown and preliminary studies (B. Redemann and E. Mendelson, unpublished data) failed to detect phosphorylation of any AAV protein. However, the modified Rep78 is primarily a nuclear protein and was more readily extracted from nuclei by extensive nuclease digestion. Thus, some Rep78 may be tightly bound to DNA or to the nuclear matrix. In contrast, about 50% of Rep52 and Rep40 is found in cytoplasmic extracts (34, 50), and the rest can be completely released from nuclei by repeated salt extractions (E. Mendelson, unpublished data). Therefore, Rep proteins encoded from p_5 and p_{19} transcription units have different biochemical properties. This is consistent with genetic analysis which showed that Rep78 (or Rep68) is specifically required for RF DNA replication (20, 43). Furthermore, recent studies (N. Chejanovsky and B. Carter, unpublished data) show that Rep52 (or Rep40) is also specifically required for SS DNA accumulation.

In addition to their functions in AAV DNA replication, the Rep proteins may mediate both positive and negative regulation of viral or heterologous genes (26, 27, 46, 48; Mendelson et al., in press). These regulatory phenomena are complex, but there are several possibilities that are consistent with our present results. The activation by Rep occurs at the level of transcription (27, 48) in the absence or presence of helper virus when cells are transfected with recombinant AAV plasmids. The role of this Rep activation function has not been assayed in AAV particle-mediated infections. The low levels of Rep78 and Rep52 that we detected in the first 10 to 12 h may mediate activation of AAV promoters during the transition to the more active growth phase thereafter. The inhibition of AAV protein synthesis late in infection may also be mediated by Rep proteins since Rep-mediated negative regulation of gene expression was observed in several systems (26, 27, 46; Mendelson et al., in press). Alternatively, changes in adenovirus gene expression later after infection may decrease AAV protein synthesis since inhibition of cellular protein occurs late after adenovirus infection.

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