Transcription-positive cofactor 4 enhances rescue of adeno-associated virus genome from an infectious clone

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While Rep proteins are required for adeno-associated virus (AAV) replication, little is known about cellular proteins that interact with Rep. We demonstrate here that transcription-positive cofactor 4 (PC4, p15) fused to Gal4-activating domain interacted with both AAV-2 and AAV-3 Rep proteins fused to Gal4 DNA-binding domain, leading to reporter activation in the yeast two-hybrid system. In addition to its coactivating function, PC4 recently has been shown to be involved in replication of simian virus 40. To study a functional role for the PC4–Rep protein interaction, 293-31 cells were cotransfected with a PC4 expression plasmid and an infectious clone of AAV-3, followed by coinfection with helper adenovirus. A significantly increased number of AAV-3 genomes were rescued in PC4 transfected cells. Our results support a possible involvement of PC4 in AAV replication and may be used in efficient production of AAV vectors for gene therapy.

Adeno-associated viruses (AAV) are classified as dependoviruses, and efficient replication of the AAV genome requires coinfection with a helper virus such as adenovirus (Ad) or herpesvirus (Atchison et al., 1965; Hoggan et al., 1966; Buller et al., 1981). Among the five serotypes of primate AAV, nucleotide sequences have been determined for AAV-2, AAV-3 and AAV-4 (Srivastava et al., 1983; Muramatsu et al., 1996; Rutledge et al., 1998; Chiorini et al., 1997). The genome of these AAVs contains two large open reading frames (ORF), rep and cap. The nonstructural Rep proteins, products of the rep gene, are required for AAV DNA replication. Rep activities include binding to the terminal hairpin structures (Ashktorab & Srivastava, 1989; Im & Muzyczka, 1989; Chiorini et al., 1994b), ATP-dependent DNA helicase activity (Im & Muzyczka, 1990), sequence-specific DNA endonuclease activity (Im & Muzyczka, 1990), covalent binding to the 5′ end of their DNA substrate (Im & Muzyczka, 1990) and the ability to replicate viral DNA in a cell-free system (Chiorini et al., 1994a; Ni et al., 1994). Rep mutants are defective for viral DNA synthesis and replication (Hermont et al., 1984; Tratschin et al., 1984). Additionally, Rep functions both as a transactivator and a transrepressor of AAV. In AAV-2, the Rep78 and Rep68 proteins (the full-length ORF products derived from transcripts initiated from the p5 promoter; Rep68 is produced by a splicing event at the 3′ end of its RNA) have been shown to repress both p5 and p19 transcription in the absence of Ad (Horer et al., 1995; Kyostio et al., 1995), and to activate p19 and p40 promoter in the presence of Ad (Labow et al., 1986; McCarty et al., 1991; Pereira et al., 1997). In studies designed to elucidate the mechanism of promoter regulation in AAV, several cellular and viral proteins were characterized. The Ad early region 1a protein (E1a) functions to activate the p5 and p19 promoters and is facilitated by interactions with the p5-bound cellular proteins YY1 and the major late transcription factor (Laughlin et al., 1982; Chang et al., 1989; Shi et al., 1991). Sp1 protein and an unknown 34 kDa protein are probably involved in the ability of Rep to transactivate the p19 promoter (Pereira & Muzyczka, 1997). Nevertheless, little is known about cellular factors which interact with Rep proteins and are involved in viral DNA replication.

We employed the yeast two-hybrid system (MATCHMAKER; Clontech) to identify cellular proteins that interact with AAV-3 Rep protein. The coding region of AAV-3 Rep protein (nucleotides 318–2189) was cloned into plasmid pGBT9, and the fusion protein of Gal4 BD and AAV-3 Rep was used as a bait for screening a human cDNA library derived from HeLa S3 cells. The cDNA was fused to the GAL4 AD in a pGADGH vector. Using the lithium acetate procedure (Gietz et al., 1992), HF7c yeast cells were sequentially transformed with pGADGH-Rep and pGADGH-cDNA-library. Cotransformants (106) were cultured on selective plates containing tryptophan (Trp)-, leucine (Leu)- and histidine (His)-deficient synthetic medium, and screened for expression of the two reporter genes, his3 and lacZ. His3+ LacZ+ yeast transformants were purified by replating on plates containing synthetic medium without Trp, Leu or His. A total of 11 double-positive colonies was obtained. The cDNA plasmids extracted from double-positive yeast colonies were transfected into E. coli.
HB101 cells in order to rescue cDNA library plasmids. Isolated clones were sequenced by the dideoxynucleotide chain termination method using either Sequenase II (Amersham) for manual sequencing or AmpliTaq DNA Polymerase FS (Perkin Elmer) for automated DNA sequencing (Applied Biosystems). Nucleotide sequences were compared with the nucleotide sequences deposited in the GENEINFOR BLAST network server at the National Center for Biotechnology Information (Bethesda, MD). Seven out of eleven cDNA plasmids carried identical inserts with poly(A) tails. Homology searching revealed that these seven sequences matched the sequence of transcription-positive coactivator 4 (PC4, p15; GenBank accession no. X79805). Sequences of the other four positive clones contained similar short sequences of either human Alu sequence, ribosomal protein L29 (accession no. U10248), cytoplasmic linker protein-170 (Restin; accession no. M97501) or mitochondrial genes for tRNAs (accession no. V00710), and were not analysed further. Further studies focused on PC4.

PC4, also designated p15, is one of the upstream stimulatory activity (USA)-derived cofactors which are required in activator-dependent transcription (Ge & Roeder, 1994; Kretzschmar et al., 1994). PC4 contains 127 amino acids with two short stretches rich in serine and acidic residues near the amino terminus. This amino-terminal region shares sequence homology with viral immediate early proteins involved in transcriptional regulation, such as IE62, ICP4 and IE180 of varicella-zoster virus, herpes simplex virus type 1 and pseudorabies virus, respectively (Kretzschmar et al., 1994). PC4 acts as an adaptor by virtue of direct interactions both with the activation domain of a conventional activator such as VP16 and with a component (TFIIA or TBP–TFIIA complex) of the general transcriptional machinery (Ge & Roeder, 1994).

To address binding specificity, full-length PC4 was cloned into pGADGH and was retransfected into HF7c cells with pGBT9/AAV-3 Rep. As controls, pGBT9 containing human laminin C_{66-230} (pLAM5') was also transfected with pGADGH/PC4. HF7c cotransformants with pGBT9/AAV-3 Rep and pGADGH/PC4 gave rise to β-galactosidase-positive colonies. No double-positive colonies were obtained using control plasmids. To further examine the specificity, similar
experiments were carried out using another yeast strain, SFY526, and by swapping domains (i.e. Rep fused to Gal4 BD and PC4 fused to Gal 4AD), and all showed comparable results (data not shown). These data demonstrate that the interaction between AAV-3 Rep fused to Gal4 BD or AD and PC4 fused to Gal4 AD or BD is specific at least in yeast cells. For quantitative liquid-culture β-galactosidase assay, AAV-3 Rep and PC4 cDNA plasmids were subcloned into the high-expression plasmids pAS2-1 and pACT2 - respectively, and transformed into yeast strain Y187 together with other control plasmids. AAV-2 Rep78 coding sequence was subcloned into pAS2-1 from pAV2 (ATCC). The truncated AAV-3 Rep constructs encoding amino acids 1–442 or 103–624 were made by cleaving AAV-3 Rep in pAS2-1 with NcoI or BglI followed by self-ligation. All assays were done with at least three independent transformants with chemiluminescence detection (Clontech) using a tube luminometer. In yeast strain Y187 cotransformed with pACT2/PC4 and pAS2-1 containing either AAV-2 Rep78 or AAV-3 Rep, marked reporter activation was detected (Fig. 1a). In contrast, pAS2-1 alone or with pACT2/PC4, pAS2-1/AAV-3 Rep alone or with pACT2, and pLAM5-1 with pACT2/PC4 all resulted in minimal reporter activity. As was expected from the high homology (88%) and the presence of a conserved ATP/GTP-binding site motif and a putative zinc-binding motif between AAV-3 Rep protein and AAV-2 Rep78 protein (Muramatsu et al., 1996), we could show that either of the two Rep proteins fused to Gal4 BD interacted with PC4 fused to Gal4 AD. However, neither N-terminal nor C-terminal truncated AAV-3 Rep proteins activated reporter (Fig. 1b), perhaps because the large deletions caused loss of structural conformation in the remaining portion of the fusion proteins and hence loss of function.

PC4 binds both single- and double-stranded (ss and ds) DNA, and as a general activator the non-specific binding properties to dsDNA are important (Ge et al., 1994; Kaiser et al., 1995). In contrast, as an ssDNA-binding protein, PC4 forms complexes with replication factor A (RPA) on ssDNA and can either inhibit or activate simian virus 40 (SV40) DNA replication depending on the concentration of RPA (Pan et al., 1996). To evaluate the function of PC4 in AAV-3 DNA replication, we analysed efficiency of viral genome rescue from the AAV-3 infectious clone pAAV3 (Muramatsu et al., 1996). Human 293-31 cells were purchased from ATCC and were maintained in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum and penicillin–streptomycin. The full-length fragment of PC4 was cloned into plasmid pcDNA3 (Invitrogen). 293-31 cells were seeded at a density of 10⁵ cells per 60 mm diameter culture dish, and transfected with 4 µg of pAAV3 and various amounts of pcDNA3/PC4, using LipofectAMINE (Gibco/BRL). In each transfection, pcDNA plasmid was used to make up the total amount of DNA when necessary. At 24 h, the LipofectAMINE medium was replaced with DMEM supplemented with 2% foetal calf serum and containing the helper adenovirus type 5 (Ad5; m.o.i. 5). At 40 h after transfection, DNA was extracted (Tissue kit; Qiagen) and digested with EcoRI. The DNA was then fractionated by electrophoresis on 0.8% agarose gel, transferred to nylon membrane, and hybridized to 32P-labelled AAV-3 DNA. Increased number of AAV-3 genomes were rescued from a constant amount of pAAV3 in PC4 transfected cells (2, 3, 4 µg). No additional enhancement was observed at the highest concentration of PC4 tested. ‘pl’ represents 293-31 cells transfected with pAA3 alone.

In summary, using the yeast two-hybrid system, we found that PC4 fused to Gal4 AD interacted with AAV Rep proteins fused to Gal4 BD. However, in our experiments direct association between AAV-3 Rep protein and PC4 could not be detected either by communoprecipitation using polyclonal antibody to PC4, or by pull-down assay using HA-tagged PC4 and Flag-tagged Rep (data not shown). This may be because PC4 can interact with Rep only in the presence of activators such as Gal4 to facilitate activation. It is known that PC4 enhances activation by various kinds of activation domains including Gal4 and Ad E1a (Ge & Roeder, 1994), and in AAV
infection E1a-mediated activation may be enhanced by PC4 in Ad-infected cells. Alternatively, it may not be possible to demonstrate coimmunoprecipitation because the associations between Rep and PC4 may be weak and transient. Such transient interactions between PC4 and RPA, which was also not detected by coimmunoprecipitation, have been shown in SV40 replication (Pan et al., 1996). Although increased concentration of PC4 enhanced the rescue of viral AAV-3, suggesting that PC4 may support the unwinding of ds AAV DNA, as has been proposed for SV40, the details of the interaction of Rep and PC4 are unknown. Recently, it was shown that RPA (and the Ad DNA-binding protein) is involved in AAV DNA replication, presumably due to interaction with one of the components of the replication complex, including Rep itself (Ward et al., 1998), and PC4 may function in this interaction. Above a certain PC4 level, excess PC4 might inhibit AAV replication by non-specific binding to ssDNA. Further studies will be needed to elucidate the details of the interaction.

AAV has been proposed as a viral vector for somatic gene therapy in non-dividing or quiescent cells such as neurons or muscles (Kapliit et al., 1994; Kessler et al., 1996). However, the use of AAV vectors has been limited by the difficulty of producing large quantities of high-quality vector. Further understanding the mechanism by which Rep proteins regulate AAV replication could provide a practical strategy for efficient production of AAV vectors in vitro.

**References**


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