JOURNAL OF VIROLOGY, Jan. 1994, p. 170–176
0022-538X/94/$04.00+0
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Vol. 68, No. 1

Site-Directed Mutagenesis of Adeno-Associated Virus Type 2 Structural Protein Initiation Codons: Effects on Regulation of Synthesis and Biological Activity

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Received 7 June 1993/Accepted 23 September 1993

It has been shown that two of the three adeno-associated virus type 2 capsid proteins, B and C, are synthesized from a single spliced transcript. Protein C arises from an AUG codon at nucleotide 2810, whereas protein B is initiated by a unique eucaryotic initiation codon (ACG) that lies 65 triplets upstream from the C origin. The third capsid component, protein A, is synthesized from a second spliced transcript which uses an alternative 3′ acceptor site. In this study we used oligonucleotide-directed mutagenesis to confirm the positions of the B initiation codon and the 3′ acceptor sites for the alternatively spliced B/C and A protein messages. We also located definitively the protein A initiation codon, an AUG triplet mapping to nucleotide 2203. Mutagenesis of the B initiator permitted a direct test of the effect of increased B initiator strength on the translational efficiencies of the B and C proteins. It was found that conversion of the relatively inefficient protein B initiator (ACG) to an AUG enhanced the level of B synthesis while abolishing the synthesis of C from its downstream AUG initiator. Protein C synthesis thus depends on the strength of the B initiator, i.e., the relatively higher levels of C (approximately 20-fold greater than B) must result from frequent readthrough of the weak B initiator. Finally, we examined the abilities of mutants deficient in the synthesis of A, B, or C to produce infectious virions. We found that at least two of the structural proteins, B and C, are required for the production of infectious virions and that sequestration of single-stranded adeno-associated virus genomes from the pool of replicating DNA molecules does not occur in the absence of either of these proteins.

The human dependovirus, adeno-associated virus type 2 (AAV2), whose replication requires either adenovirus (Ad) or herpesvirus gene products (4, 22), produces its capsid proteins from two mRNAs which are derived from a primary transcript (unspliced p40 2.6-kb RNA [11]) by alternative splicing (Fig. 1) (2, 6, 30). Both spliced transcripts (2.3-kb RNAs) differ only with respect to their 3′ acceptor sites, which are apparently located only 27 nucleotides (nt) apart (at nt 2200 and 2227). The largest protein (A [90 kDa]) is generated from the slightly larger message ($2.3kb_A$), whereas the other two proteins (B [72 kDa] and C [60 kDa]) are translated from the smaller message ($2.3kb_{B/C}$), by alternative use of an ACG initiator encoded at nt 2615 (protein B) and a downstream AUG initiator that maps to nt 2810 (protein C) (3). Translational initiation at a non-AUG codon is rare among higher eucaryotes, and other examples have now been reported (12, 24). All three AAV capsid proteins share overlapping amino acid sequences (i.e., C is contained within B, and B and C are contained within A) and terminate at a TAA codon located at nt 4322 (Fig. 1) (10, 20). Subspecies of two of the capsid proteins (A and C) have also been detected, however, and these may arise by variable readthrough of several successive in-frame termination codons at the 3′ termini of AAV mRNAs (21). Earlier studies have indicated that the molecular proportions of the three AAV structural proteins are 20:1:1 and that each AAV virion would thus be composed of 60 molecules of C and 3 or 4 molecules each of A and B (26).

In a previous report, Becerra et al. (2) demonstrated that an intronless plasmid construct did not synthesize protein A, whereas A was synthesized when the last 34 bases of the intervening sequence were present. Additionally, A was shown to be initiated in reading frame 1 (along with B and C), implying that its initiator was the AUG codon that maps to nt 2203. Trempe and Carter (30) also made a similar deduction, but, again, without direct evidence for the position of the A start site. In the present study we have used oligonucleotide-directed mutagenesis to definitively map the A initiator and to confirm the positions of the B initiation codon and the 3′ acceptor sites for the alternatively spliced B/C and A protein transcripts. We also demonstrate that expression of the major repeating unit of the capsid structure, protein C, can be regulated by the strength of the protein B initiation codon. In vivo analysis of initiation codon mutants of the A and B capsid proteins revealed that at least B and C are required for sequestration of single-stranded progeny genomes from the DNA synthetic pool.

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MATERIALS AND METHODS

Viruses and cells. Human 293-31 cells, an established line of Ad type 5 (Ad5)-transformed human embryonic kidney cells (9), were grown at 37°C in monolayers in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 2× vitamins and amino acids. AAV2, and Ad2, and Ad5 were propagated in KB cells, and Ad5 DNA was prepared as described previously (4). HeLa cells were grown in monolayers in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Plasmids and molecular cloning. Plasmids were generated in *Escherichia coli* HB101 cells grown in Luria-Bertani broth containing 50 μg of ampicillin per ml and were purified by a

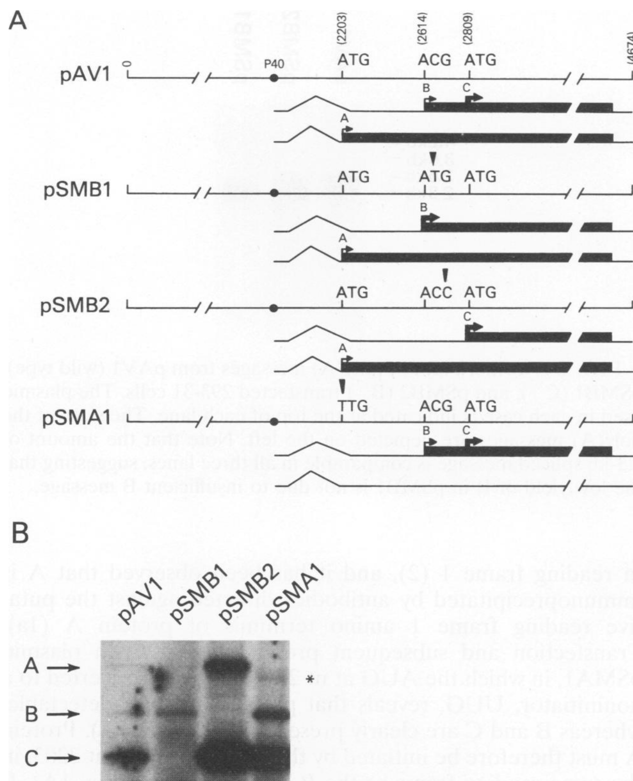

FIG. 1. In vivo expression of AAV capsid proteins in 293-31 cells transfected with initiator mutants of proteins B and A. (A) Organization of the transcription units of the AAV DNA plasmids pAV1 (wild type), pSMB1, pSMB2, and pSMA1. Only the AAV DNA sequence of each plasmid is represented. The positions of the p40 promoter and the initiation codons for the capsid proteins are depicted on each line. Nucleotide numbers are given in parentheses. Structures of the predicted spliced mRNAs are represented by lines, with splices indicated by carets. The specific nucleotides mutated are indicated by solid triangles. Note that the splice acceptor sites for the two mRNA structures are separated by 27 nucleotides and that the ATG for protein A falls within this short segment. (B) Autoradiogram of [^{35}S]methionine-labelled AAV capsid proteins resolved by SDS-PAGE. Unless otherwise noted, in this and subsequent experiments, all transfections with AAV DNA plasmids were carried out in conjunction with Ad5 DNA for helper functions (see Materials and Methods). The capsid proteins were purified from nuclear extracts by immunoprecipitation. Plasmids used in each case are indicated at the top of each lane.

modified Birnboim and Doly method as described by Maniatis et al. (19). Plasmid pAV1 is an infectious AAV2 clone that contains the entire viral sequence inserted at a *Bgl*II site in a modified pBR322 vector (18). We derived the pSM1 plasmid from pAV1 by deleting a 2.2-kb *Kpn*I fragment (nt 1906 to 4153), which was then cloned in M13mp18 for oligonucleotide mutagenesis. Fragments with various mutations were cloned back into pSM1 to generate the following mutant pAV1 clones: (i) pSMA1 (the proposed ATG initiation codon for protein A at nt 2203, changed to TTG); (ii) pSMB1 (the ACG initiation codon for B at nt 2614 changed to an ATG); (iii) pSMB2 (the ACG initiator changed to an ACC); (iv) pSMA2 (the putative A message splice acceptor site at nt 2200 changed from --CCAG-- to --CCTT--); and (v) pSMBC (the splice acceptor for the B/C message at nt 2227 changed from --CCAG-- to --CCTT--).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (17), with a Bio-Rad kit. Mutants were screened by DNA sequencing, and sequencing reactions were carried out with a Sequenase kit (United States Biochemical Corporation).

Transfection of cells and detection of AAV capsid proteins. By using the calcium phosphate procedure (31), all transfections were performed with the CellPhect Kit (Pharmacia), according to the manufacturer's protocol. 293-31 cells ($\sim 3 \times 10^6$) were transfected in 60-mm-diameter petri dishes at 75 to 80% confluency with 10 μg of plasmid DNA and were either cotransfected with 50 μg of Ad5 DNA or preinfected with Ad2 at a multiplicity of infection of 10 1 h before transfection. Proteins were labelled with [^{35}S]methionine (>1,000 ci/mmol; Amersham) at 48 h posttransfection for 2 h at 37°C, and the capsid proteins were immunoprecipitated with a polyclonal antibody and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (14).

RNA analysis. In vivo-synthesized AAV RNA was obtained from 293-31 cells which were infected with Ad2 (multiplicity of infection of 10) and transfected with wild-type or mutant AAV DNA plasmid constructs (10 μg). Cells were harvested 48 h posttransfection, and poly(A) mRNA was isolated and purified by using an mRNA purification kit (Stratagene). The final RNA pellet was dissolved in 50 μl of distilled water treated with diethylpyrocarbonate. For Northern (RNA) blotting, a 15-μl sample was electrophoresed into a 1% formaldehyde-agarose gel which was then blotted on to a GeneScreen nylon membrane (New England Nuclear) and hybridized with a ^{32}P-labelled *Bgl*II-restricted AAV2 DNA probe.

In vitro translation. In vitro translation was performed in 35 μl of nuclease-treated rabbit reticulocyte lysates (Amersham) programmed with in vivo-synthesized AAV RNA, and proteins were labelled with [^{35}S]methionine (Amersham). After 1 h of incubation at 30°C, 5-μl aliquots were analyzed by SDS-PAGE. The remaining samples were immunoprecipitated with polyclonal antibodies against the capsid proteins and analyzed by SDS-PAGE.

Negative hybrid selection (hybrid arrest). The procedure was as described by Ozawa et al. (23). An antisense oligonucleotide complementary to the first 21 nt of the proposed alternatively spliced 2.3-kb message was used for hybrid arrest. Following RNase H treatment, samples were translated in vitro in rabbit reticulocyte lysates and analyzed by SDS-PAGE.

Detection of AAV replication. Crude lysates of Ad and AAV cotransfected or infected-transfected cells were prepared as follows. Cells were harvested at 48 h postinfection or posttransfection, subjected to three freeze-thaw cycles, and centrifuged at $400 \times g$ at 4°C for 10 min to sediment the cell debris. The cleared lysates were treated with RQ1 DNase (RNase-free; Promega) at 37°C for 15 min. The AAV titer was determined by both a dot blot assay and an indirect immunofluorescence assay. For the dot blot assay, lysates (prepared as above) were used to infect 293-31 cells. At 48 h postinfection, viral DNA was extracted by a modified Hirt procedure (5), digested with *Dpn*I to remove input AAV DNA, serially diluted, and transferred to a GeneScreen membrane in a dot blot apparatus. The membrane was then probed with ^{32}P-labelled AAV DNA. The relative titer of AAV was determined from the highest dilution yielding a positive signal on the blot. For the indirect immunofluorescence assay, HeLa cells were grown in monolayers in Labtek chamber slides (5×10^5 cells per chamber) and were infected with 0.2 ml of an appropriate dilution of the cleared lysate plus Ad2 at a multiplicity of infection of 5 and incubated at 37°C for 1 h to allow adsorption. The inoculum was then

removed, 0.2 ml of fresh medium containing 10% fetal calf serum was added, and the incubation continued at 37°C. At 30 h postinfection, cells were fixed in 95% methanol and incubated with anti-AAV2 guinea pig serum for 45 min at 37°C. The cells were then incubated with fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin G (ICN Immunobiologicals) for 45 min at 37°C and examined for fluorescence with a Zeiss fluorescence microscope.

Analysis of DNA replication and single-stranded DNA production. Monolayers of 293-31 cells were transfected with Ad DNA and either the wild-type plasmid, pAV1, or individual mutant plasmids deficient in the synthesis of capsid proteins, as follows: A, pSMA1; B, pSMB2; and C, pSMB1. At 40 h after cotransfection, viral DNA was extracted according to the modified Hirt procedure, treated with *Dpn*I, and electrophoresed into a 1% agarose gel. The DNA was then transferred to a GeneScreen nylon membrane and hybridized with a ^{32}P-labelled AAV DNA probe.

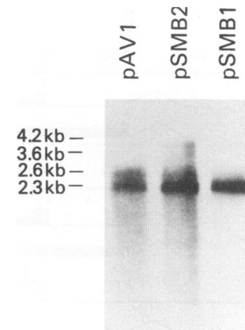

FIG. 2. Northern blot of poly(A) messages from pAV1 (wild type), pSMB1 (C^-), and pSMB2 (B^-) transfected 293-31 cells. The plasmid used in each case is indicated at the top of each lane. The sizes of the poly(A) messages are depicted on the left. Note that the amount of 2.3-kb spliced message is comparable in all three lanes, suggesting that the low yield of B in pSMB1 is not due to insufficient B message.

RESULTS

Mutagenesis of the protein B initiation codon. Proteins B and C are translated with a B/C ratio of approximately 1:20 from the same spliced message, both in vivo and in vitro (2). Initially, protein B's ACG initiator was changed either to an AUG (pSMB1) or to a noninitiator triplet, ACC (pSMB2). Interestingly, although ACG normally specifies threonine, in its initiation function it apparently specifies a methionine (16). Conversion to ACC also yields a threonine codon. Figure 1A diagrammatically illustrates these substitution mutations and their specific locations. The proteins synthesized by the mutants are visualized in an autoradiogram following SDS-PAGE (Fig. 1B). First, conversion of ACG to ACC (pSMB2) abolished the synthesis of B, confirming that B is initiated by the ACG codon that maps to nt 2614. Second, conversion of ACG to an AUG (pSMB1) enhanced the relative level of B synthesis when compared with that of the wild type (pAV1, compare A with B) and abolished the synthesis of C from its downstream AUG initiation codon at nt 2809. Not only do these results confirm the location of the B initiation codon, but they also demonstrate directly that the ratio of protein B to protein C is regulated by the B initiator. Differences in intensity of corresponding bands between lanes are presumably due to technical factors, as variations occurred from experiment to experiment. However, relative differences between bands generated with each construct were reproducible from experiment to experiment. It should be noted that the observed increase in B was somewhat less than expected when the B initiator was converted to an AUG (i.e., compare with pAV1, protein C). This is not explained by initiator placement, since the AUG triplet lies in the most favorable initiation context (16). To be certain that the mutation was not additionally producing an inhibitory effect at the level of transcription or at the level of splicing, poly(A) mRNAs were isolated from 293-31 cells preinfected with helper Ad2 followed by transfection with pAV1 (wild type), pSMB1 (ACG to AUG), or pSMB2 (ACG to ACC) and analyzed by Northern blotting. Results are shown in Fig. 2. With both mutants there was no apparent decrease in the amount of spliced (2.3-kb) message, indicating that neither transcriptional nor splicing defects are responsible for the less than expected level of B when driven by the AUG initiator.

Protein A is initiated by the AUG codon at nt 2203. As previously noted, there are, essentially, two possible initiation sites for protein A, i.e., the AUG mapping to nt 2203 in frame 1 or the AUG mapping to nt 2213 in frame 2 (2). The former site is more likely, because most of A is known to be translated in reading frame 1 (2), and it has been observed that A is immunoprecipitated by antibodies directed against the putative reading frame 1 amino terminus of protein A (1a). Transfection and subsequent protein analysis with plasmid pSMA1, in which the AUG at nt 2203 has been converted to a noninitiator, UUG, reveals that protein A is not detectable, whereas B and C are clearly present (Fig. 1B, lane 4). Protein A must therefore be initiated by the AUG codon at nt 2203, in the same reading frame as the B and C proteins (Fig. 1A). It should be noted that the faint band seen in Fig. 1B, lane pSMA1 (marked by an asterisk), represents a protein that migrates slightly faster than A. A similar band is also faintly present in lanes pSMB1 and pSMB2. This protein is of uncertain origin but could represent a viral protein (VP1a) previously observed by Trempe and Carter (30) (see Discussion).

The splice acceptor site at nt 2200 is essential for synthesis of protein A. In a preliminary experiment, negative hybrid selection was used to additionally demonstrate that A arises from an alternatively spliced message. Poly(A) mRNA from Ad-infected and pAV1-transfected cells was hybridized with an antisense oligonucleotide complementary to the first 21 nt downstream from the reported 3′ splice acceptor site for the A message (nt 2200). An autoradiogram of the translation products before and after oligonucleotide hybridization is shown in Fig. 3. In the presence of the antisense oligonucleotide, protein A is not detected, whereas the B and C proteins are clearly seen (lane 2). Furthermore, a longer exposure of the autoradiogram did not reveal the presence of A. We therefore conclude that the A protein is synthesized from an mRNA that contains a nucleotide tract not present in B/C mRNA and this tract lies within a 27-base segment just upstream from the B/C acceptor site.

In eucaryotes, the process of RNA splicing is complex, involving several critical sequences, numerous factors, and several discrete steps (27). A critical sequence, which occurs at the splice acceptor site, consists of a pyrimidine tract ending in a conserved CAG trinucleotide (U/C_{16}NCAG:G) (27). Substitution of the dinucleotide AG in this sequence by pyrimidines has been shown to obliterate splicing (27). To confirm the location of the A and B/C mRNA splice acceptor sites, two mutants were constructed. Plasmid pSMBC has the dinucleotide AG substituted by UU at the previously identified splice acceptor site for the B/C message (nt 2227; Asp-to-Tyr conversion in protein A). Plasmid pSMA2 has a similar mutation

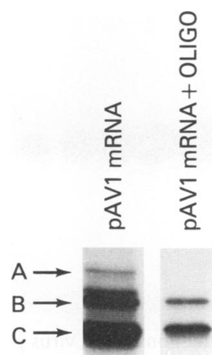

FIG. 3. In vitro translation of poly(A) messages purified from 293-31 cells infected with Ad2 and transfected with plasmid pAV1 (wild type). The left lane shows translation products of pAV1 poly(A) mRNA prior to negative hybrid selection. The positions of the capsid proteins A, B, and C are indicated by arrows. The right lane shows translation products of pAV1 mRNA following incubation with an antisense oligonucleotide (OLIGO) directed towards the first 21 nt of the alternatively spliced 2.3-kb message ($2.3kb_A$). Note that protein A is not made. In vitro translation in rabbit reticulocyte lysates was carried out in the presence of [^{35}S]methionine. Translation products were immunoprecipitated with anticapsid antisera and analyzed by SDS-PAGE.

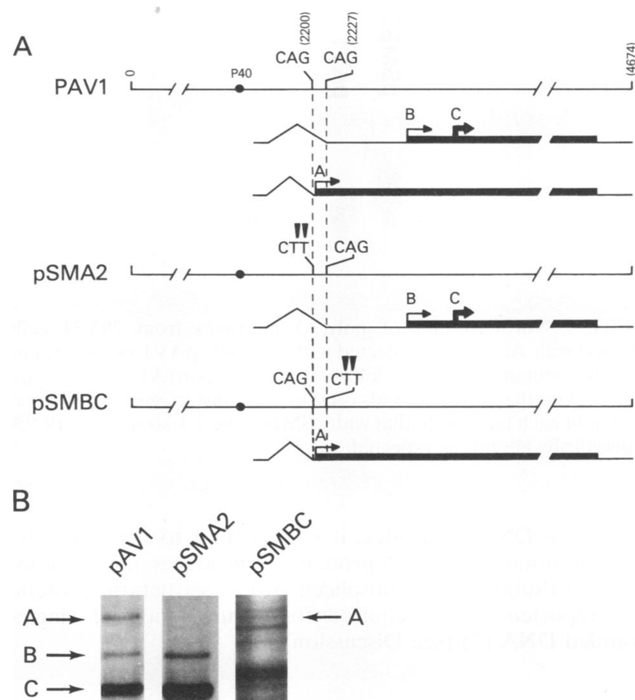

FIG. 4. In vivo expression of capsid proteins in 293-31 cells transfected with AAV DNA constructs containing mutations at the A and B/C mRNA splice acceptor sites. (A) Organization of the transcription units of the AAV DNA plasmids pAV1 (wild type), pSMA2, and pSMBC. The top line for each plasmid represents the AAV DNA sequence only. The positions of the p40 promoter and the two 3′ splice acceptor sites are indicated on each line. Nucleotide numbers are given in parentheses. Structures of the predicted spliced mRNAs are represented by lines, with the splices designated by carets. The specific nucleotides mutated are indicated by solid triangles. Note that there are two splice acceptor sites (nt 2200 and nt 2227). Changing two bases, A and G, in the consensus sequence (...CAG]GT..) of an acceptor site is expected to abolish it, leaving the other acceptor site available for use. (B) Autoradiogram of [^{35}S]methionine-labelled capsid proteins resolved by SDS-PAGE. Transfections and purification of capsid proteins were as described before (see legend to Fig. 1). Plasmids used in each case are indicated at the top of each lane. Note that a longer exposure of the pSMBC channel was required to detect A and that B and C are still undetectable.

in the proposed splice acceptor site for the A message (nt 2200). A schematic representation of these mutations is shown in Fig. 4A, and an autoradiogram depicting the proteins synthesized by each mutant is shown in Fig. 4B. As anticipated, when the known splice acceptor site for the B/C message was altered, proteins B and C were not visible, and a longer exposure (shown only in the pSMBC lane) confirmed the finding. Protein A, however, was apparently detected only on the longer exposure. The diminished presence of protein A could relate to mutational interference with the A acceptor site or to the inability of our anticapsid antiserum to effectively precipitate free A (i.e., A unassociated with B and/or C), a feature previously observed by Trempe and Carter (30). When the presumed splice acceptor site at nt 2200 was altered (pSMA2), the synthesis of protein A only was abolished, indicating that this site is required for the synthesis of A mRNA. This result also demonstrates that there are no other upstream splice acceptor sites that generate significant amounts of functional A message in vivo. It should be noted that, as in Fig. 1B, a band of uncertain origin is seen just below the presumptive A band in the pSMBC lane and that a corresponding faint band is also seen in the pSMA2 lane.

The mechanism that regulates the frequency of splicing at the alternative acceptor site is unknown. In the absence of a functional splice acceptor for the B/C message, it is possible that an enhancement of $2.3kb_A$ mRNA could have occurred, even though we were unsure of the extent of A synthesis (Fig. 4B, lane pSMBC). However, a Northern blot analysis of poly(A) RNA purified from AD2-infected and pSMBC-transfected cells (Fig. 5) revealed little, if any, detectable 2.3-kb RNA (i.e., spliced $2.3kb_A$ message) and a large accumulation of 2.6-kb RNA (precursor RNA; compare with pAV1). Although this suggests that the production of the A message is not reciprocally regulated at the level of B/C splicing, we still cannot exclude the possibility that the B/C splice acceptor site mutation has interfered with production of A message.

Inability of initiator mutants to produce infectious AAV particles. In transfections with initiator mutants unable to synthesize the A, B, or C proteins, we could not detect the propagation of infectious AAV particles by either immunofluorescence (Fig. 6) or a dot blot assay (Fig. 7). This suggests that all three structural proteins are necessary for the assembly of infectious AAV virions. However, it is possible that this result was due to an additional restriction(s) of viral DNA synthesis. Hirt extracts prepared from 293-31 cells transfected with each mutant were therefore analyzed for DNA replication (Fig. 8). Interestingly, duplex monomers and dimers were detected with all three mutants, whereas unit-length single-stranded genomes were absent in each case (compare with the wild type [pAV1]). The fact that synthesis of single-stranded AAV genomes was not detectable with any of the mutants strongly suggests that these mutants would be incapable of generating any DNA-containing particles. In a complementation assay, in which cells were doubly transfected with pSMA1 (A minus) and pSMB2 (B minus), the single-stranded component was restored. Although this implies that in virion assembly all three structural proteins are necessary for the sequestration of single-stranded genomes, i.e., their capture from the pool of

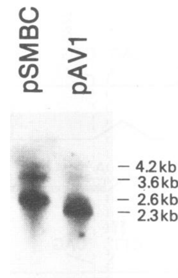

FIG. 5. Northern blot of poly(A) messages from 293-31 cells infected with Ad2 and transfected with plasmids pAV1 (wild type) or pSMBC (mutant). Sizes (in kilobases) of the poly(A) messages are indicated on the right. Plasmids used in the transfections are shown at the top of each lane. Note that with pSMBC, the 2.3-kb spliced mRNA is practically absent, as expected.

replicating DNA molecules, it is important to note that the initiator mutation of the A protein also produces a one-amino-acid substitution in the unspliced p19 nonstructural protein, now reported to be required for accumulation of single-stranded DNA (7) (see Discussion).

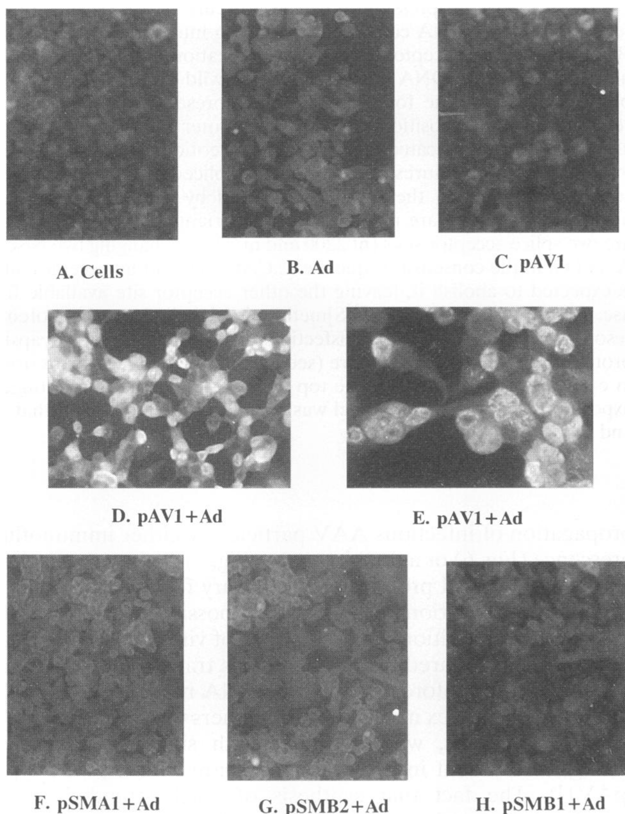

FIG. 6. Detection of infectious AAV virus particles by using immunofluorescence. Crude extracts prepared from 293-31 cells transfected with plasmid pAV1 (wild type), pSMA1 (A^-), pSMB2 (B^-), or pSMB1 (C^-) were used to infect HeLa cells in the presence of Ad. At 48 h postinfection, cells were fixed and assayed by indirect immunofluorescence for the presence of AAV virus particles. The DNA used in the initial transfection is shown below each panel. Note the nuclear localization of the AAV capsid proteins in panel E.

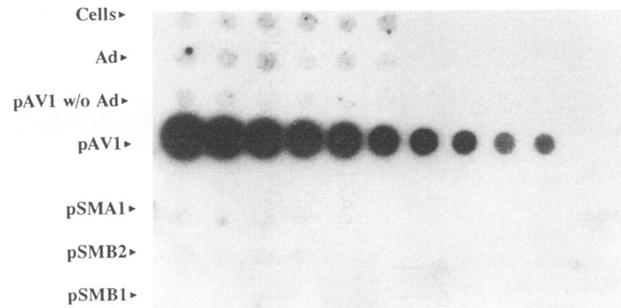

FIG. 7. Detection of infectious AAV virus particles by using a dot blot assay. Crude extracts prepared from 293-31 cells transfected with plasmid pAV1 (wild type), pSMA1 (A^-), pSMB2 (B^-), or pSMB1 (C^-) were used to infect 293-31 cells. At 24 h postinfection, viral DNA was extracted by a modified Hirt procedure and analyzed by a dot blot assay. The DNA used in the transfection is depicted by arrows on the left. The wells represent serial twofold dilutions of DNA from left to right.

DISCUSSION

On the basis of sequence alterations introduced by site-directed mutagenesis, we have mapped initiation codons of the three AAV2 structural proteins and the splice acceptor sites responsible for generating the two mRNAs from which these proteins arise. Although the initiator for protein A was deduced previously as the AUG at nt 2203 (2, 6), it has now been definitively mapped by demonstrating that conversion of this AUG to a UUG codon (plasmid pSMA1) selectively abolished the synthesis of A. Protein A thus initiates in the first reading

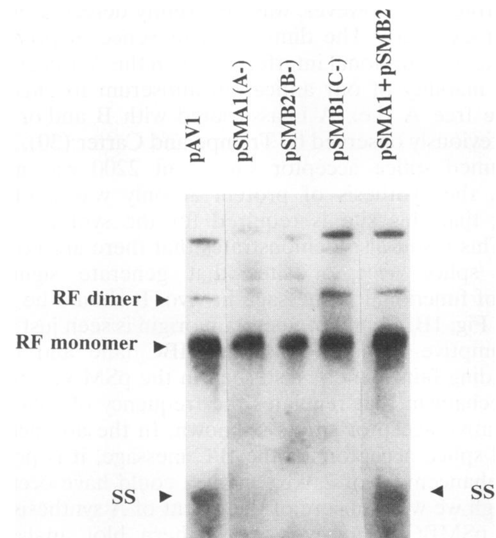

FIG. 8. Southern blot of viral DNA extracted from 293-31 cells. Cells were transfected with plasmid pAV1 (wild type), pSMA1 (A^-), pSMB2 (B^-), or pSMB1 (C^-) or cotransfected with pSMA1 and pSMB2, and the viral DNA was extracted at 36 to 40 h posttransfection by a modified Hirt procedure. The replicative form (RF) double-stranded monomer, RF double-stranded dimer, and the single-stranded (SS) forms of AAV DNA are depicted by arrows on the left. The bands migrating more slowly than the RF dimer represent higher concatameric forms of DNA. The plasmid(s) used in the transfection is shown above each lane. The lanes with the mutants pSMA1, pSMB2, and pSMB1 represent a longer exposure and do not reveal any single-stranded DNA.

frame, extending directly into the open reading frame of B and C. As already noted, the faint band migrating slightly faster than A (Fig. 1B, asterisk) could correspond to another viral protein (VP1a) reported by Trempe and Carter (30). They suggested that VP1a originates at an ACG (nt 1987) upstream from the splice donor site and that it is translated from the $2.3kb_{B/C}$ RNA species. Although they did not detect this protein in purified virions, a possible corresponding protein has been observed by McPherson and Rose (21) in purified preparations of AAV1 and AAV2 virions. The appearance of a similarly positioned band with the $2.3kb_{B/C}$ RNA splice acceptor site mutant, pSMBC (Fig. 4B), was unexpected, but this band could represent a contaminant made visible by the longer exposure shown for the pSMBC mutant, since most of the visible bands in this channel were absent in the shorter exposure used for the pSMA2 and pAV1 channels. The very faint candidate band which is seen with the pSMA2 mutant would be expected. Possible reasons for the apparent decrease or even absence of detectable A have been mentioned (see Results). The less than anticipated increase in the amount of protein B, when its ACG initiator was converted to AUG (Fig. 1B, pSMB1), might also be explained by inefficient precipitation by our anticapsid antiserum in the absence of C (30).

We believe that the results depicted in Fig. 1B provide a direct demonstration that the ratio of protein B to protein C is regulated by protein B's relatively weak ACG initiator. The natural use of ACG as an initiation codon in eucaryotes has been described in only one other instance. Curran and Kolakofsky (8) reported that an ACG initiates synthesis of a Sendai virus nonstructural protein (protein B). As is the case for AAV, this ACG codon also lies in an optimal context for AUG initiation, with a purine at position -3 and a G at $+4$. Furthermore, the sequence positions $+5$ to $+10$ (with the exception of $+7$) are identical for both viruses. In addition, there are two artificial situations in which an ACG codon has been shown to initiate protein synthesis. In the bacteriophage T7 mRNA, a naturally occurring AUG has been mutagenized to an ACG, and translation of the corresponding protein was diminished to 12 to 18% of the wild type (1). In another example, the natural AUG of the mouse dihydrofolate reductase gene was changed to an ACG and, upon transfection into COS cells, was found to synthesize the dihydrofolate reductase protein at about 5% of the wild-type level (24). Translational initiation at yet another non-AUG codon, a CUG, has been observed in the c-Myc proteins (12). The c-*myc* gene has three exons with an AUG-initiated open reading frame in exon 2 and a CUG-initiated open reading frame beginning in exon 1, both being read in the same reading frame. In Burkitt's lymphoma, removal or mutation of exon 1 in c-*myc* translocations has been correlated with the suppression of the synthesis of the larger c-Myc protein initiated by CUG in exon 1, contributing perhaps to the oncogenic activation of c-*myc* (12). Thus, the use of ACG and other non-AUG codons as translational initiators may well be a means for regulating the synthesis of critical or even potentially toxic proteins required in relatively small amounts. Our results concerning regulation of translation of the AAV2 C protein are consistent with a scanning mechanism for translational initiation in eucaryotes previously proposed by Kozak (16), i.e., C synthesis does not result from internal initiations but occurs because the bound initiation complex frequently scans past a weak ACG initiator (2; this study).

Mutational analysis of the previously identified splice acceptor sites (2, 6, 30), involving a dinucleotide substitution in the consensus sequence ($Py_{16}NCAG:G$ to $Py_{16}NCTT:G$), confirmed their roles in alternative splicing (Fig. 4). Again, as noted by Becerra et al. (2), there was no evidence for the existence of any other message capable of synthesizing A. The use of alternative splicing to generate the largest capsid protein has also been observed in studies with autonomous parvoviruses, although the strategy used in these cases generally differs from that observed with AAV, which uses one donor and two acceptor sites (2, 6, 15, 25, 30).

In a recent report, Smuda and Carter (28) described two conditional lethal mutants of AAV having amber mutations affecting either the A protein (AAV VP1*am*) or all three capsid proteins (AAV Cap*am*). Neither mutation overlaps *rep* gene sequences. In nonpermissive cells, AAV VP1*am* produced a low level of infectivity, whereas no infectivity was detected with AAV Cap*am*. In the present study, genomic clones that contained initiator mutations which abolished the synthesis of A, B, or C (Fig. 1) did not generate infectious AAV, as assayed by passage of extracts from transfected 293-31 cells into HeLa cell cultures (Fig. 6). In the case of the A mutant, however, conversion of its AUG initiator to UUG would also produce a mutation (Tyr to Phe) in the *rep* gene proteins read from the nonspliced versions of the p5 and p19 mRNAs. Although this mutation substitutes a structurally similar amino acid (i.e., a semiconservative mutation) and might not be expected to impair, completely, the activities of these proteins, we cannot rule out the possibility that it actually does interfere with the accumulation of single-stranded DNA progeny molecules (7). Thus, while we can conclude that both B and C must be present for sequestration of single-stranded viral genomes (and hence virion infectivity [Fig. 6, 7, and 8]), we are not certain whether B and C together are capable of carrying out this function, that is, whether there is either a partial or absolute requirement for protein A. While our results have now defined a sequestration requirement for protein B, the role of A still remains unclear. Although others have suggested that protein A may not be absolutely necessary for genomic sequestration or even virion infectivity, these studies have not excluded completely the production of modified and potentially active A proteins (13, 28, 29). Finally, it should be noted that our findings exclude the possibility that protein C has evolved because of a natural mutation in the B initiator (i.e., AUG to ACG). If this were the case, mutant pSMB1 should have been infectious.

ACKNOWLEDGMENTS

We thank E. Sebring and F. Koczot for many helpful discussions.

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