Virology **256,** 351–359 (1999)
Article ID viro.1999.9626, available online at http://www.idealibrary.com on IDEAL®

Transcription Mapping and Characterization of 284R and 121R Proteins Produced from Early Region 3 of Bovine Adenovirus Type 3[1]

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Received November 3, 1998; returned to author for revision December 8, 1998; accepted January 27, 1999

We established the transcription map of early region (E) 3 of bovine adenovirus 3 (BAV-3) by Northern blot, S1 nuclease protection assays, cDNA sequencing, and RT-PCR analysis. Five major classes of mRNAs were identified, which shared the 3′ ends. Four classes of mRNAs transcribed from the E3 promoter also shared the 5′ end, while one major class of mRNA transcribed from the major late promoter contained a tripartite leader sequence at the 5′ end. These five transcripts have the potential to encode four proteins, namely 284R, 121R, 86R, and 82R. To identify the proteins, rabbit antiserum was prepared using a bacterial fusion protein encoding 284R or 121R protein. Serum against 284R immunoprecipitated protein of 26–32 kDa in *in vitro* translated and transcribed mRNA and three proteins of 48, 67, and 125 kDa from BAV-3-infected cells. Western blots and enzymatic digestions confirmed that the 284R protein is a glycoprotein, which contains only N-linked oligosaccharides, both high mannose (48 kDa) and complex types (67 kDa). Serum against 121R immunoprecipitated a protein of 14.5 kDa from *in vitro* translated and transcribed mRNA and BAV-3-infected cells. Although 121R protein shows limited sequence similarity to a 14.7-kDa protein of human adenovirus 5, the 284R protein appears to be unique to BAV-3. Since proteins encoded by the E3 region appear to influence adenovirus pathogenesis, the 284R protein may contribute to the unique pathogenic properties of BAV-3.

Adenovirus genomes are organized into complex transcriptional units, which can be divided into early, intermediate, and late regions. The early (E) region contains four transcriptional units, E1, E2, E3, and E4. The E3 region is transcribed rightward on the "r" strand and different mRNAs synthesized from the same promoter arise by alternate splicing of a common primary mRNA. The E3 region codes for a group of proteins, which appear to help adenovirus to evade the host immune response (Wold and Gooding, 1991). Although the E3 region has been maintained throughout evolution, it is a highly variable region among different adenoviruses infecting both humans and animals.

The E3 region of human adenovirus (HAV)-2 and -5 has been studied extensively and shown to be nonessential for virus growth in cell culture (Wold, 1995). At least nine overlapping mRNAs, which have the potential to code for nine proteins, are produced from this region (Wold, 1995). Although seven of these proteins, including gP19, 14.7K, 14.5K, 12.5K, 10.4K, and 11.6K, have been identified, few have been characterized at the functional level (Gooding *et al.,* 1988, 1991; Rawle *et al.,* 1989; Tollefson *et al.,* 1991, 1996). The predominant protein from the E3 region of HAV is a 19K glycoprotein (gp19), which is retained in the rough endoplasmic reticulum (Paabo *et al.,* 1987). Gp19 binds to major histocompatibility (MHC) class I antigens in the rough endoplasmic reticulum and prevents their transport to the cell surface (Kvist *et al.,* 1978). This may prevent virus-infected cells from being recognized and subsequently lysed by HAV-specific cytotoxic T lymphocytes (Rawle *et al.,* 1989). The E3 14.7K and 10.4K/14.5K proteins protect the virus-infected cells from cytolysis induced by tumor necrosis factor (TNF) (Gooding *et al.,* 1988, 1991; Dimitrov *et al.,* 1997). The E3 10.4K/14.5K proteins are also required (i) to down-regulate the epidermal growth factor receptor in HAV-infected cells (Tollefson *et al.,* 1991) and (ii) to mediate loss of CD95 and resistance to CD95-induced apoptosis (Shisler *et al.,* 1997; Chen *et al.,* 1998). The E3 11.6K proteins have the ability to promote the lysis of HAV-infected cells (Tollefson *et al.,* 1996). In addition to selectively characterizing the role of individual proteins *in vitro,* different small animal models have been used to elucidate the role of E3 in HAV pathogenesis *in vivo* (Cox *et al.,* 1994; Ginsberg *et al.,* 1989).

In contrast to HAVs, the E3 region of other adenoviruses has been less thoroughly studied. To expand this knowledge, we have been characterizing bovine adenovirus type 3 (BAV-3) at the molecular level with the goal of developing BAV3 as a recombinant vector for human gene delivery and for veterinary vaccine applications.

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0042-6822/99 $30.00

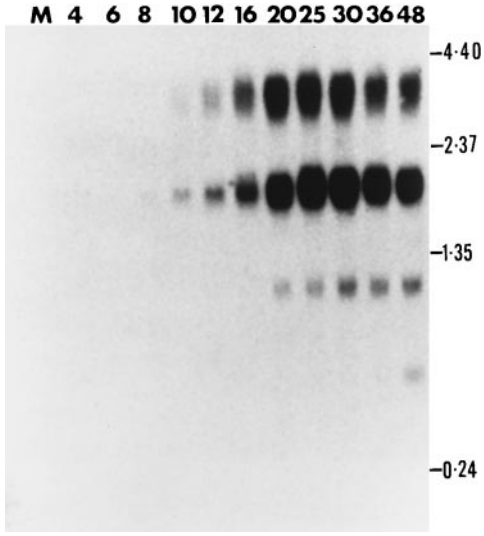

FIG. 1. Northern blot analysis of the E3 region of BAV-3. Total cellular RNA isolated from mock-infected (M) or BAV-3-infected MDBK cells, collected at different time points (4 to 48 h) postinfection in the presence of AraC, was analyzed by Northern blot as described under Materials and Methods. The probe used was a random primer-labeled 1.2-kb fragment (nt 26,472–27,683). The positions of the RNA molecular weight markers are indicated on the right.

Recently, we determined the entire nucleotide sequence of BAV-3 (Baxi *et al.,* 1998; Lee *et al.,* 1998; Reddy *et al.,* 1998), which suggested that the overall size and genome organization of BAV-3 were similar to those of HAVs. However, the E3 region of BAV-3 is half the size of the E3 region of HAV5 (Mittal *et al.,* 1993). In addition, only one (121R) of the four putative E3 proteins shows similarity to E3 14.7K and 15.3K proteins encoded by HAVs (Hong *et al.,* 1988; Mittal *et al.,* 1993). Since the E3 region of BAV-3 showed little homology to the E3 region of other adenoviruses including BAV-1 (Evans *et al.,* 1998), we are beginning to analyze this region in detail. In this report, we present the establishment of a transcriptional map and identification and characterization of two proteins encoded by the E3 region of BAV-3.

RESULTS

Identification of E3 transcripts. Northern blot analysis was performed to determine the transcripts originating from the E3 region of BAV-3. A 1.2-kb DNA probe (nt 26,472 to 27,683; nt numbers are based on BAV-3 genome sequence; GenBank Accession No. AF030154) was hybridized to mRNA isolated at various times from mock- or BAV-3-infected cells. As shown in Fig. 1, the DNA probe hybridized to four transcripts of 0.9, 1.2, 2.0, and 4.0 kb. Two transcripts (4 and 2 kb) appeared at 8 h postinfection and the third transcript (1.2 kb) appeared at 20 h postinfection and did not decrease in abundance for the remaining course of infection. The fourth transcript (0.9 kb) was detected at 48 h postinfection. Since the cells were treated before and during infection with a viral DNA synthesis inhibitor, all these transcripts appear to be of the early class.

To determine the E3 transcriptional start and stop site(s), S1 nuclease protection assays were performed. For 5′ end mapping, a 462-bp fragment (nt 25,857 to 26,319) was 5′ end-labeled with γ-^{32}P. When this probe was hybridized to BAV-3 RNA and digested with S1 nuclease, a major fragment of 165 bp was protected (Fig. 2A). This suggests that the transcription in the E3 region initiates 25 bp (nt 26,185) downstream of the TTTAAA (nt 26,155–26,160). For 3′ end-mapping of E3 mRNAs, a 583-bp (nt 27,376 to 27,959) fragment was labeled at the 3′ end with α-^{32}P and hybridized to BAV-3 RNA. After digestion of the hybridized probe with S1 nuclease, a major fragment of 388 bp was protected (Fig. 2B). This indicates that the transcripts generated from the E3 region terminate at nt 27,776, which is 15 bp downstream from the 3′ end of the E3 polyadenylation signal.

Two strategies were used to determine the structure of E3 mRNAs. First, a cDNA library was constructed using

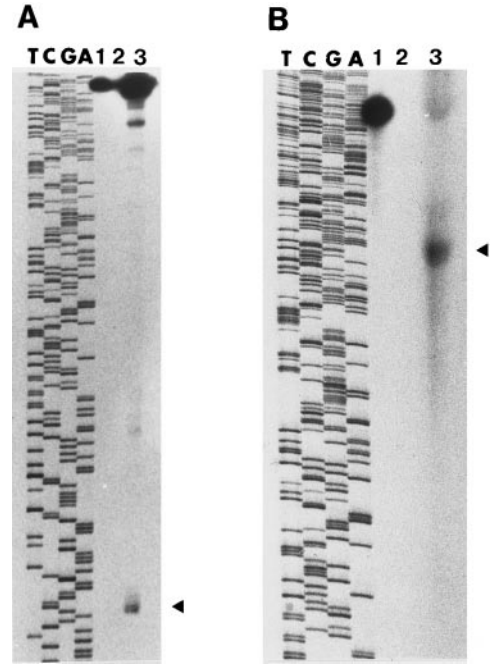

FIG. 2. S1 nuclease mapping of BAV-3 E3 mRNA. RNA was isolated from mock-infected or BAV-3-infected MDBK cells at 18 and 24 h postinfection. (A) The 462-bp (nt 25,857–26,319) fragment was used as a probe to map the 5′ end. (B) The 583-bp (nt 27,376–27,959) fragment was used as a probe to map the 3′ end. Lanes T, C, G, and A represent sequence ladder as a marker. Lane 1, probe; lanes 2 and 3 represent S1 nuclease protected fragments with RNA extracted from mock- or BAV-3-infected cells, respectively. The position of the protected fragment is indicated by an arrowhead.

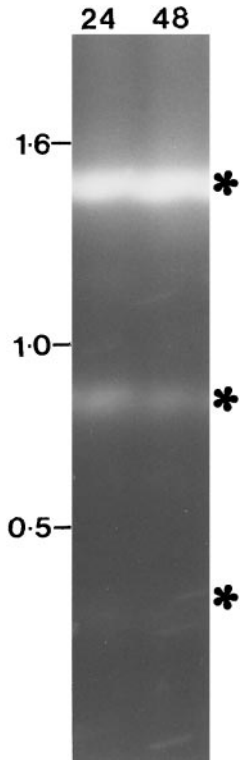

FIG. 3. RT-PCR analysis of the E3 region of BAV-3. Total RNA isolated from MDBK cells at 24 and 48 h postinfection was reverse-transcribed using oligo(dT) as a primer. The E3-specific cDNAs were amplified using primer 1, CTGCTTAAGACCAGATGGAG, and primer 2, GGAACAGGAACAGCGGATGC, in PCR and analyzed on a 0.6% agarose gel.

mRNA extracted from BAV-3-infected cells, and 34 clones, which hybridized to a E3 probe of 1.2 kb (nt 26,472 to 27,683), were completely sequenced. Second, RT-PCR analysis using common E3 5′ and 3′ primers (based on S1 nuclease analysis) was used. RT-PCR produced cDNAs of three different sizes (Fig. 3), which were completely sequenced. The results of these studies are summarized in Fig. 4. The 1591-nt transcript did not show any splicing and may code for 284R protein. The 899-nt transcript has an intron with a splice donor site at nt 26,458 and an acceptor site at nt 27,151, and it has the potential to code for a 121R protein. The 877-nt transcript has an intron with a splice donor site at nt 26,436 and an acceptor site at nt 27,151, and it has the potential to code for a 121R protein. The 438-nt transcript has an intron with a splice donor site at nt 26,299 and an acceptor site at nt 27,452, and it has the potential to code for 86R and 82R proteins. All these transcripts originate from the E3 promoter and were 5′ and 3′ coterminal. The 729-nt transcript appears to be transcribed from the major late promoter (MLP; Reddy *et al.,* 1998). This transcript contains a tripartite leader sequence (TPL) of 205 bp (nt 5587 to 5626, 6642 to 6719, and 9076 to 9162) at the 5′ end with a splice acceptor site at nt 27,151 in the E3 region. All transcripts have a 3′ end corresponding to the poly(A) site at nt 27,776, which is the same as that corresponding to the poly(A) site determined by S1 nuclease analysis.

Production of antiserum. In order to identify and characterize the proteins encoded by the E3 region of BAV-3, we made anti-GST-protein fusion serum. A 0.5-mg quantity of gel-purified fusion protein(s) in Freund's complete adjuvant was injected into each rabbit. Subsequently booster immunizations were given with 0.2 mg of fusion protein(s) in Freund's incomplete adjuvant at 4-week intervals. Sera collected after the fourth boost were analyzed in detail.

In vitro translation and immunoprecipitation of 284R and 121R. In order to determine the identity of 284R and 121R ORFs, and to check whether the antibodies against these gene products recognized their respective proteins, two *in vitro* expression plasmids, pSP64-284R and pSP64-121R, were generated, in which the coding sequence of 284R and 121R, respectively, was placed downstream of the SP6 promoter. *In vitro* translation of pSP64-284R RNA resulted in the synthesis of a protein of 26–32 kDa (Fig. 5A, lane a), which was recognized by anti-284R serum (Fig. 5A, lane c). Similarly, *in vitro* translation of pSP64-121R RNA resulted in the synthesis of a polypeptide of 14.5 kDa (Fig. 5B, lane a), which was recognized by anti-121R serum (Fig. 5B, lane b). These proteins were not immunoprecipitated with anti-284R (Fig. 5A, lane b) or anti-121R serum (Fig. 5B, lane c) from reactions in which pSP64polyA (negative control plasmid) RNA was translated *in vitro.*

In vivo expression of 284R and 121R. To further characterize the proteins and to confirm the specificity of the antisera, radioimmunoprecipitation assays were performed. Anti-284R serum detected three proteins of 48, 67, and 125 kDa in BAV-3-infected (Fig. 6A, lane c) but not in mock-infected cells (Fig. 6A, lane a). The 48-, 67-, and 125-kDa proteins were detectable at 24 h postinfection (Fig. 6A, lane c) but not at 12 h (Fig. 6A, lane b) or 48 h postinfection (Fig. 6A, lane d). Anti-121R serum detected a protein of 14.5 kDa in BAV-3-infected (Fig. 6B, lanes c–e) but not in mock-infected cells (Fig. 6B, lane a). The 14.5-kDa protein was detected at 24 h postinfection and remained detectable throughout the infection (Fig. 6B, lanes c–e). None of these bands were detected when BAV-3-infected cell lysates were immunoprecipitated with preimmune sera (data not shown). The 46-kDa nonspecific band observed in different lanes is presumably actin. The 96-kDa (Fig. 6A, lanes c and d) and other high-molecular-weight bands (Fig. 6B, lanes c–e), which are observed late in infection, appear to be BAV-3-specific proteins, since these proteins are not immunoprecipitated from BAV-3-infected cells grown in the presence of AraC (Zakhartchouk, personal communication).

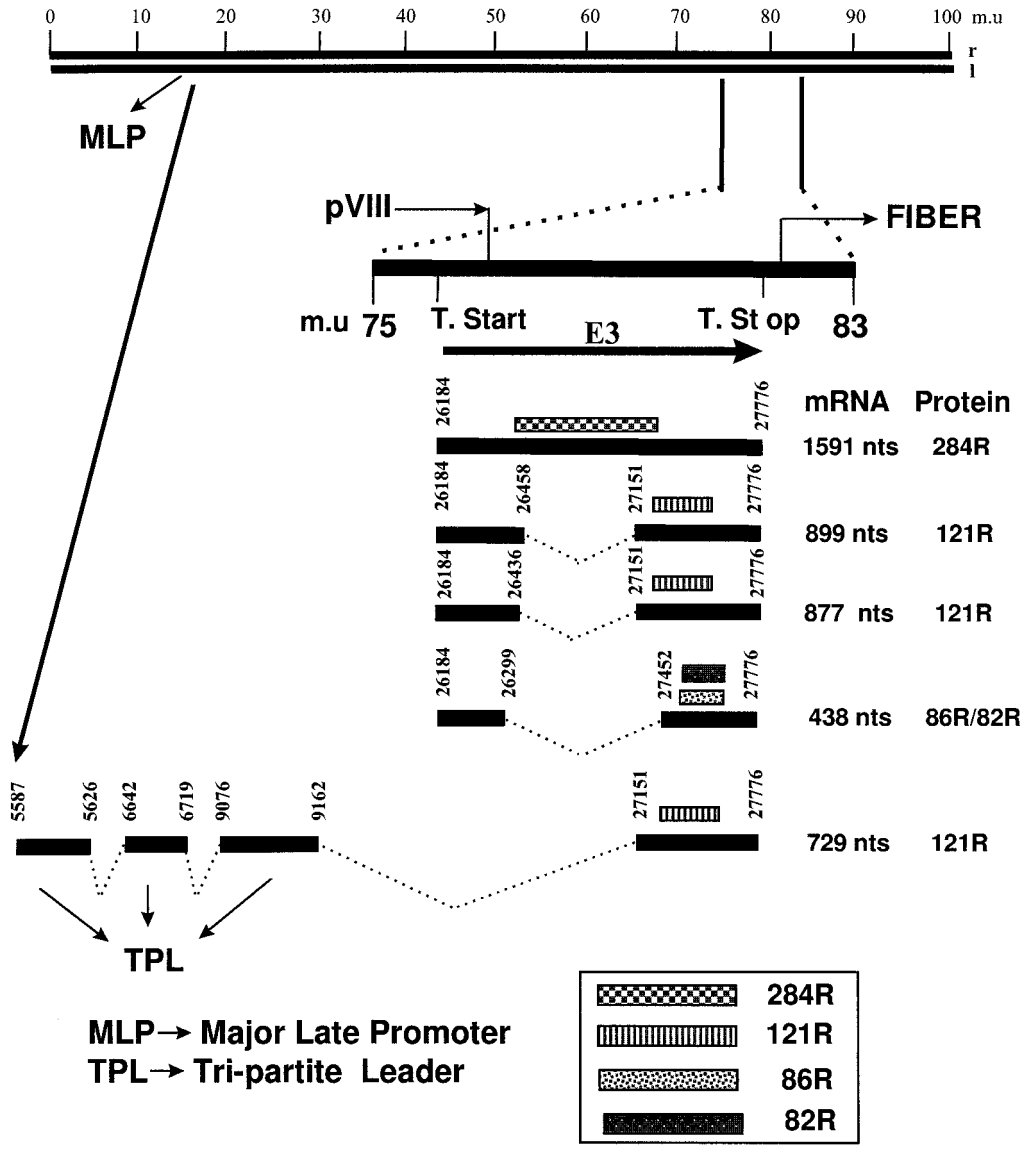

FIG. 4. Location of E3 transcripts on the BAV-3 genome. The upper double line represents the BAV-3 genome with map units indicated. The region from map unit 75 to 83 is expanded, showing the location of the E3 region. The five major classes of mRNAs are diagrammed. The thick horizontal lines and carets represent the exons and introns, respectively. The sizes of mRNA and encoded protein are indicated on the right of each mRNA. The start, splice, and polyadenylation sites are indicated in nt numbers below the appropriate mRNA.

Glycosylation of 284R and 121R. To determine whether the proteins are modified by the attachment of oligosaccharides, we labeled the proteins with [^{3}H]glucosamine. The three proteins of 48, 67, and 140 kDa synthesized in BAV-3-infected (Fig. 7A, lane b) but not in mock-infected (Fig. 7A, lane a) cells were labeled with [^{3}H]glucosamine (Fig. 7A, lane b), suggesting that 284R protein is glycosylated. However, the 121R protein synthesized in BAV-3-infected cells was not labeled by [^{3}H]glucosamine, suggesting that 121R is not glycosylated (data not shown).

To determine what type of oligosaccharides are attached to the 284R protein, we first treated the 284R protein with different glycosidases. The PNGase F treatment increased the mobility of both 48- and 67-kDa proteins (Fig. 7B, lane d). However, endo H treatment increased the mobility of only the 48-kDa protein (Fig. 7B, lane e). Neuraminidase and *O*-glycanase treatment did not increase the mobility of the 284R protein (data not shown). Second, we labeled the proteins produced in BAV-3-infected cells with [^{3}H]glucosamine or [^{3}H]mannose. As seen in Fig. 7C, the 67-kDa protein was strongly labeled with [^{3}H]glucosamine (lane a) and was resistant to endo H digestion (lane b). In contrast, the 48-kDa protein was heavily labeled with [^{3}H]mannose (lane c) and was sensitive to endo H digestion (lane d).

To confirm whether different bands (48 and 67 kDa) observed in the immunoprecipitation studies represent

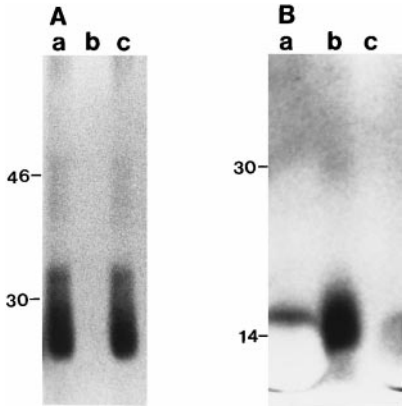

FIG. 5. Immunoprecipitation of proteins synthesized by *in vitro* transcription and translation of plasmids. (A) [^{3}H]leucine labeled *in vitro* transcribed and translated pSP64.284R (lanes a, c) and pSP64polyA (lane b) products before (lane a) or after immunoprecipitation with anti-284R serum (lane b, c) were separated on 10% SDS–PAGE under reducing conditions. (B) [^{3}H]leucine labeled *in vitro* transcribed and translated pSP64.121R (lanes a, b) and pSP64polyA (lane c) products before (lane a) or after immunoprecipitation with anti-121R serum (lane b, c) were separated on 12.5% SDS–PAGE under reducing conditions. The positions of the molecular weight markers are shown to the left of each panel.

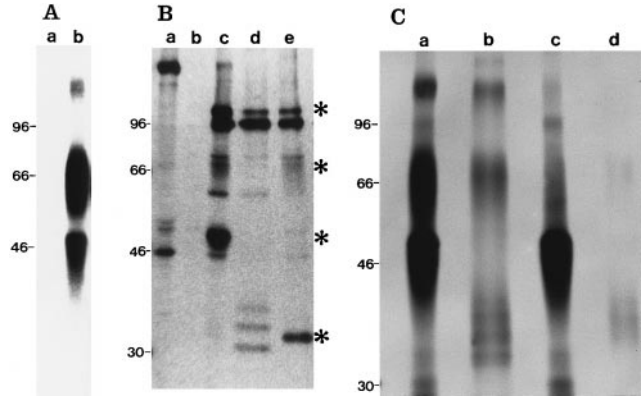

FIG. 7. Glycosylation of 284R protein. (A) Proteins from lysates of [^{3}H]glucosamine labeled mock-infected (lane a) or BAV-3-infected (lane b) MDBK cells were immunoprecipitated with anti-284R serum and analyzed on 10% SDS–PAGE under reducing conditions. (B) [^{3}H]leucine labeled proteins from lysates of mock-infected (lane a) or BAV-3-infected MDBK cells were immunoprecipitated with anti-284R serum and analyzed before (lane c) and after digestion with PNGase F (lane d) or endo H (lane e) on 10% SDS–PAGE under reducing conditions. (C) Proteins from lysates of [^{3}H]glucosamine (lanes a, b) or [^{3}H]mannose (lanes c, d) labeled BAV-3-infected MDBK cells were immunoprecipitated with anti-284R serum and analyzed before (lanes a, c) and after digestion with endo H (lanes b, d) on 10% SDS–PAGE.

posttranslationally modified forms of 284R protein or cellular/viral proteins that coimmunoprecipitate with the 48-kDa protein, we carried out a Western blot assay. As seen in Fig. 8, two bands of 48 and 67 kDa were observed in BAV-3-infected cells (Fig. 8, lane b) but not in mock-infected cells (Fig. 8, lane a). These bands were similar to the 48- and 67-kDa bands detected by immunoprecipitation.

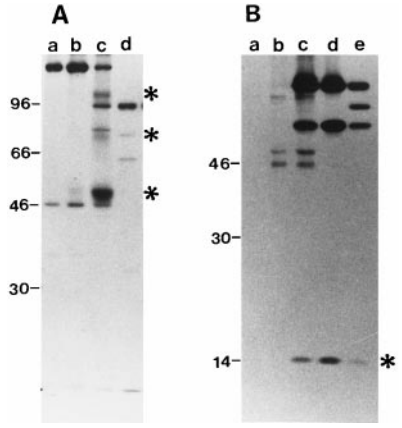

FIG. 6. *In vivo* expression of 121R and 284R proteins. (A) Proteins from the lysates of [^{3}H]leucine labeled mock-infected (lane a) or BAV3-infected (lane b, 12 h postinfection; lane c, 24 h postinfection; lane d, 48 h postinfection) MDBK cells were immunoprecipitated with anti-284R serum and separated on 10% SDS–PAGE under reducing conditions. (B) Proteins from the lysates of [^{3}H]leucine labeled mock-infected (lane a) or BAV3-infected (lane b, 12 h postinfection; lane c, 24 h postinfection; lane d, 36 h postinfection; lane e, 48 h postinfection) MDBK cells were immunoprecipitated with anti-121R serum and separated on 12.5% SDS–PAGE under reducing conditions. The positions of the specific proteins are indicated by asterisks. The positions of the molecular weight markers are indicated to the left of each panel.

DISCUSSION

Although the E3 region is not essential for adenovirus replication (Kelly and Lewis, 1973; Ginsberg *et al.,* 1989), most adenovirus genomes contain a well-defined E3 region located usually between the genes encoding pVIII and fiber proteins. In addition, the sizes of the region, the RNA transcripts, and the proteins encoded by the E3 region of different adenoviruses differ considerably

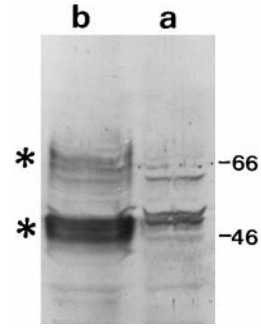

FIG. 8. Western blot analysis of 284R. Proteins from mock-infected (lane a) or BAV-3-infected (lane b) MDBK cells were separated on 10% SDS–PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blots by anti-284R serum. The positions of the molecular weight markers are shown to the left.

(Beard *et al.,* 1990; Beard and Spindler, 1995; Evans *et al.,* 1998; Wold, 1995). This has led to the suggestion that the E3 region may be needed for other functions such as host virus interaction and thus may determine the pathogenic properties of an adenovirus (Bailey and Mautner, 1994). In order to precisely dissect the structure and function of different proteins encoded by BAV-3, we recently determined the entire DNA sequence of the BAV-3 genome (Baxi *et al.,* 1998; Lee *et al.,* 1998; Reddy *et al.,* 1998). In this report, we describe the construction of a transcriptional map and characterization of two proteins encoded by the E3 region of BAV-3.

The entire BAV-3 E3 transcription unit was found to be 1591 bp in length, which is smaller than the E3 region of HAV-5 (Wold, 1995) and BAV-1 (Evans *et al.,* 1998), but larger than the E3 of MAV-1 (Beard *et al.,* 1990) and porcine adenoviruses (PAV) (Reddy *et al.,* 1996). Like other adenoviruses, the promoter of the BAV-3 E3 region is also located in the coding sequence of the pVIII virion protein. However, a detailed transcriptional map of the E3 region of BAV-3 showed marked differences when compared with those of other adenoviruses (Beard *et al.,* 1990; Evans *et al.,* 1998; Wold, 1995). Four major classes of mRNAs transcribed from the E3 promoter were identified in BAV-3, which is more than the 1 to 3 observed in BAV-1 (Evans *et al.,* 1998), MAV-1 (Beard *et al.,* 1990), and PAV-3 (Reddy *et al.,* 1996), but less than the 7 to 10 observed in HAV-5 (Wold, 1995). Similar to MAV-1 (Beard *et al.,* 1990), BAV-3 mRNA was found to be 5′ and 3′ coterminal. In contrast, HAV-5 E3 mRNAs share the 5′ end sequence but utilize at least two different poly(A) sites (Wold, 1995). In BAV-3, one mRNA, which is 3′ coterminal with other E3 mRNAs, originates from the MLP, since the 5′ end of this transcript has a TPL sequence similar to one found spliced to the 5′ end of the late region mRNAs of BAV-3 (Reddy *et al.,* 1998). Similar mRNAs have been identified in HAVs (Wold, 1995). In addition, the BAV-3 E3 mRNAs were 3′ coterminal with late (L6) mRNAs (Reddy *et al.,* 1998), which is similar to the results reported for MAV-1 (Beard *et al.,* 1990). However, in HAV-5 (Wold, 1995), the 3′ end of the late (L4) mRNAs lies in the first intron of the E3 mRNAs.

Like HAV-2 (Wold, 1995), BAV-3 E3 transcripts originating from the E3 promoter contain internal introns. In addition, a late E3 transcript originating from BAV-3 MLP utilizes the same splice acceptor site used by a transcript initiating from the E3 promoter. This suggests that multiple E3 proteins arise by differential splicing of BAV-3 E3 primary transcripts. A similar mechanism has been proposed for the expression of multiple E3 proteins in HAV-2 (Wold, 1995). Surprisingly, no introns have been found in the E3 transcript of BAV-1, which has led to the speculation that differential splicing may not be used to generate multiple E3 proteins in BAV-1 (Evans *et al.,* 1998). However, this requires further confirmation as the technique used may not be sensitive enough to identify the structure of mRNAs present in small quantities.

The E3 mRNAs of BAV-3 have the potential to code for four proteins including 284R and 121R. Protein 284R has the sequence features of a type I membrane protein. It contains an N-terminal signal sequence, a hydrophobic transmembrane anchor sequence near the carboxyl terminus, and seven potential sites for the addition of N-linked oligosaccharides. Antiserum directed against the 284R protein immunoprecipitated four bands of 48, 67, and 125/140 kDa from BAV-3-infected cells but not from mock-infected cells. The same 48- and 67-kDa bands were also detected in a Western blot, suggesting that they represent posttranslationally modified forms of 284R and are not coimmunoprecipitating proteins. *In vitro* transcription and translation of the 284R gene yielded protein of 26–32 kDa, confirming that two bands (48 and 67 kDa) are produced due to posttranslational modification. The 48-kDa form of 284R was sensitive to endo H and PNGase F, indicating that it contains exclusively high-mannose oligosaccharides and probably resides in the rough endoplasmic reticulum. The 67-kDa form was sensitive to PNGase F but not to endo H, indicating that it contained complex rather than high-mannose-type oligosaccharides. These data indicate that 284R contains N-linked oligosaccharides of both high mannose and complex types.

Based on molecular mass, the 140-kDa protein appears to be a homodimer of the 67-kDa protein; and the 125-kDa protein appears to be a heterodimer of the 67- and 48-kDa proteins; however, we were not able to detect these proteins in Western blots. Whether it is a dimer or contains a 284R protein complexed with other protein remains to be determined.

The E3 region of HAVs encodes a number of glycoproteins, namely 6.7K (Wilson-Rawls and Wold, 1993), gp19K (Persson *et al.,* 1979), 11.6K (Scaria *et al.,* 1992), 20.5K (Hawkins and Wold, 1995), and 14.5K (Tollefson *et al.,* 1990). Recently, the E3 region of MAV-1 has also been shown to encode a glycoprotein (Beard and Spindler, 1995). However, none of these proteins showed any significant homology to the 284R glycoprotein of the BAV-3 E3 region. In addition, 284R is transported to the cell surface instead of being retained in the rough endoplasmic reticulum. This suggests that 284R may be functionally different from glycoproteins encoded by the E3 region of other adenoviruses. This is not surprising as the E3 regions of different adenoviruses including different BAV serotypes show little homology (Evans *et al.,* 1998; Wold and Gooding, 1991; Wold, 1995). Since the E3 proteins may reveal the pathogenic potential of an adenovirus, determining the role of 284R protein in BAV-3 infection of cattle may reveal new mechanisms of adenovirus–host interactions.

Antisera produced against 121R immunoprecipitated a

single protein of 14.5 kDa from BAV-3-infected but not from mock-infected MDBK cells. *In vitro* transcription and translation of the 121R gene also yielded a protein of 14.5 kDa, suggesting that 121R is not posttranslationally modified. The 121R protein shows limited homology to the 14.7K protein encoded by the E3 region of HAV-5 (Mittal *et al.,* 1993; Reddy *et al.,* 1998), which has been shown to counteract the effect of tumor necrosis factor (Gooding *et al.,* 1988). The homologs (serological and functional) of the 14.7K protein have been found in different adenoviruses (Horton *et al.,* 1990). The BAV-3 121R and HAV-5 14.7K proteins are also serologically related, as antiserum against the 14.7K protein of HAV-5 immunoprecipitates a protein of 14.5K (faint band) from BAV-3-infected MDBK cells (Zakhartchouk, personal communication). This suggests that 121R encoded by the E3 region of BAV-3 may be functionally related to the 14.7K protein encoded by HAV-5.

As the E3 region proteins have been implicated in mediating virus–host interactions, especially those involving the immune system, the characterization of the BAV-3 E3 region should help in determining the role each E3 protein plays in infection of its natural host (cattle).

MATERIALS AND METHODS

Cells and virus. Madin Darby bovine kidney (MDBK) cells and fetal bovine retina cells (FBRC) were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. Virus stocks of the WBR-1 strain of BAV-3 were prepared in MDBK cells.

Northern blots. The total RNA from MDBK or FBR cells (60 PFU/cell) infected with BAV-3 in the presence of AraC (125 μg/ml) was extracted as described (Chomczynski and Sacchi, 1987). The RNA (10 μg/lane) from mock- or BAV-3-infected FBR cells was separated on 1% agarose–formaldehyde gels and transferred to Nytran membranes as described (Sambrook *et al.,* 1989). The blots were baked and processed as described previously (Sambrook *et al.,* 1989). Finally, the membranes were allowed to air-dry and then subjected to autoradiography.

cDNA cloning. The generation of a BAV-3 mRNA-specific cDNA library has been described (Reddy *et al.,* 1998). Briefly, the cDNA library was generated using poly(A) RNA extracted from BAV-3-infected cells at 8–10 and 18–24 h postinfection and cloned into Lambda ZAP vectors (Stratagene). The plaques were screened with the isolated BAV-3 E3 1.2-kb fragment (nt 26,472 to 27,683). The selected plaques were used to excise the plasmids containing cDNAs. The resulting plasmids were divided into groups based on restriction enzyme profiles and sequenced by the dideoxy chain-termination method (Sanger *et al.,* 1977).

Nuclease protection assay. To prepare the probe, selected fragments of DNA were digested with the appropriate enzyme. They were either labeled with [γ-^{32}P]dATP, using polynucleotide kinase, or 3′ end-labeled with [α-^{32}P]dCTP using the Klenow fragment. The desired labeled DNA was isolated from the gel after digesting with a second enzyme to remove the label at one end. S1 nuclease protection assays were performed using the procedure described previously (Berk and Sharp, 1977; Sambrook *et al.,* 1989).

RT-PCR analysis of mRNA. Total RNA extracted from mock- or BAV-3-infected MDBK cells was used to synthesize first-strand cDNA using oligo(dT) as a primer in the presence of MuLV reverse transcriptase according to the supplier's directions (Perkin–Elmer). Using two primers (Fig. 3) the cDNAs were amplified in the thermal cycler (Perkin–Elmer) under the following conditions: denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for 2 min. The amplified cDNA products were blunt end repaired with T4 DNA polymerase and cloned into pGEM3Z(+) plasmid and sequenced as described (Sanger *et al.,* 1977).

Plasmid construction. A 1.056-kb *Bam*HI–*Nco*I fragment containing the E3 284R gene (Reddy *et al.,* 1998) was excised from plasmid pSM14 (containing the *Bam*HI "D" fragment of the BAV-3 genome; Mittal *et al.,* 1995) and ligated to *Bam*HI–*Nco*I-digested pSL301, generating plasmid pSL.284R. A 0.95-kb *Afl*II–*Nco*I fragment (blunt end repaired with T4 DNA polymerase) derived from pSL.284R was inserted into *Sma*I-digested pSP64polyA, generating plasmid pSP64.284R. A 0.698-kb *Xho*I–*Nco*I fragment (blunt end repaired with T4 DNA polymerase) derived from pSL.284R was ligated to *Sal*I-digested (blunt end repaired with T4 DNA polymerase) plasmid pGEX-5X-3, generating plasmid pGEX.284R.

A 0.585-kb *Nco*I–*Hpa*I fragment containing the E3 121R gene (Reddy *et al.,* 1998) was excised from plasmid pSM14 (Mittal *et al.,* 1995) and ligated to *Nco*I–*Hpa*I-digested pSL301, generating plasmid pSL.121R. The same 0.585-kb *Nco*I–*Hpa*I fragment was blunt end repaired with T4 DNA polymerase and ligated to *Hin*cII-digested pSP64polyA, generating plasmid pSP64.121R, and to *Sal*I-digested (blunt end repaired with T4 DNA polymerase) pGEX-5X-3, generating plasmid pGEX.121R.

Antisera. The coding regions of 284R (C-terminal 220 amino acids) and 121R were fused individually to the gene encoding GST in plasmid pGEX-5X-3 (Pharmacia), creating plasmids pGEX.284R and pGEX.121R, respectively. The junctions of sequences encoding GST-284R or GST-121R were sequenced to ensure that the coding domains were in-frame. The competent *Escherichia coli* strain BL21 was transformed with pGEX.284R or pGEX.121R plasmids. The fusion protein(s) was induced by addition of 0.1 M isopropyl-β-D-thiogalactoside and purified using sodium dodecyl sulfate (SDS)–polyacryl-

amide gels (PAGE). Rabbits were immunized subcutaneously with gel-purified GST-284R or GST-121R fusion proteins in Freund's complete adjuvant followed by four injections in Freund's incomplete adjuvant at 4-week interval.

In vitro transcription/translation. Plasmid DNA (0.5–5 μg) (pSP64.284R or pSP64.121R) was *in vitro* transcribed with SP6 polymerase as described by the supplier (Promega). RNA was translated *in vitro* for 2 h at 30°C using a rabbit reticulocyte lysate containing 50 μCi [^{3}H]leucine. After synthesis, the *in vitro* translated proteins were analyzed on SDS–PAGE with or without immunoprecipitation.

Radiolabeling of cells and immunoprecipitation. For immunoprecipitation, MDBK cells were infected with BAV-3 at a multiplicity of infection of 5. After 90 min of adsorption, the cells were washed and incubated in MEM containing 2% FBS. At different times postinfection, the cells were incubated in leucine-free medium for 2 h before being labeled with [^{3}H]leucine (100 μCi/ml). Similarly, the infected cells were incubated in glucose-free medium for 2 h before being labeled with [^{3}H]glucosamine (100 μCi/ml) or [^{3}H]mannose (100 μCi/ml). After 4 h of labeling, the proteins were immunoprecipitated from infected cells lysed with modified radioimmunoprecipitation assay buffer and analyzed by SDS–PAGE as described previously (Tikoo *et al.,* 1993).

Western blot analysis. The Western blotting technique was performed as described (Sambrook *et al.,* 1989). Briefly, after electrophoresis, mock- or BAV-3-infected cell lysates were electrophoretically transferred to nitrocellulose sheets. The membranes were treated with 1% bovine serum albumin fraction V followed by rabbit anti-284R serum. Finally, the membranes were washed, exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase, and developed using an HRP color development kit.

Enzyme treatments. The immunoprecipitated proteins were eluted from protein A–Sepharose in 0.5% SDS and digested with endo H, PNGase F, neuraminidase, and/or *O*-glycosidase as described previously (Tikoo *et al.,* 1993). Finally, the proteins were analyzed by SDS–PAGE.

ACKNOWLEDGMENTS

We thank other members of our laboratory including Dr. Alexandre Zakhartchouk, Dr. Mohit Baxi, Yan Chen, and Caron Pyne for help and advice; Transgene, Strasbourg, France for their help and advice; and the staff of the Animal Care Unit of Veterinary Infectious Disease Organization for their help with animal work. This work was supported by grants from Natural Science and Engineering Research Council of Canada, Saskatchewan Agriculture Development Fund, Saskatchewan Beef Development Fund, Western Economic Diversification, and Alberta Agriculture Research Institute. P.S.R. was the recipient of a postdoctoral research fellowship from the Health Services Utilization and Research Commission, Saskatoon, Saskatchewan, Canada.

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