Relative Influence of the Adeno-Associated Virus (AAV) Type 2 p5 Element for Recombinant AAV Vector Site-Specific Integration[▽]

Mickaël Guilbaud,[1,2] Gilliane Chadeuf,[1] Fabio Avolio,[3] Achille François,[1] Philippe Moullier,[1,2] Alessandra Recchia,[3,4] and Anna Salvetti[1,5]*

INSERM, U649, Nantes F-44000, France[1]; EFS des Pays de la Loire, Nantes F-4400, France[2]; Istituto Scientifico H. San Raffaele, Gene Therapy Program, Milano, Italy[3]; University of Modena and Reggio Emilia, Department of Biomedical Sciences, Modena, Italy[4]; and INSERM, U758, Lyon F-69007, France[5]

The p5 promoter region of the adeno-associated virus type 2 (AAV-2) *rep* gene has been described as essential for Rep-mediated site-specific integration (RMSSI) of plasmid sequences in human chromosome 19. We report here that insertion of a full-length or minimal p5 element between the viral inverted terminal repeats does not significantly increase RMSSI of a recombinant AAV (rAAV) vector after infection of growth-arrested or proliferating human cells. This result suggests that the p5 element may not improve RMSSI of rAAV vectors in vivo.

Adeno-associated virus type 2 (AAV-2) was originally found to be able to integrate into the AAVS1 locus of human chromosome 19 in a site-specific manner (6a), and since then, several studies have been conducted that confer this capacity to recombinant AAV (rAAV) vectors. It was recently reported that a 138-bp p5 region (nucleotides 151 to 289) of the AAV-2 genome is an essential *cis*-acting element required for Rep-mediated site-specific integration (RMSSI) of plasmids, both in the presence and in the absence of the viral inverted terminal repeats (ITRs) (10, 11). This viral region, which includes the promoter of the *rep68/78* gene, was previously characterized as a *cis*-acting replication element (8, 9, 13), and recent analyses defined a 55-bp p5 region (p5D10) as a minimal replication element. This region contains the TATA box, the Rep binding site, the terminal resolution site, the Yin Yang 1 (YY1) binding site located at position +1, YY1 and a downstream 17-bp sequence that could potentially form a hairpin structure (HP) with to the terminal resolution site at the top of the loop (3). These studies also indicated that the TATA box flanking the Rep binding site was absolutely required for in vivo replication of the p5 element, likely through TATA binding protein-mediated enhancement of Rep-dependent binding and nicking. Similarly, Murphy et al. recently confirmed that mutating the TATA box severely compromised replication of the p5 region and also found that RMSSI of p5-containing plasmids was sensitive to the same mutations as those affecting replication of this element (7). However, these studies, like the previous ones, were performed by analyzing stable cell clones derived from actively dividing cells, such as HeLa or 293 cells, transfected with p5-containing plasmids. Therefore, the fundamental question that remained to be answered concerned the

effect of the p5 element on RMSSI in the context of the ITRs and during viral infection of human cells.

Validation of the minimal 55-bp p5 element using a rAAV plasmid-based assay. A first study was conducted to determine if the minimal 55-bp p5 element, identified as an efficient replication origin (3), was also able to increase RMSSI of rAAV plasmid as previously documented for the entire p5 region. To answer this question, we used the procedure that was previously employed by other investigators (7, 10, 11), consisting of the analysis of stable cell clones isolated from HeLa cells transfected with the rAAV plasmid containing different versions of the p5 element inserted between the ITRs (Fig. 1). The wild-type (wt) 163-bp p5 region covers the entire p5 promoter from the major late transcription factor binding region to position 353, downstream from the HP region. The
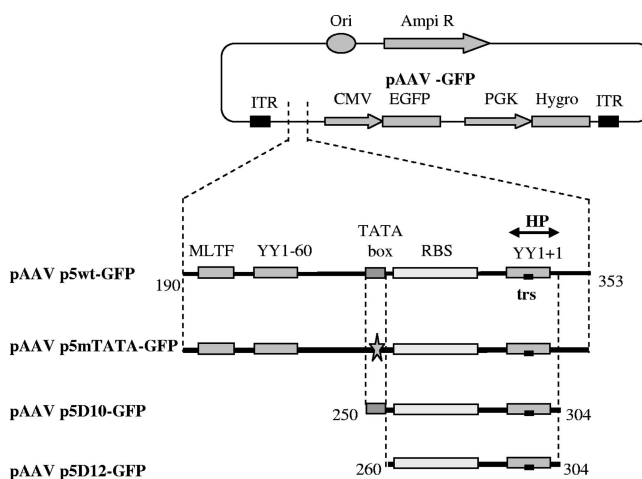

FIG. 1. Schematic view of the pAAV-GFP plasmids containing different versions of p5. The star indicates the presence of a mutated TATA box, and HP indicates the position of the putative HP (3). Positions refer to the wt AAV-2 genome (GenBank no. NC_001401). The control pAAV-GFP plasmid contains a nonrelevant 150-bp region instead of the p5 element.

* Corresponding author. Mailing address: INSERM U758, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, F-69007 Lyon, France. Phone: 33 472728401. Fax: 33 472728137. E-mail: anna .salvetti@ens-lyon.fr.

TABLE 1. Analysis of HeLa cell clones transfected with AAV-GFP plasmids

Vector plasmid	Total no. of clones	No. of GFP-positive clones[a] (%)	No. of clones with AAVS1 rearranged[b]/no. of GFP-positive clones (%)	No. of clones with RMSSI[c]/no. of GFP-positive clones (%)
pAAV-GFP	87	17 (19)	5/17 (29)	1/17 (6)
pAAVp5wt-GFP	81	32 (39)	15/32 (47)	12/32 (37)
pAAVp5mTATA-GFP	64	15 (23)	8/15 (53)	3/15 (20)
pAAVp5D10-GFP	71	19 (27)	8/19 (62)	5/19 (26)
pAAVp5D12-GFP	85	40 (48)	14/40 (35)	5/40 (12)

[a] As determined by PCR analysis and GFP expression.
[b] As determined by Southern blotting using an AAVS1 probe.
[c] Number of clones with AAV sequences integrated in AAVS1 region as determined by Southern blot analysis using *gfp* and AAVS1 probes.

minimal 55-bp p5 region (p5D10) includes only the four elements previously described as essential for in vivo and in vitro replication (Fig. 1) (3). Since our previous analysis indicated a critical role of the TATA box for in vivo replication, two additional replication-defective p5 elements containing either a mutation (p5mTATA) or a deletion (p5D12) of this binding site were inserted into the rAAV vector plasmid. Each rAAV plasmid was cotransfected into HeLa cells together with a plasmid expressing Rep under the control of doxycycline-inducible promoter (pBGTetOCMVRepTS) (12). Two weeks after transfection and culture in the presence of doxycycline, green fluorescent protein (GFP)-positive cells were sorted by a fluorescence-activated cell sorter and cloned by limiting dilution. After expansion, each clone was analyzed for the presence of the *gfp* sequence by PCR and visualization of GFP expression. As expected, only a portion of the selected clones had integrated the *gfp* sequence (Table 1), and all the clones containing this gene also expressed the protein, although at different levels (data not shown). In each of these GFP-stable cell clones, RMSSI was detected by Southern blot analysis performed for EcoRI-digested DNA by using, first, an AAVS1 probe to detect rearrangements of this chromosomal region and then, after dehybridization of the membrane, a *gfp* probe to detect vector-containing bands (Fig. 2). Site-specific integration of the rAAV vector plasmid was defined by the colocalization of the *gfp* and AAVS-1 bands, as in previous studies of RMSSI. As expected from previous studies, the presence of the wt p5 element significantly increased site-specific integration of the rAAV plasmid compared to results with the pAAV-GFP vector (Table 1). Insertion of the minimal p5D10 element also had a positive effect on RMSSI, similar to that observed

for the entire p5 region. In this context, the use of long or short replication-defective p5 elements (p5mTATA and p5D12, respectively) also resulted in an increase of RMSSI, although at a level that was apparently lower than that observed with their respective wt counterparts. Altogether, the results of this plasmid-based assay indicate that the minimal 55-bp region can increase RMSSI of the rAAV plasmid to a level similar to that observed with the wt p5 region and that replication of this element is not strictly required to exert this activity.

Effect of the wt and minimal p5 element during viral infection of human cells. We next asked whether an effect of the p5 element on RMSSI could similarly be observed during viral infection of human cells. To determine this, pAAV-GFP plasmids containing or not containing the p5 element were used to produce rAAV particles using a *rep*-expressing plasmid with the cytomegalovirus promoter substituting for the p5 promoter to avoid any recombination events with the p5-containing rAAV genome. The first model used for this study was composed of human hepatocyte-like cells derived after differentiation of the HepaRG hepatoma cell line (1, 4). This model was chosen because it represented human growth-arrested cells displaying a quasi-normal karyotype, in particular concerning chromosome 19, thus resembling conditions found during wt and rAAV infection in vivo. For the experiments described below, the cells were infected with rAAV-GFP particles 15 days after being cultured in conditions inducing differentiation (4). The expression of Rep was achieved by coinfection with a gutless adenovirus expressing the *rep78* gene under the control of a doxycycline-inducible promoter (Ad-Rep) (12). Quantitative PCR was then used to analyze RMSSI of each rAAV vector in differentiated HepaRG cells, 5 days postinfection.
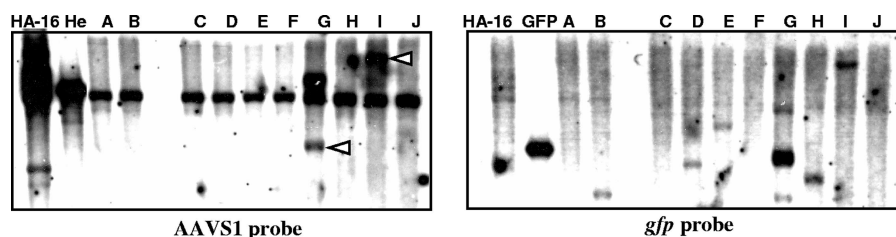

FIG. 2. Representative Southern blot analysis of RMSSI in stably transfected HeLa cells clones. HeLa cells (He) were cotransfected with each pAAV-GFP plasmid (Fig. 1) and with a second construct encoding Rep78 under the control of a doxycycline-inducible promoter (pBGTetOC MVRepTS) (12). Whole genomic DNA extracted from of each cell clone was digested by EcoRI and analyzed by Southern blotting using an AAVS1 probe and, after dehybridization, a *gfp* probe. Genomic DNA from HA-16 cells and the pAAV-GFP plasmid (GFP) served as positive controls for AAVS1 disruption and *gfp* probe hybridization, respectively. The black arrow indicates the expected EcoRI restriction fragment containing the AAVS1 sequence. The white arrows indicate AAVS1-disrupted bands that colocalize with *gfp*-containing bands.
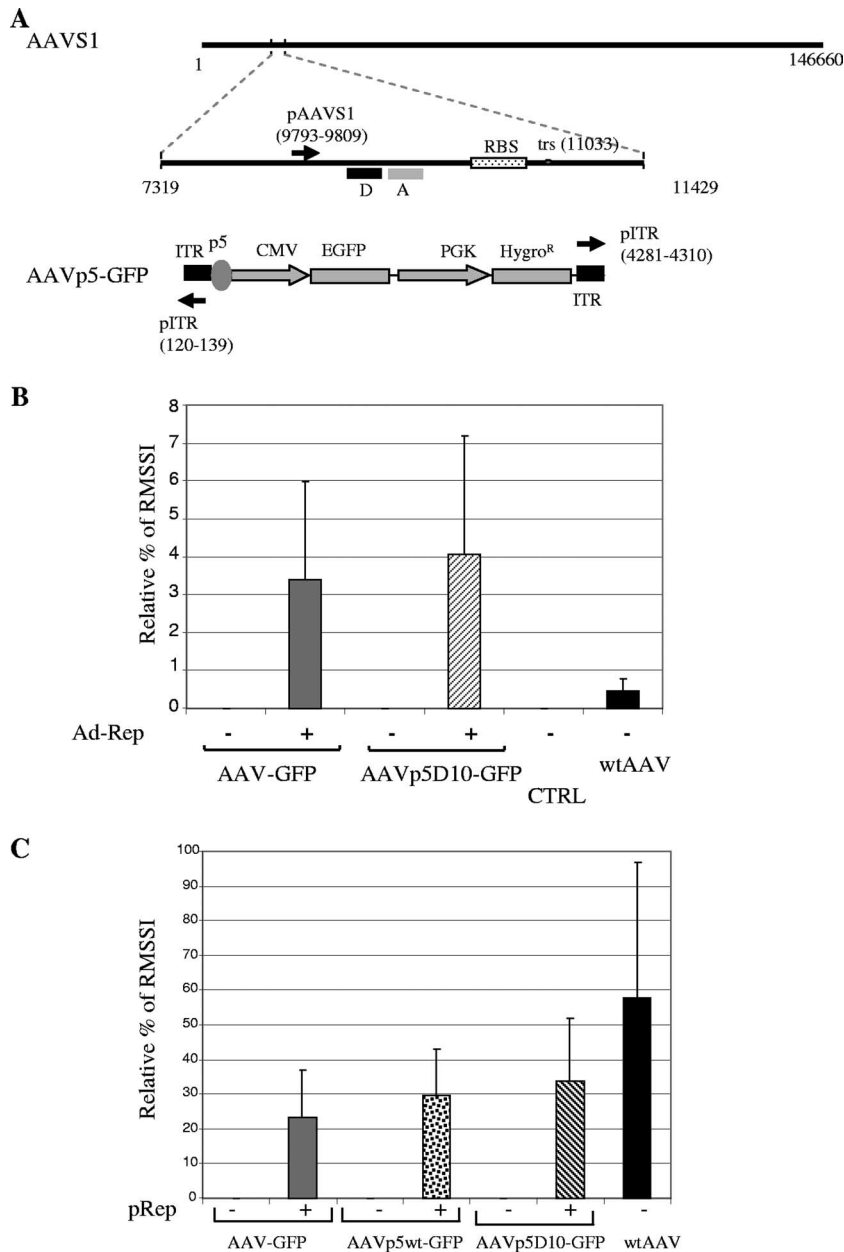
FIG. 3. Quantification of RMSSI in transduced HepaRG cells. (A) Human chromosome 19 region (GenBank accession no. AC010327) and position of primers pAAVS1 (5′-TCAGAGGACATCACGTG-3′) and pITR (5′-TTAACTACAAGGAACCCCTA-3′) used for the amplification of AAVS1-AAV junctions by quantitative PCR. D and A indicate the positions of the donor (5′-TGTTGCTGCCCAAGGATGCT-FL; TIB Molbiol) and acceptor (5′-LC Red640-TTTCCGGAGCACTTCCTTCTCG-p; TIB Molbiol) probes, respectively. The number of integrated AAV copies in the AAVS1 locus was measured by using as a standard different copies of the pAAVS1-ITR control plasmid that carries an artificial AAVS1-chromosome 19 junction fragment (5, 6) and was further calibrated by using genomic DNA extracted from HA-16 cells that contain the wt AAV-2 genome integrated into the AAVS1 site in a head-to-tail configuration (2). Results are expressed as the percentage of AAVS1-ITR junctions found in each sample relative to the number of junctions found using equivalent amounts of HA-16 cells. (B) Differentiated HepaRG cells were transduced with AAV-GFP or AAVp5D10-GFP in the presence or in the absence of a gutless adenovirus encoding Rep78 under the control of a doxycycline-inducible promoter (12). Alternatively, the cells were infected with wt AAV-2 particles (multiplicity of infection, 4,000 physical particles/cell and 20 infectious particles/cell for rAAV and adenoviral vectors, respectively). The percentage of RMSSI was measured for HepaRG cells 5 days after transduction. CTRL, genomic DNA from nontransduced HepaRG cells. (C) HeLa cells were transfected with the Rep-expressing plasmid (pBGTetOCMVRepTS) and then infected with rAAV. Alternatively, the cells were infected with wt AAV2 particles. Both infections were performed at a multiplicity of infection of 4,000 particles/cell. RMSSI was measured by quantitative PCR 3 days after infection and culture in the presence of doxycycline.

This assay, previously described by Hüser et al. (5, 6), allowed the detection of rAAV vector integration in both orientations (Fig. 3A). By use of this protocol, detectable levels of RMSSI integration were measured in cells coinfected with AAV-GFP vectors and Ad-Rep in the presence of doxycycline but not in the absence of Rep (Fig. 3B). Surprisingly, however, the use of the AAVp5D10-GFP vector did not significantly increase RMSSI compared with results for the AAV-GFP virus (mean

integration frequencies of 3.4 ± 2.5% and 4.0 ± 3.1%, respectively). To examine whether higher levels of site-specific integration could be achieved using a viral genome with a p5 element and the *rep* gene on the same DNA molecule, we measured RMSSI after infection of differentiated HepaRG cells with wt AAV-2 particles. The integration frequency using wt AAV2 particles was approximately 0.5 ± 0.3%, thus indicating that RMSSI in this cell type could not be enhanced even using this optimal substrate (Fig. 3B). Altogether, these results indicated that insertion of the minimal p5 element did not enhance RMSSI of a rAAV vector during infection of growth-arrested cells.

To determine whether this result could be attributed to the cell type chosen for this assay, a similar experiment was conducted using HeLa cells that were transfected with the doxycycline-inducible Rep plasmid (pBGTetOCMVRepTS) and then infected with AAV-GFP particles containing either the full-length or the minimal p5 element. RMSSI was measured by quantitative PCR for the cell population 3 days after infection in the presence of doxycycline. By using this protocol, a higher level of RMSSI was detected when HeLa cells were infected with wt AAV2 particles than with HepaRG cells (Fig. 3C). When cells were infected with rAAV vectors, detectable levels of RMSSI events were measured only in cells infected in the presence of Rep. However, as previously observed for HepaRG cells, the insertion of the wt or minimal p5 element in the rAAV vector did not significantly increase RMSSI compared with results for the AAV-GFP virus (mean integration frequencies of 23.3 ± 13.5%, 29.5 ± 13.2%, and 33.9 ± 18.0% for AAV-GFP, AAVp5wt-GFP, and AAVp5D10-GFP, respectively).

Altogether, this study documents for the first time the effect of the p5 element in the context of a viral infection. We show that insertion of the p5 region, in either its wt or its minimal version, cannot significantly increase RMSSI of a rAAV vector during infection of proliferating or quiescent human pseudo-primary cells. This result contrasts with the effect previously observed in this and other studies analyzing RMSSI of p5-containing rAAV plasmids after transfection into individual cell clones. This differential effect may depend upon the rAAV substrate introduced into the cells (i.e., a plasmid versus a linear molecule) or the selection procedure performed to obtain individual cell clones. Further studies should be conducted to determine whether, likewise, insertion of the p5 element is dispensable for enhancing RMSSI of rAAV vectors in vivo.

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