The Rep68 Protein of Adeno-Associated Virus Type 2 Stimulates Expression of the Platelet-Derived Growth Factor B c-*sis* Proto-Oncogene

RAMANI S. WONDERLING AND ROLAND A. OWENS*

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

Rep68 protein, encoded by adeno-associated virus type 2 (AAV), has been previously shown to bind to specific sequences within the viral genome and in human chromosome 19. The effect of AAV Rep protein on human cellular genes is of interest because AAV is being developed as a gene therapy vector. We have identified sequences related to the Rep recognition sequence in the AAV P_5 promoter in or near the c-*sis* proto-oncogene and the genes coding for a hepatocyte glucose transporter, α-A-crystallin, and carcinoma marker GA733-1. The ability of Rep68 to bind to these sites was established by gel shift assays, and the effect of Rep68 on the expression of these genes was tested by semiquantitative reverse transcriptase PCR. Rep68 enhances the expression of the c-*sis* proto-oncogene, which codes for the B polypeptide of platelet-derived growth factor, a multifunctional growth factor that is involved in embryonic development, tissue regeneration, osteogenesis, fibrosis, atherosclerosis, and neoplasia.

Adeno-associated virus type 2 (AAV) is a nonpathogenic human parvovirus which normally requires coinfection with helper viruses such as adenovirus, herpesvirus, or vaccinia virus for productive infection (7, 24). AAV is being aggressively developed as a gene therapy vector (15, 29). Attractive features of AAV include its nonpathogenicity and preferential integration of its genome into a region of human chromosome 19 (16, 23). The first-generation AAV vectors have most of the AAV genome excised except for the inverted terminal repeats, which are required in *cis* for replication and packaging (7). When these vectors turned out to lack preferential integration into chromosome 19, it was discovered that the AAV *rep* gene is required for this specific integration (29).

The AAV *rep* gene encodes four Rep proteins which play a vital role in AAV replication and have varied effects on the host cell. Rep proteins, mainly Rep68 and Rep78, regulate gene expression from the P_5, P_{19}, and P_{40} promoters of AAV (6, 17, 18) and also possess site- and strand-specific endonuclease activity (13) as well as DNA-DNA (13) and RNA-DNA helicase (28) activities. Rep proteins have been shown to bind to Rep recognition sequences (RRSs) in the inverted terminal repeats of AAV (13, 21), the P_5 promoter of AAV (19), the preferred AAV integration locus in human chromosome 19 (27), the human immunodeficiency virus type 1 long terminal repeat (4), and the c-H-*ras* promoter (5) by recognition of an imperfect GCTC or GAGC repeating motif. Rep proteins have been shown to down-regulate human c-*fos*, c-*myc* (12), and H-*ras* gene expression (11). Rep proteins inhibit neoplastic transformation of human cells by bovine papillomavirus (10) or by adenovirus E1a plus an activated *ras* oncogene (14). Rep proteins also inhibit human immunodeficiency virus type 1 replication (3, 20, 22). The mechanism by which Rep proteins accomplish these functions has not yet been fully elucidated.

With the development of AAV as a gene therapy vector and the potential use of the Rep proteins as anticancer and antiviral agents, it is imperative to understand all of the effects of Rep proteins on the human cellular machinery.

We performed a BLASTN homology search (2) using the RRS in the AAV P_5 promoter (GCCCGAGTGAGCACGC) as the query sequence and searched PDB, GB update, GenBank, EMBL update, and EMBL databases. The search parameters set were as follows: expectancy, 1,000; cutoff, 60; matrix, default; genetic code, standard; strand, both; and filters, none. The search revealed the presence of sequences homologous to the AAV P_5 RRS in or near several human cellular genes (Fig. 1A): the c-*sis* proto-oncogene (Y00389), a hepatocyte glucose transporter gene (K03195), the α-A-crystallin gene (J04152), and the gene coding for a carcinoma marker (GA733-1/Trop-2) (X77753 and S7945) (GenBank accession numbers are shown in parentheses). The aim of this search was to identify RRSs matching closely the P_5 RRS, since the latter has been shown to be involved in gene regulation (17). However, because of its stringency, the search did not identify all of the RRSs in the human genome (including the chromosome 19 RRS). The putative RRSs in the c-*sis* proto-oncogene and the hepatocyte glucose transporter gene are located between the mRNA start site and the beginning of the coding region, while the putative RRS in the carcinoma marker gene is located in the coding sequence 271 bp downstream of the ATG which encodes the first methionine (Fig. 1B). In the case of the α-A-crystallin gene, the putative RRS is 1,721 bp upstream of the transcription start site.

The ability of Rep68 to bind to these putative RRSs was tested in electrophoretic mobility shift assays. The assays were performed as described previously (17), using nuclear extracts of 293 cells transfected with plasmids coding for either a truncated nonfunctional mutant of Rep68 (pHIVrep*am*), wild-type Rep68 (pSK9), or the nucleoside triphosphate [NTP] mutant of Rep68 (which has a lysine-to-histidine substitution in the NTP binding region but still has the ability to bind to other RRSs) (pSK9/NTP) as the source of Rep protein. Plasmids pHIVrep*am*, pSK9, and pSK9/NTP are described in detail

* Corresponding author. Mailing address: Laboratory of Molecular and Cellular Biology, NIDDK, National Institutes of Health, Bldg. 8, Rm. 309, 8 Center Dr. MSC 0840, Bethesda, MD 20892-0840. Phone: (301) 496-3359. Fax: (301) 402-0053. Electronic mail address: rolando@bdg8.niddk.nih.gov.
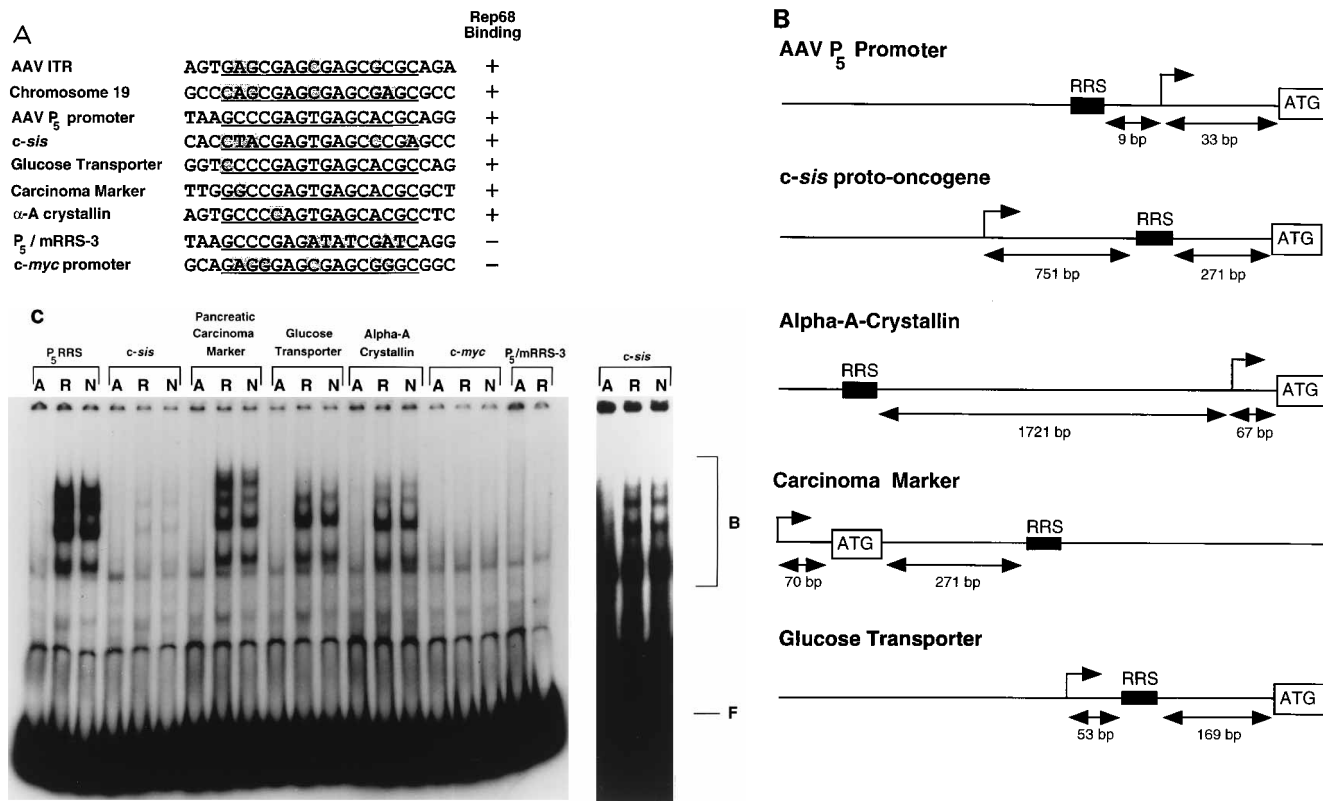
FIG. 1. Rep68 binds to the putative RRSs identified in various cellular genes. (A) Sequences tested for Rep68 binding compared with RRSs in AAV and human chromosome 19. The shading denotes nucleotides that are different compared with the AAV P_5 RRS. The core imperfect repeating GAGC motif is underlined. (B) Locations of RRSs within various genes. ATG represents the ATG which encodes the first methionine codon, and the bent arrow represents the transcription start site. The figure is not to scale. (C) Gel mobility shift assays. A, R, and N represent reactions performed with nuclear extracts from cells transfected with plasmids coding for an amber truncated mutant of Rep68 (Repam), wild-type Rep68, and the NTP binding-site mutant of Rep68, respectively. B and F indicate bound and free DNA, respectively. The last three lanes show the c-sis lanes exposed longer.

elsewhere (3, 17, 21). The substrates consisted of 77-bp radio-labeled DNA fragments containing the different RRSs (as shown in Fig. 1A). A mutant version of the AAV P_5 promoter (P_5/mRRS-3) was included as a negative control (17). As seen in Fig. 1C, both Rep68 and Rep68/NTP bind to the putative RRSs in all four human sequences identified by the BLASTN search. However, Rep binds to the RRS in the c-sis gene with lower affinity compared with the other RRS sequences. Rep68 did not bind to a sequence in the human c-myc P_1 promoter which was very similar to the AAV inverted terminal repeat.

Next we looked at the effect of Rep68 on the expression of these genes that contain RRSs, using semiquantitative reverse transcriptase PCR (RT-PCR) assays. Human 293 cells were transfected with a plasmid coding for either wild-type Rep68 or a truncated nonfunctional mutant of Rep68. Cytoplasmic RNA was isolated 48 h later, and expression of the genes containing RRSs was analyzed by RT-PCR assays using various amounts of cytoplasmic RNA as instructed in the protocol for the Gene Amp RNA-PCR kit (Perkin-Elmer, Foster City, Calif.). A small amount of ^{32}P-labeled forward primer (10^6 cpm) was added to the reaction mixtures to aid in the quantitation of the PCR products. RT-PCR products were analyzed on a 4% poly-acrylamide gel and quantitated with a densitometer (Protein and DNA Imageware Systems, Huntington Station, N.Y.). Sequences of the forward and reverse primers used to detect mRNA encoded by various genes are shown in Table 1. As seen in Fig. 2A, Rep68 increases c-sis mRNA levels by two- to fourfold ($P < 0.001$ in a paired t test) but does not have a

significant effect on hepatocyte glucose transporter gene expression. Neither carcinoma marker nor α-A-crystallin messages could be detected in 293 cells (data not shown).

To control for the RT and PCR steps, the semiquantitative RT-PCR assays for c-sis messages were also done in the presence of c-sis mimic cRNA. c-sis mimic cRNA was produced as follows. A 377-bp unrelated PCR product was made by using primers specific for β-tubulin mRNA. The 377-bp PCR product was subsequently amplified by using forward and reverse composite primers (50-mers). The 5′ half of each composite primer was the same as a c-sis primer, and the 3′ half was specific for the 377-bp β-tubulin PCR product (Table 1). The resultant PCR product was then amplified by using c-sis mRNA-specific primers and blunt-end cloned into plasmid pSP64 poly(A) (Promega, Madison, Wis.) at the SmaI site to produce psisSP64poly(A). c-sis mimic cRNA was produced by using psisSP64poly(A) linearized with EcoRI, SP6 polymerase, and an in vitro transcription kit (Promega). RT-PCR assays done in the presence of the c-sis mimic cRNA also indicated that Rep68 increased the steady-state level of c-sis mRNA (Fig. 2B).

In this study, we have demonstrated for the first time that the Rep68 protein of AAV can up-regulate the expression of a human cellular gene, the c-sis proto-oncogene (which codes for the B chain of platelet-derived growth factor [PDGF]). Alexander et al. (1) have shown that the transduction efficiency of rep⁻ AAV vectors is stimulated by DNA-damaging agents. PDGF has been shown to raise the intracellular levels of hy-

TABLE 1. Primers used in RT-PCR

Primer	Sequence
c-*sis* forward	5′ ATGAGATGCTGAGTGACCACTCGAT 3′
c-*sis* reverse	5′ GTGCCGTCTTGTCATGCGTGTGCTT 3′
Glucose transporter forward	5′ TTGGCTACAACACTGGAGTCATCAA 3′
Glucose transporter reverse	5′ TGGTGGCATACACAGGCTGCTGCAC 3′
Carcinoma marker forward	5′ CTGCTCCACGCTGACCTCCAAGTGT 3′
Carcinoma marker reverse	5′ CCAGTTCCTTGATCTCCACCTTCTT 3′
α-A-crystallin forward	5′ TGAACATGGACGTGACCATCCAGCA 3′
α-A-crystallin reverse	5′ TCCACAAAGTCGTCCTGCACCTTCA 3′
β-Tubulin forward	5′ GGTTGATTCTGTCCTGGATGTGGTA 3′
β-Tubulin reverse	5′ TGGTGTGGTCAGCCTCAGAGTGCGG 3′
c-*sis*-mimic forward composite	5′ ATGAGATGCTGAGTGACCACTCGATGGTTGATTCTGTCCTGGATGTGGTA 3′
c-*sis*-mimic reverse composite	5′ GTGCCGTCTTGTCATGCGTGTGCTTTGGTGTGGTCAGCCTCAGAGTGCGG 3′

drogen peroxide (26). We speculate that the Rep-dependent integration of AAV into its preferred integration site might actually be aided by the increased production of PDGF. Induction of PDGF by Rep68 may lead to an increase in H_2O_2 levels, which may damage DNA via free radicals, which in turn may lead to the induction of repair polymerases that would aid in the integration of the AAV genome.

Wild-type AAV integrates preferentially into an 8-kb region in human chromosome 19, presumably mediated by an RRS and Rep68 or Rep78 (8, 16, 23, 27, 29). Approximately 30% of the time, AAV integrates into other chromosomal locations (23). The c-*sis* proto-oncogene (chromosome 22q12.3-q13.1), hepatocyte glucose transporter (chromosome 22q13.1), carcinoma marker (chromosome 1p32), and α-A-crystallin (chromosome 21) genes might be alternate AAV integration sites.

Human PDGF is the major growth factor of human serum. It is a dimer of two homologous polypeptide chains, A and B,

found in three different combinations (AA, BB, and AB) to create biologically active PDGF. It appears to be an important factor in early development and also appears to modulate tissue regeneration and remodeling during wound healing and osteogenesis. The inappropriate expression of PDGF genes and their mitogenic products has been linked to several proliferative disorders such as fibrosis, atherosclerosis, and neoplasia (9, 25). The ability of Rep68 to up-regulate the c-*sis* proto-oncogene may imply a role for wild-type AAV in these diseases.
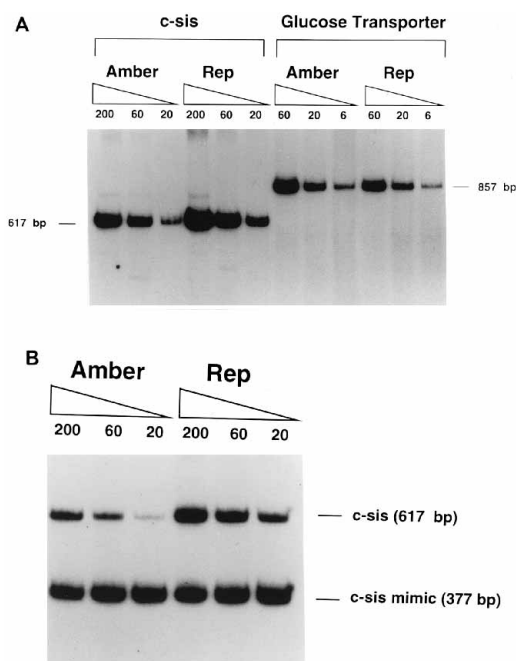

FIG. 2. Rep enhances expression of the c-*sis* proto-oncogene. (A) Amber and Rep represent reactions using RNA isolated from 293 cells transfected with pHIVrep*am* and pSK9, respectively. The amount (in nanograms) of cytoplasmic RNA added in each reaction is indicated above each lane. (B) RT-PCR assays to quantitate c-*sis* messages were done in the presence of 5×10^{-20} mol of c-*sis* mimic cRNA, which served as an internal control for the RT and PCR steps.

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