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Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA

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ABSTRACT **AAV is unique among eukaryotic viruses in the ability of its DNA to integrate preferentially into a specific region of the human genome. Understanding AAV integration may aid in developing gene therapy systems with predictable integration sites. Using a gel mobility-shift assay, we have identified a DNA sequence within the AAV integration locus on human chromosome 19 which is specifically bound by the AAV Rep78 and Rep68 proteins. This Rep recognition sequence is a GCTC repeating motif very similar to sequences within the inverted terminal repeats of the AAV genome which are also bound by Rep78 and Rep68. Cloned oligonucleotides containing the recognition sequence can direct specific binding by Rep proteins. Binding assays with mutant Rep proteins show that the amino-terminal portion of Rep78 and Rep68 can direct binding to either the AAV terminal repeat hairpin DNA or chromosome 19. This human genomic DNA can be complexed with AAV DNA by Rep proteins as demonstrated by a dual-label (^{32}P/biotin) assay. These results suggest a role for Rep in targeting viral integration.**

AAV type 2 is being developed as a vector for human gene therapy (1–5). An attractive feature of AAV is that the viral DNA preferentially integrates within a defined region of the cellular genome, thus reducing the risks of insertional mutagenesis associated with vectors that integrate randomly. AAV is a nonpathogenic human parvovirus which usually requires adenovirus or herpesvirus as a helper to replicate efficiently. In the absence of helper virus the AAV genome integrates stably into host-cell genomic DNA at high frequency (6–8). Analyses of flanking sequences from latently infected cells have revealed integration of the AAV genome into a specific locus in 60–70% of cases (9, 10). The integration locus (AAVS1) has been sequenced and localized to human chromosome 19q13.3–qter (11–13). Sites of integration have been mapped to multiple points within AAVS1 (9, 10). The AAV genome is a single-stranded linear DNA molecule with terminal repeats at both ends which fold into hairpin structures and function as origins of replication (Fig. 1) (15, 16). AAV vectors containing only the viral terminal repeats are proficient for integration but have not been observed to target to 19q (5, 10). AAV vectors which contain an intact *rep* gene as well as terminal repeats integrate into AAVS1 at high frequencies (A. N. Shelling and M. G. Smith, personal communication). Targeting of integration may therefore involve AAV *rep* gene products. The *rep* open reading frame encodes four overlapping Rep proteins (Fig. 1) necessary for DNA replication (16–19). The larger Rep proteins (Rep78 and Rep68) bind specifically to AAV terminal hairpin DNA (20–22) and possess helicase and site-specific endonuclease activities required for AAV replication (23).

We investigated the role of Rep proteins in specific integration by using an electrophoretic mobility-shift assay to detect Rep binding sites within the integration locus. We show that Rep78 and Rep68 specifically bind to a 109-bp DNA fragment from human chromosome 19 near sites of viral integration in AAVS1. We have identified a Rep recognition sequence (RRS) which is similar to the Rep-binding site on AAV hairpin DNA (14). We also demonstrate that Rep proteins can mediate complex formation between the DNA of human chromosome 19 and AAV hairpin DNA.

METHODS

Plasmids. The 109-bp *Sma* I fragment designated P1 begins at nucleotide 354 of the AAVS1 sequence (13) and was cloned into the *Sma* I site of pUC18 to generate the plasmid pMAT50. Oligonucleotide pairs containing the wild-type RRS or a mutant version (mRRS) were synthesized, annealed, and cloned into the multiple cloning site (MCS) of pBluescript II SK(+) (Stratagene). The oligonucleotides contained *Bam*HI-compatible sequences at both ends of the following core sequences; RRS, 5′-GC(GCTC)$_3$GCTGGG-3′; mRRS, 5′-GC(CCTC)$_3$CCTCGG-3′.

Electrophoretic Mobility-Shift Assay. Assays were performed with 3′-^{32}P-end-labeled DNA fragments essentially as described previously for AAV hairpin DNA (21, 22). Reaction mixtures (20 μl) contained 50 mM NaCl, 25 mM Hepes/KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2% (vol/vol) glycerol, 0.5 μg of bovine serum albumin, 0.01% Nonidet P-40, and 1 μg of poly(dI-dC). The presence and relative abundance of Rep proteins of the expected sizes were tested by SDS/PAGE analysis for all nuclear extracts and *in vitro* translations. A rabbit antibody against the S18K oligopeptide (Rep78 amino acids 516–533) was added in a volume of 1 μl where indicated (19). Nuclear extracts were diluted 10-fold (14). Two microliters of rabbit reticulocyte lysate was added to reaction mixtures with *in vitro* translated proteins, as described (14).

Methylation Interference Assays. These assays were performed as described (14). The substrate used was the P1-containing *Hin*dIII–*Eco*RI fragment from pMAT50. The fragment was labeled at the 3′-end of either strand with DNA polymerase I Klenow fragment and [α-^{32}P]dATP by digestion with one restriction enzyme followed by the Klenow fill-in reaction prior to digestion with the second restriction enzyme.

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Abbreviations: AAV, adeno-associated virus; MCS, multiple cloning site; RRS, Rep recognition site; mRRS, mutant Rep recognition site.

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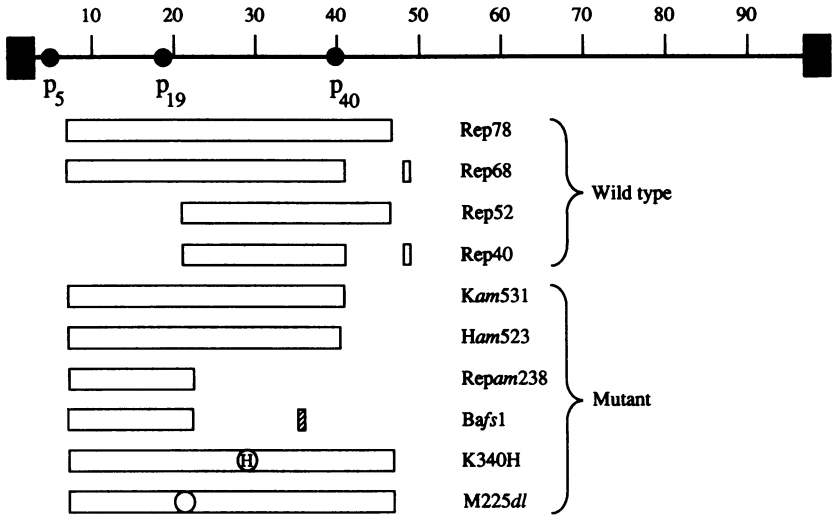

FIG. 1. The AAV genome (4680 nucleotides) is shown as a single bar on a 100-map-unit scale. Filled boxes represent the inverted terminal repeats, and filled circles indicate transcription promoters. Wild-type and mutant Rep proteins (14) are represented by open boxes. The hatched box depicts missense amino acid sequences in the Ba*fs*1 frameshift mutant, and open circles indicate mutagenesis of single amino acids. Lys-340 was replaced with a histidine in K340H. Met-225 was deleted in M225*dl*.

Assay for Rep-Mediated DNA Complexes. Biotinylated AAV hairpin DNA substrate was prepared similarly to the previously described ^{32}P-labeled substrate (22). The hairpin terminal repeat was 3′ labeled by a fill-in reaction with DNA polymerase I Klenow fragment and biotin-14-dATP (GIBCO/BRL) plus unlabeled dCTP, dGTP, and dTTP. The substrate was purified from an 8% polyacrylamide gel and the DNA concentration determined with the BluGENE nonradioactive nucleic acid detection system (GIBCO/BRL). Biotinylated AAV hairpin DNA (0.1 ng) was incubated with nuclear extracts from human 293 embryonic kidney cells in the standard binding reaction (14). After 10 min of incubation on ice, 100,000 cpm of ^{32}P-labeled DNA was added and reactions continued for a further 20 min. Streptavidin-agarose beads (20 μl) prewashed three times in wash buffer [binding reaction mixture without poly(dI-dC)] were added and mixtures were incubated for 1 hr at 4°C with gentle agitation. The beads were pelleted and washed four times with 1 ml of ice-cold wash buffer. The resin was resuspended in 20 μl and treated with proteinase K (20 μg) in a reaction mixture containing 10 mM EDTA (pH 7.5) and 0.2% SDS and incubated at 37°C for 1 hr. The DNA fragments were separated in a nondenaturing 6% polyacrylamide gel with 90 mM Tris/borate/2 mM EDTA, and the gel was dried for autoradiography.

RESULTS

Binding of Rep to the Integration Locus. Cloned DNA sequences from AAVS1 were ^{32}P-end-labeled and screened for binding with Rep proteins. The proteins either were synthesized *in vitro* or were in nuclear extracts of human 293 cells transfected with constructs containing the *rep* gene under control of the long terminal repeat promoter of human immunodeficiency virus type 1 (14). Binding was detected for a 109-bp *Sma* I fragment (designated P1) of AAVS1 with nuclear extracts containing Rep78 and Rep68 (Fig. 2*A*). The P1 fragment is within 455 bases of the sites mapped as recombination junctions between AAV and human DNA (10, 13). We screened 1.5 kb of the AAVS1 sequence and no other DNA fragment was bound by Rep. Binding was also shown with *in vitro* translated Rep78 and Rep68 (Fig. 2*C*) but was not detected for the two shorter Rep proteins, Rep52 and Rep40 (Fig. 2*C*). An antibody made against a specific oligopeptide (anti-S18K) of the Rep protein (19) caused a supershift of Rep78- or Rep68-dependent complexes when included in the binding assay mixture (Fig. 2*A*). Mutant Rep78 derivatives (Fig. 1) were tested for DNA binding to delineate the protein sequences required for binding to human genomic DNA (Fig. 2*B*). Truncation mutants with deletions at the carboxyl terminus (to amino acids 531 and 523) bound the P1 fragment. A mutant protein with an amino acid substitution in the consensus nucleoside triphosphate (NTP) binding site (K340H) bound P1 suggesting that NTP-binding was not required for complex formation. The M225*dl* mutant Rep (with Met-225 deleted) bound weakly to P1. A truncated protein with a frameshift mutation at the *Bam*HI site of the *rep* gene (Ba*fs*1) produced a single shifted band, whereas a mutant protein with an amber mutation at residue 238 (Rep*am*238) failed to bind DNA. The pattern of binding for all

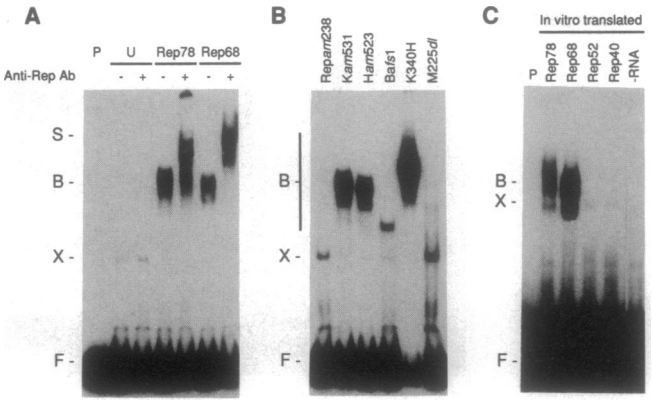

FIG. 2. Binding of Rep proteins to the AAVS1 integration site in the mobility-shift assay. Lane P contained only the ^{32}P-labeled P1 fragment. Positions of free DNA (F), the Rep-specific bound complexes (B), the antibody-supershifted complexes (S), and complexes with cellular factor(s) (X) are indicated. (*A*) Analysis of nuclear extracts from human 293 cells that were either untransfected (U) or transfected with pARtat plus plasmids encoding Rep78 or Rep68 under control of the long terminal repeat of human immunodeficiency virus (14). Reactions were performed without (−) or with (+) anti-Rep S18K antibody (Ab) (19). (*B*) Analysis of nuclear extracts from 293 cells expressing mutant Rep proteins (Fig. 1). (*C*) Analysis of Rep proteins synthesized *in vitro* in a rabbit reticulocyte lysate. A lysate that was not programmed with RNA was included as a negative control (−RNA).

mutant Rep proteins mirrored their relative abilities to bind to AAV hairpin DNA (14), including the presence or absence of the previously observed pattern of multiple shifted bands (14, 20, 22).

Identification of a RRS. Previous footprint analyses of Rep binding to AAV DNA indicated sequences involved in binding, which included four methylation-sensitive guanine residues within the A′ region of the hairpin (14, 20, 21) that were part of a GCTC repeating motif. Protein contact points within the P1 fragment were studied by the methylation interference assay. The P1 fragment was partially methylated *in vitro* and incubated with Rep68 in the mobility-shift assay. Both bound and free DNA fragments were recovered, cleaved at methylated guanine residues, and analyzed by electrophoresis in a sequencing gel. Assays of both strands were performed three times and showed only modest differences between Rep78-bound and free DNA (Fig. 3*A*). In all cases there were four guanine residues, within a GCTC motif, whose methylation interfered with Rep78 binding. Slightly weaker interference was seen with the complementary strand (Fig. 3 *A* and *B*). It was difficult to obtain a clear footprint region, due to the use of nuclear extracts. No other reproducible footprint pattern specific to the Rep protein was found, although this region does contain binding-site motifs for other proteins that might be present within the nuclear extracts (13). The protein contact points for Rep on the P1 sequence (Fig. 3*B*) were almost identical to those identified on the AAV terminal hairpin DNA (14) (Fig. 3*B*) and agreed with DNase I footprint data on the P1 substrate (not shown).

The methylation interference results were consistent with the model that the guanine residues within the GCTC motif (Fig. 3*B*) are important for binding, but the results were not conclusive. We inserted this GCTC motif into a different background to see whether it could confer binding. An annealed oligonucleotide pair containing this RRS was cloned into the MCS of pBluescript II SK(+). A fragment encompassing the entire MCS and the cloned oligonucleotides was radiolabeled and used as a substrate in the electrophoretic

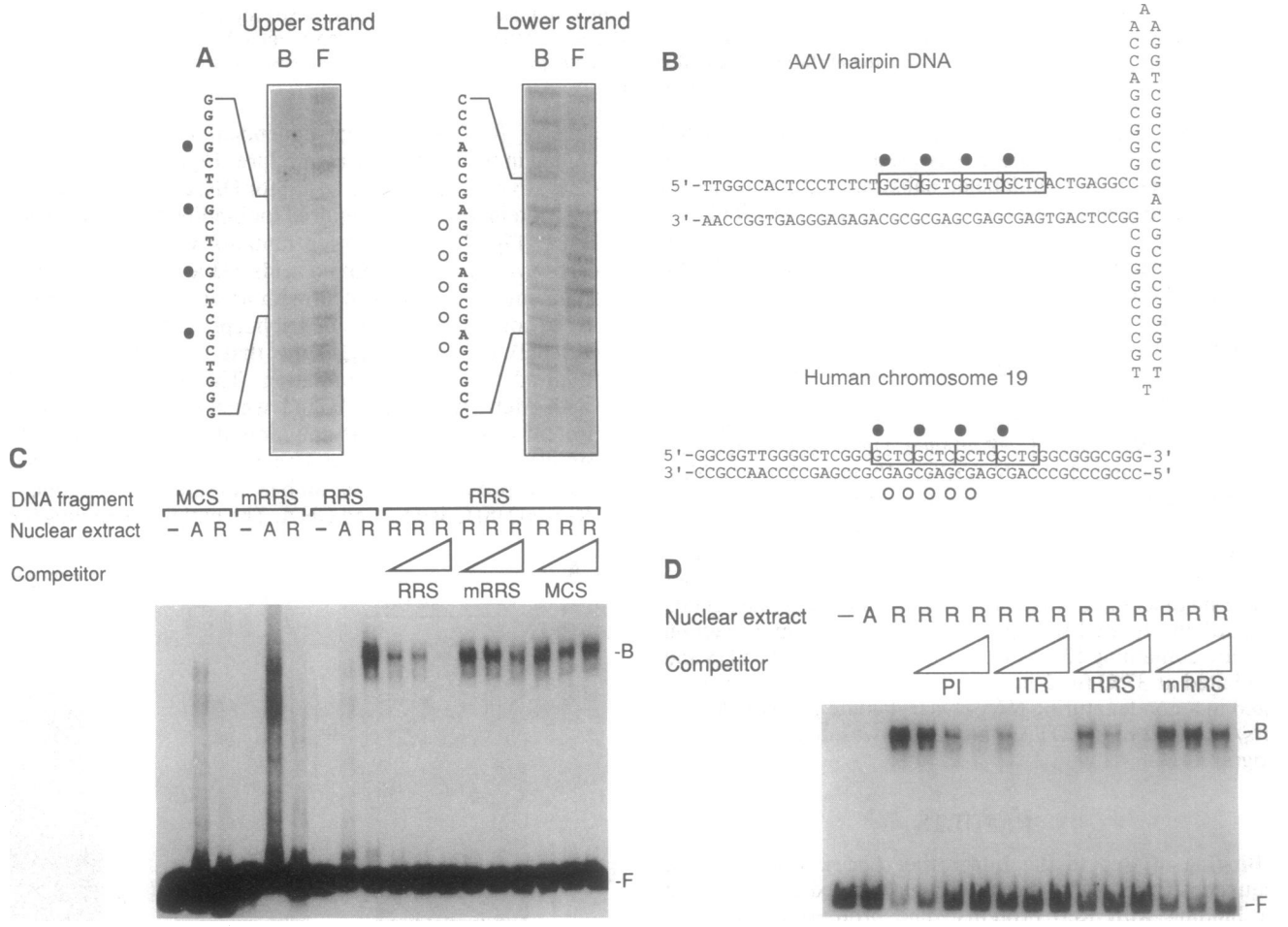

FIG. 3. Identification of a Rep recognition sequence. (*A*) The P1 fragment was end-labeled on either strand and partially methylated at guanine residues by dimethyl sulfate. Methylated DNA was incubated with nuclear extracts containing Rep78. Bound DNA (B) was separated from free DNA (F) in a nondenaturing gel. DNA was eluted and cleaved with piperidine, and samples containing equal counts were electrophoresed in an 8% polyacrylamide denaturing gel. Circles represent methylation-sensitive sites (○, partial interference; ●, strong interference). Assay-to-assay variation in band intensities occurred, and only the reproducible changes are marked. Bands above the marked methylation-sensitive area on the assay of the upper strand appear to be weaker for the bound DNA in this gel, but this was not observed in other trials. Upper and lower strands refer to the human P1 sequence as depicted in *B*. (*B*) Sequences bound by Rep on the AAV terminal repeat (14) and the P1 fragment of human chromosome 19 (13). GCTC repeats are boxed and circles are as in *A*. This region was resequenced and is shown with corrections to the published sequence (13). (*C*) The *Bss*HII fragment encompassing the MCS was isolated from pBluescript II SK(+) (MCS fragment was 173 nucleotides long) or from plasmids containing the RRS or mRRS cloned into the *Bam*HI site of the MCS. Fragments were 3′-end-labeled and used in the mobility-shift assay alone (−) or with nuclear extract containing Rep68 (R) or the Rep*am*238 mutant (A). Increasing molar ratios (5, 10, and 100) of unlabeled DNA fragments to labeled fragment were added as competitors. Positions of free (F) and Rep-bound (B) DNA are indicated. (*D*) The P1 fragment was 3′-end-labeled and used in the mobility-shift assay alone (−) or with Rep68 (R) or Rep*am*238 (A) nuclear extract. Increasing molar ratios (10, 40, and 160) of unlabeled P1 fragment (PI), AAV inverted terminal repeat hairpin DNA (ITR), or the *Bss*HII fragment of either the cloned RRS or the cloned mRRS were added as competitors.

mobility-shift assay. The Rep protein specifically bound to a DNA fragment containing the RRS, whereas no Rep-dependent complex was detected for the MCS alone (Fig. 3*C*). No Rep-dependent complex was detected for a fragment containing a mRRS in which the guanine residues shown by the methylation interference assay to be critical for Rep binding were replaced with cytosine residues (Fig. 3*C*). Rep binding to the RRS was blocked by an excess of unlabeled DNA fragment containing the RRS but not by the MCS alone or by the mRRS (Fig. 3*C*). The cloned RRS fragment and AAV terminal repeat hairpin DNA, but not the cloned mRRS fragment, competed with radiolabeled P1 fragment for binding to Rep68 (Fig. 3*D*). An excess of unlabeled fragment from an adjacent region of AAVS1 failed to compete (data not shown). The annealed synthesized oligonucleotides without flanking MCS sequences could not be bound by Rep protein in the mobility-shift assay (data not shown).

Rep-Mediated Complex Formation. Since Rep68 and Rep78 can bind to both AAV and human genomic DNA, it seemed plausible that Rep proteins could mediate an interaction between viral hairpin DNA and the insertion target. To test this hypothesis, we developed an assay in which streptavidin-agarose beads are used to isolate complexes formed between biotinylated AAV hairpin DNA and radiolabeled human DNA in the presence of Rep proteins. This assay is similar to other streptavidin-biotin assays (24) and is shown schematically in Fig. 4*A*. The amount of ^{32}P-labeled P1 fragment precipitated by the streptavidin-agarose beads was greatly enhanced by the presence of nuclear extract containing Rep68 (Fig. 4*B*). The truncated Rep*am*238 derivative could not form a complex between the AAV hairpin DNA and the P1 fragment (data not shown). A ^{32}P-labeled *Alu* I DNA fragment comprising nucleotides 510–677 of AAVS1 was used as a negative control fragment. This fragment has a G+C content similar to P1, but only trace amounts were recovered in this assay even in the presence of Rep protein (Fig. 4*B*).

DISCUSSION

The presence of the AAV *rep* gene has been correlated with the targeting of AAV DNA integration into human chromosome 19. We used DNA binding assays as a first step toward defining the possible role of Rep proteins in targeting viral integration. Rep78 and Rep68 were found to bind to a specific DNA sequence from chromosome 19 in the vicinity of the sites of viral integration. This ability to bind to human chromosomal DNA at the integration locus provides direct support for the involvement of Rep in the specific integration of AAV. This work also demonstrates the binding of Rep to non-AAV DNA sequences and to a linear duplex DNA molecule. It was previously shown that Rep78 and Rep68 could bind to AAV terminal repeat hairpin DNA (20–22), but it was suggested that Rep could form a complex with the terminal repeat only when it was in the hairpin configuration (21). The ability of Rep to bind to the P1 linear DNA fragment from chromosome 19 demonstrates that the secondary structure is not essential for DNA recognition and binding. This has also been shown by a synthetic truncated terminal repeat lacking the T-structure of the hairpin (Δitr), which can be bound by a maltose-binding protein–Rep fusion in electrophoretic mobility-shift assays (25).

Using a series of mutant Rep proteins, we previously showed that the amino-terminal portion of Rep78 and Rep68 contains a domain which can direct binding to AAV terminal hairpin DNA (14). Here we have determined that the amino-terminal portion of Rep78 and Rep68 can also direct binding to the P1 fragment of chromosome 19. A pattern of multiple shifted bands was observed for Rep binding to the P1 fragment in the mobility-shift assay (Fig. 2 *A* and *C*), as was previously observed for Rep binding to AAV hairpin DNA (14, 22). The bands may represent multiple Rep molecules

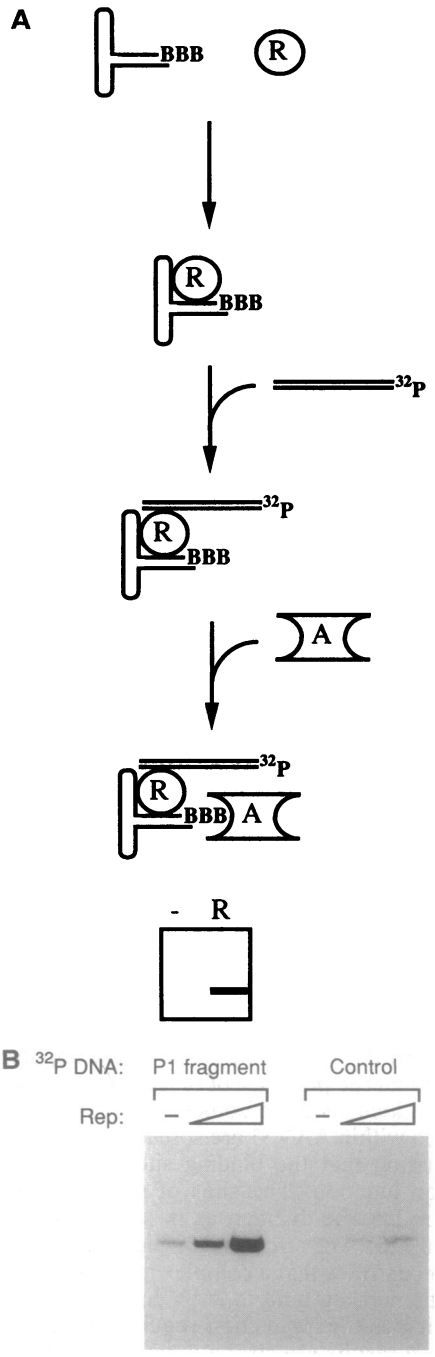

FIG. 4. Rep-mediated DNA complexes. (*A*) Schematic of the assay. Biotinylated AAV terminal hairpin DNA was incubated with nuclear extract from human 293 embryonic kidney cells containing Rep protein (R) or with control extract. Fragments from AAVS1 were added as the ^{32}P-labeled DNA species. Streptavidin-agarose beads (labeled A) were added, pelleted, and washed in wash buffer. Proteinase K released the radiolabeled DNA from the complex. Gel electrophoresis and autoradiography detected radiolabeled DNA that had been specifically isolated through the protein complex on the biotinylated AAV hairpin DNA. (*B*) Complex formation between biotinylated AAV hairpin DNA and either ^{32}P-labeled P1 fragment or a control fragment. All reaction mixtures contained the same amount of nuclear extract from human 293 cells that were transfected with pARtat plus either pGEM-4Z(−) (Promega) or pSK9 (Rep) (14). Increasing amounts of Rep extract were added [left to right: no Rep extract plus 2 μl of GEM extract (−); 1 μl of Rep extract plus 1 μl of GEM extract; 2 μl of Rep extract.

bound to the DNA or conformational changes of the DNA–protein complex.

Methylation interference assays identified a binding motif for the Rep proteins near the AAV integration site on chromosome 19. This motif consists of a GCTC repeating unit and does not resemble any known DNA motifs recognized by other DNA-binding proteins. This GCTC motif resembles sequences on the AAV terminal hairpin DNA contacted by Rep (Fig. 3*B*). When cloned into the MCS of the plasmid pBluescript II SK(+), this sequence imparted the ability to be bound by Rep protein. Competition with unlabeled DNA fragments showed the specificity of this binding. The methylation interference assay and the inability of the mRRS to be recognized by Rep suggest that the guanine residues are important for binding. The annealed oligonucleotides alone were insufficient for Rep binding, suggesting that flanking sequences are required to stabilize DNA binding. This suggestion is supported by DNase I footprint analysis, which showed that a large area of the AAV hairpin was protected by Rep (21). A screen of 1993 Genbank (Release 80) has revealed that this sequence is present at multiple locations within the human genome, and it will be of interest to determine whether Rep binds to this sequence at additional sites and what are the effects. The specificity for integration into AAVS1 is not absolute, since 20–30% of integration events occur elsewhere. It will be interesting to find out whether this Rep recognition sequence is present near other sites of integration.

Our data suggest that the primary binding and recognition site for Rep78 and Rep68 on the AAV hairpin is 16 bases away from the terminal resolution site where the proteins make an ATP-dependent endonuclease cut. There are at least three examples of prokaryotic type III restriction endonucleases (*Eco*P1, *Eco*P15, and *Hin*fIII) which are ATP-dependent and cleave 25–27 nucleotides away from their nonpalindromic recognition sites (26). A recent investigation of the substrate requirements of Rep in the *trs* endonuclease reaction suggested that the sequence at the cut site was not involved in recognition of the terminal repeat for specific binding, and also demonstrated a greatly reduced efficiency of cleavage when the cut site was moved an additional 8 bases away from the GCTC motif (27). Rep proteins may nick the DNA within the human integration locus but we detected no endonuclease activity with the P1 substrate (M.D.W., unpublished data). We doubt that the presence of the Rep binding site within the integration locus is mere coincidence. As the sites for viral integration within AAVS1 are spread over >1-kb (9, 10), it is not surprising that the binding site is not precisely at an integration junction. The sites of specific recognition and integration may be distinct, as is seen with bacterial transposon Tn*7* (28). The streptavidin–biotin assay that we developed demonstrates that a complex mediated by Rep proteins can simultaneously bind to AAV hairpin and human DNA, establishing the juxtaposition required for specific integration. These results are strong *in vitro* evidence for a role of the Rep proteins in targeting AAV integration. The complex may be composed of multiple Rep molecules and may include cellular proteins. Within the P1 fragment there are other sites recognized by DNA-binding proteins (e.g., Sp1 and upstream binding factor 1) (13) which may also play a role in AAV integration. Understanding the role of Rep proteins in AAV integration will be important for exploiting AAV as a gene therapy vector.

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