E1A promoter of bovine adenovirus type 3
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Conserved motifs of eukaryotic gene promoters, such as TATA box and CAAT box sequences, of E1A of human adenoviruses (e.g. human adenovirus 5) lie between the left inverted terminal repeat (ITR) and the ATG of E1A. However, analysis of the left end of the bovine adenovirus 3 (BAdV-3) genome revealed that the conserved sequences of the E1A promoter are present only in the ITR. As such, the promoter activity of ITR was tested in the context of a BAdV-3 vector or a plasmid-based system. Different regions of the left end of the BAdV-3 genome initiated transcription of the red fluorescent protein gene in a plasmid-based system. Moreover, BAdV-3 mutants in which the open reading frame of E1A was placed immediately downstream of the ITR produced E1A transcript and could be propagated in non-E1A-complementing Madin–Darby bovine kidney cells. These results suggest that the left ITR contains the sole BAdV-3 E1A promoter.

Bovine adenovirus 3 (BAdV-3) is being characterized at the molecular level to develop gene-transfer vector(s) for gene delivery to animals (Reddy et al., 1999c; Zakhartchouk et al., 1998, 1999) and humans (Rasmussen et al., 1999). Recently, the nucleotide sequence and the transcription map of the BAdV-3 genome have been determined (Baxi et al., 1999; Idamakanti et al., 1999; Reddy et al., 1998c, 1999a; Zheng et al., 1999). The E1 region of BAdV-3, located at the left end of the viral genome, consists of the E1A and E1B transcription units (Reddy et al., 1999a; van Olphen et al., 2002). The E1A transcriptional unit, located between 0·8 and 10·5 map units (m.u.), produces several different transcripts that encode phosphoproteins of 43, 57 and 65 kDa. The E1B transcriptional unit, located between 4·2 and 10·5 m.u., overlaps that of E1A and encodes phosphoproteins of 19 and 48 kDa. In this report, by using E1A 5′-flanking region mutants, we evaluated the promoter activity of the left inverted terminal repeat (ITR) of BAdV-3.

The ITRs of human, canine, porcine and simian adenoviruses (members of the genus Mastadenovirus) contain conserved sequence motifs that bind cellular transcriptional factors Sp1 and ATF (Dán et al., 2001), which are responsible for the promoter activity of the ITR (Hatfield & Hearing, 1991). However, E1A of these adenoviruses also contains promoter-like elements located between the left ITR and the ATG of E1A (Davison et al., 2003).

Unlike in other members of the genus Mastadenovirus, including human adenovirus 5 (HAdV-5; Hearing & Shenk, 1983, 1986), porcine adenovirus 3 (PAdV-3; Xing & Tikoo, 2004, 2005), mouse adenovirus 1 (Meissner et al., 1997) and canine adenovirus 1 (Morrison et al., 1997), we could not find conserved motifs of eukaryotic gene promoters, such as TATA box and CAAT box sequences, between the left ITR and the ATG of E1A of BAdV-3. The software program I (Reese et al., 1996) suggests that the E1A gene is under the control of a TATA-less promoter that is located mainly in the ITR (between nt 94 and 211). The region between nt 94 and 211 contains GC-rich sequences, which are believed to be Sp1-binding sites (Hatfield & Hearing, 1993; Kadonaga et al., 1987). However, deletion of 72 bp between nt 89 and 162, overlapping most of the potential promoter sequence predicted by program I, did not seem to have any effect on the kinetics of virus replication compared with those of wild-type BAdV-3 (van Olphen & Mittal, 2002), suggesting that GC-rich sequences between nt 94 and 211 (Fig. 1a) could not be the major promoter region of E1A. Program II (Hctata; Milanesi & Rogozin, 1998; Milanesi et al., 1995, 1996, 1999) also suggests that the E1A gene promoter is located in the ITR, but it may contain a TATA box (Hampsey, 1998) with the sequence ‘TATGA’ between nt 68 and 72. Additionally, the CAAT element of eukaryotic promoters was found between nt 46 and 49 (Fig. 1a). Interestingly, although there are conserved motifs of eukaryotic-like promoters between the left ITR and the ATG of E1A of BAdV-1 and BAdV-2 (Fig. 1a), there are no such sequence motifs present in the ITR of BAdV-1 and BAdV-2 (genus Mastadenovirus). In contrast, conserved motifs of eukaryotic-like promoters are present in the ITR of BAdV-4 (genus Atadenovirus), which does not possess a recognizable homologue of E1A (Davison et al., 2003).

The promoter activity in the ITR of adenoviruses is well established (Hatfield & Hearing, 1991; Ooyama et al., 1989; Xing & Tikoo, 2005; Yamamoto et al., 2003). To test the

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Fig. 1. Analysis of the BAdV-3 promoter. (a) Left ITR (filled box) and 5'-flanking sequences of BAdV E1A ORF (thin line). Numbers designate the nucleotide positions relative to the left terminus of the respective BAdV genomes [BAdV-1 (GenBank accession no. NC_006324); BAdV-2 (AF252854); BAdV-3 (AF030154); BAdV-4 (AF036092)]. (b) Left ITR (filled box), 5'-flanking sequences of BAdV-3 E1A ORF (thin line) and cis-acting packaging domain (open box). Numbers designate the nucleotide positions relative to the left terminus of the BAdV-3 genome. (c) PAdV-3 or BAdV-3 genome sequences (open arrows). Numbers designate the nucleotide positions relative to the left terminus of the corresponding virus genome. Arrows also indicate the direction of mRNA transcription. (d) Products of RT-PCR using DNase-treated RNA isolated from cells transfected with indicated plasmid DNA were synthesized by using a primer pair specific for RFP as described in the text. RNA with reverse transcriptase (+); RNA without reverse transcriptase (−). MassRuler DNA ladder mix DNA (Fermentas) was used as size marker (M). The expected band size is shown on the right. (e) The cells were transfected with 2 μg pDsRed2-N1 (P), pDsRed2-NP (N), pDsRedpav1 (1), pDsRedpav2 (2), pDsRedbav1 (3) or pDsRedbav2 (4) plasmid DNA. After 48 h, transfected cells were analysed by fluorescence microscopy. (i) Fluorescence; (ii) phase contrast.
in vitro promoter activity of BAdV-3 E1A transcriptional-control sequences (Fig. 1b), we constructed plasmids in which the red fluorescence protein (RFP)-encoding gene was placed under the control of BAdV-3 DNA sequences (Reddy et al., 1998c), between nt 1 and 224 (pDsRedbav1) or between nt 1 and 601 (pDsRedbav2) (Fig. 1c). Plasmid pDsRed2-N1 contains the RFP gene under the control of the human cytomegalovirus immediate-early promoter and plasmid pDsRed2-NP contains the RFP gene without any transcriptional-control element at the 5′ end. For comparison purposes, we also constructed plasmids in which the RFP gene was under the control of PAdV-3 DNA sequences (Reddy et al., 1998a, b) between nt 1 and 151 (pDsRedpav1) or nt 1 and 495 (pDsRedpav2) (Fig. 1c). Initially, the promoter activity was analysed by RT-PCR using specific primers. Different cells were transfected with individual plasmids. At 72 h post-transfection, the cells were collected and total RNA was isolated and analysed as described previously (Zakhartchouk et al., 2004), using RFP gene-specific primers (RFPF, 5′-ATGGCCTCCTCCGAGAACGTCTATG-3′; RFPR, 5′-CTACAGGAACAGGTGGTGGC-3′). As seen in Fig. 1(d), an RFP gene-specific RT-PCR product of the expected size could be detected in cells transfected with plasmid pDsRedpav2 (lane +) or plasmid pDsRedbav2 (lane +). The DNA sample controls with no reverse transcriptase did not show any bands (lane –), indicating that the 680 bp fragment was amplified from RFP mRNA and not from the residual DNA. Moreover, no such product could be detected in cells transfected with pDsRedpav1 or pDsRedbav1 (data not shown). To confirm expression of the RFP protein, the transfected cells were analysed by fluorescence microscopy. Representative results are shown in Fig. 1(e). Expression of RFP could be detected in human 293 (Graham et al., 1977), bovine VIDO R2 (Reddy et al., 1999c) and porcine VIDO R1 (Reddy et al., 1999b) cells transfected with plasmid pDsRedpav2 (column 2), but not with plasmid pDsRedpav1 (column 1). Similarly, expression of RFP could be detected in cells transfected with plasmid pDsRedbav2 (column 4), but not with pDsRedbav1 (column 3). These results suggest that, as in PAdV-3 (Xing & Tikoo, 2005), the 5′-flanking sequences of the BAdV-3 E1A display promoter activity in vitro. Transcription from some regions of the left end of the HAdV-5 genome has not been detected in a plasmid-based system (Hatfield & Hearing, 1991).

As the conserved sequences of eukaryotic promoters are located in the ITR (Fig. 1a, b), we investigated the promoter activity of the BAdV-3 left ITR by constructing and analysing a series of BAdV-3 mutants (Fig. 2a), designated Bav16 (Bav3-224/468), Bav18 (Bav3-224/541), Bav110 (Bav3-224/552) and Bav112 (Bav3-224/560) (Xing et al., 2003).

To analyse the transcription of E1A, Madin–Darby bovine kidney (MDBK) cells were infected with wild-type or mutant BAdV-3 at an m.o.i. of 40 p.f.u. in the presence of AraC (125 μg ml⁻¹). Total RNA isolated at 7 h post-infection was separated in 1% formaldehyde/agarose gel and analysed by Northern blotting using a 32P-labelled E1A-specific DNA probe (nt 560–1156). Although E1A mRNA could be detected in wild-type BAdV-3-infected cells, the
E1A mRNA produced in mutant virus-infected cells was undetectable (data not shown). This could be due to the sensitivity limitation of Northern blot analysis. Therefore, MDBK cells were infected with mutant BAdV-3 at an m.o.i. of 15 p.f.u. in the absence of AraC. Total RNA isolated at 36 h post-infection was analysed by Northern hybridization using a 32P-labelled E1A-specific DNA probe (nt 560–1156). As expected, E1A-specific mRNA could be detected in wild-type BAdV-3-infected cells (Fig. 2b). Similarly, E1A-specific mRNA could be detected in cells infected with mutants Bav16, Bav18, Bav110 or Bav112. However, the level of E1A mRNA in infected cells differed between mutant viruses. Mutant Bav16, carrying deletion of sequences between nt 224 and 468, displayed nearly the same level of E1A mRNA as wild-type BAdV-3. Cells infected by mutant Bav18 (nt 224–541), Bav110 (nt 224–552) or Bav112 (nt 224–560) displayed a correspondingly gradual reduction in the accumulation of E1A mRNA compared with wild-type or Bav16-infected cells. This could be due to deletion of sequences between nt 224 and 382, which reduces the steady-state level of E1A/E1B mRNAs significantly and also affects the mRNA levels of E2A, E3 and E4 in virus-infected MDBK cells (unpublished data). These results suggested that the left ITR directs the transcription of BAdV-3 E1A, which is regulated by downstream sequences between the left ITR and the ATG of E1A. Previously, different regions of the left end of the HAdV-5 genome have been shown to induce different levels of transcription of a reporter gene in vivo (Yamamoto et al., 2003).

To determine the E1A transcription start site in these mutants, MDBK cells were infected with mutant BAdVs at an m.o.i. of 15 p.f.u. At 36 h post-infection, total RNA was isolated and amplification of cDNA 5' ends was performed with a 5'-RACE (rapid amplification of cDNA ends) kit (Invitrogen) as described previously (Xing & Tikoo, 2005). cDNAs were synthesized by using BAdV-3 E1A gene-specific primers (reverse, nt 1327–1344; reverse, nt 1882–1899). Polydeoxynucleotide-tailed cDNAs were amplified by using a nested gene-specific reverse primer (nt 852–869) and a 5'-RACE AAP primer. DNA sequence analysis of PCR products (Fig. 2c) indicated that transcription of the E1A gene was initiated at both nt 286 and nt 560 in wild-type BAdV-3.
However, mutants Bav18 (nt 224–541) and Bav112 (nt 224–560) use only one transcription-initiation site at nt 560. In mutant Bav16 (nt 224–468), transcription of the E1A gene was initiated at nt 500–501, as well as at nt 560. In Bav110 (nt 224–552), E1A transcription starts at nt 554 (Zheng et al., 1999). Interestingly, transcription activity of different regions of the left end of HAdV-5 varied in different organs (Yamamoto et al., 2003).

E1A gene products are required for transactivation of other adenoviral early genes and, consequently, control adenovirus replication (Russell, 2000). To determine whether the level of E1A gene expression directed by the ITR in the absence of other potential transcriptional-control sequences (between nt 224 and 560) is enough to support virus replication in a non-E1A-complementing cell line, MDBK cells were infected with wild-type or mutant BAdV-3 at an m.o.i. of 5 p.f.u. At different times post-infection, infected cells were harvested and amounts of infectious virus progeny were determined by plaque assay in VIDO R2 cells. Wild-type BAdV-3 reached a titre of approximately $5 \times 10^7$ p.f.u. ml$^{-1}$ at 48 h post-infection (Fig. 3a). BAdV-3 mutants displayed a lag in viral growth to varying degrees. Moreover, Bav16, Bav18, Bav110 and Bav112 grew to 0-9, 1-5, 1-7 and 2-0 logs lower than the wild-type BAdV-3, respectively (Fig. 3a). These results suggested that E1A expression regulated by ITR and its downstream sequences (between nt 224 and 560) supports the replication of mutant BAdV-3 in MDBK cells. An earlier report suggested that the same region of the HAdV-5 ITR initiated reporter-gene expression differently in cancer cells and in normal organs in vivo (Yamamoto et al., 2003).

To study further the replication of BAdV-3 mutants, we examined viral DNA accumulation in mutant virus-infected cells. MDBK cells were infected with mutant viruses at an m.o.i. of 5 p.f.u. per cell. Total DNA isolated at 9, 17 and 33 h post-infection was digested with EcoRI and analysed by Southern blot analysis using a $^{32}$P-labelled DNA probe (nt 828–1651). As expected, all BAdV-3 mutants could replicate in MDBK cells, albeit with varying efficiency (Fig. 3b, c). The level of BAdV-3 DNA accumulation (Fig. 3b, c) appears to be correlated directly to the level of E1A mRNA (Fig. 2b) in MDBK cells.

Although the ITRs of HAdV-5 and PAdV-3 display promoter-like activity, the core elements of the E1A promoter are located between the left ITR and the ATG of E1A (Xing & Tikoo, 2005; Yamamoto et al., 2003). Our results suggest that the core elements of the BAdV-3 E1A promoter are located in the left ITR. Several lines of evidence support the fact that the left ITR of BAdV-3 contains the core promoter element of E1A. First, DNA sequence analysis of the left ITR showed the presence of a CCAAT box (nt 45–49), a TATA-like box (nt 68–72) and most of a GC box (nt 108–209). Second, in the absence of sequences between nt 224 and 560 (Bav112), the promoter activity of the ITR is sufficient to express E1A, which supports the replication of Bav112 and the production of progeny virions in non-E1A-complementing MDBK cells. Third, transcriptional analysis of the E1 region identified E1A transcriptional start sites near the left ITR (Reddy et al., 1998c; 1999a; Zheng et al., 1999). Further understanding of the different mechanisms of transcriptional-control regions of E1A should help to develop improved adenovirus vectors for gene delivery.

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