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Subgroup B Adenovirus Type 35 Early Region 3 mRNAs Differ from Those

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Adenovirus type 35 (Ad35) is a member of Ad subgroup B, DNA homology cluster B2. The B2 Ads are unique in that they are isolated most frequently from immunosupressed individuals such as AIDS patients and bone marrow transplant recipients and in that they have a tropism for the urinary tract. One region of the Ad genome which may influence serotype specific pathology is early region 3 (E3). E3 of subgroup C Ad2 and Ad5 has been shown to encode proteins which counteract the immune response to Ad infection. While a great deal is known about gene expression of the subgroup C Ad E3s, little is known about the E3 gene expression from the subgroup B Ads. Although some E3 open reading frames (ORFs) are shared between subgroups B and C, there are additional ORFs that appear in subgroup B. This paper demonstrates the results of an analysis of gene expression from the Ad35 E3 and describes differences in splicing and polyadenylation between the Ad35 and Ad2 E3s. RT–PCR, cDNA sequencing, RNase protection, 3′ RACE, and Northern blotting techniques were utilized to identify, quantify, and determine the structure of six Ad35 E3 mRNAs predicted to encode at least seven proteins. A common intron that is removed during splicing of the subgroup C E3 mRNAs is not removed from Ad35 E3 mRNAs, and only one E3 polyadenylation signal is present in the Ad35 E3 while two polyadenylation signals are used in the formation of subgroup C E3 mRNAs. The quantity of individual mRNAs encoding homologous proteins for Ad35 and Ad2 also differ substantially, presumably because of the absence in Ad35 of *cis*-acting signals which have been shown to be important for regulation of Ad2 E3 pre-mRNA processing. Such information should contribute to an understanding of the role the E3 plays in determining subgroup B Ad pathogenesis in general and Ad35 pathogenesis in particular.

INTRODUCTION

Adenovirus type 35 (Ad35), which is most frequently isolated from immunosuppressed hosts such as renal transplant recipients, bone marrow transplant patients, or AIDS patients (DeJong *et al.,* 1983; Horwitz *et al.,* 1984; Flomenberg *et al.,* 1987, 1994), is classified as a subgroup B Ad. Subgroup B, which has been divided into clusters B1 and B2 based on genomic DNA homology (Wadell, 1984), is one of six subgroups of human adenoviruses (designated A–F) and contains several of the more virulent adenovirus types (Horwitz, 1990). The B1 Ads include Ad3 and Ad7 which can cause fatal, epidemic pneumonia and Ad21 which can cause pneumonia and hemorrhagic cystitis. The B2 Ads include the urinary tract pathogens Ads11, Ad34, and Ad35 as well as the respiratory tract pathogen Ad14 (Horwitz, 1990). The molecular determinants which influence the type of disease caused by different Ad serotypes and subgroups are poorly understood. However, early region 3 (E3) of human adenoviruses appears to encode proteins which may influence Ad pathogenesis by modulating the immune response to Ad infection (Wold, 1995).

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The E3 of the subgroup C Ad2 encodes several proteins, four of which have been shown to affect the host immune response. The E3 proteins which have been detected in infected cells are the 12.5-, 6.7-, 19-, 11.6-, 10.4-, 14.5-, and 14.7-kDa proteins, named, respectively, 12.5K, 6.7K, 19K, 11.6K, 10.4K, 14.5K, and 14.7K. The 12.5K protein is produced in small amounts early and late in infection and has no known function. (Hawkins and Wold, 1992). The 11.6K protein is produced at low levels early in infection and at increased levels late in infection (Scaria *et al.,* 1992; Tollefson *et al.,* 1992). The 11.6K protein appears to facilitate release of virus particles from the infected cell (Wold, 1995). The 6.7K and 19K proteins are both endoplasmic reticulum (ER) resident proteins (Wilson-Rawls *et al.,* 1990; Hawkins *et al.,* 1993). While the 6.7K has no known function, the 19K protein is a glycoprotein (known as gp19K) which binds to and retains class I MHC in the ER of infected cells, thus preventing presentation of viral antigen to $CD8^+$ T-cells (Burgert *et al.,* 1987; Pääbo *et al.,* 1987; Tanaka and Tevethia, 1988; Cox *et al.,* 1990). The 14.7K protein is a soluble cytoplasmic and nuclear protein (Tollefson and Wold, 1988) which prevents lysis of infected cells by TNFα (Gooding *et al.,* 1988, 1990; Horton *et al.,* 1991). The 10.4K and 14.5K proteins are integral membrane proteins which localize to the plasma membrane and have been shown to be involved in two functions. Coexpression of 10.4K

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and 14.5K protects some cells from lysis by TNFα (Gooding *et al.,* 1991). Additionally, expression of 10.4K and 14.5K by Ad2 has been shown to cause downregulation of type I and type II tyrosine kinase receptors including the epidermal growth factor receptor (EGF-R) and the insulin receptor (Carlin *et al.,* 1989; Kuivinen *et al.,* 1993). Expression of 10.4K alone from a retroviral expression vector has been shown to be sufficient to achieve downregulation of the EGF-R surface expression (Hoffman *et al.,* 1990). Net EGF-R downregulation results from the targeting of the EGF-R to the prelysosomal multivesicular bodies and its subsequent degradation (Hoffman and Carlin, 1994).

Early in Ad infection, expression of E3 genes is driven by the E3 promoter (Berk and Sharp, 1977). Late in infection, E3 genes can be expressed via the major late transcription unit since the E3 lies between two families of late Ad transcripts, the L4 and L5 families (Bhat and Wold, 1986). Of note, the polyadenylation signal for the L4 transcript family lies within the E3 region, but in Ad2-infected cells, this L4 poly(A) site is active only in the late phase of viral gene expression (Brady *et al.,* 1992).

At least seven alternatively spliced mRNAs are produced by the E3 of the subgroup C Ad2 (Wold, 1995). The *cis*-acting signals required for proper Ad2 E3 splicing have been thoroughly analyzed and include two polyadenylation signals, an atypical AUUAAA, and a typical AAUAAA (Bhat and Wold, 1985). Four regions containing *cis*-acting sequences which also regulate Ad2 E3 splicing have been identified (Bhat and Wold, 1985; Deutscher *et al.,* 1985; Bhat *et al.,* 1986; Bhat and Wold, 1986; Brady *et al.,* 1992; Scaria and Wold, 1994). Like the two poly(A) sites, these regions are important for maintaining the normal balance of E3 mRNA production. Analysis of Ad2 E3 RNAs has not only helped explain how the complex E3 transcription unit functions, but has also facilitated analysis of E3 protein functions. Viruses containing deletions within one E3 ORF often have unpredictably altered E3 mRNA expression levels and thus protein expression (Deutscher *et al.,* 1985).

Little is known about the gene expression from the subgroup B E3 regions. The subgroup B1 and B2 E3s contain ORFs homologous to the Ad2 12.5K, gp19K, 10.4K, 14.5K, and 14.7K proteins (Signäs *et al.,* 1986; Flomenberg *et al.,* 1988; Hong *et al.,* 1988; Mei and Wadell, 1992, 1993; Basler *et al.,* 1995). Ad35, a subgroup B2 Ad, has been shown to produce a homologue of the Ad2 gp19K (Flomenberg *et al.,* 1987). It has also been demonstrated that the B1 Ads produce proteins homologous to the Ad2 gp19K and 14.7K (Pääbo *et al.,* 1986; Horton *et al.,* 1990). In addition to these ORFs, a 20.5K protein has been shown to be produced after Ad3 infection (Hawkins and Wold, 1995), and its 20.6K homologue is produced after Ad35 infection (unpublished data). The B1 and B2 E3s also contain ORFs potentially encoding proteins of approximately 15.0K and 20.3K which are not encoded by the subgroup C E3s (Signäs *et al.,* 1986; Flomenberg *et al.,* 1988; Hong *et al.,* 1988; Mei and Wadell, 1992, 1993; Basler *et al.,* 1995). The B1 Ads also encode an ORF homologous to that which encodes the Ad2 11.6K protein (Signäs *et al.,* 1986; Hong *et al.,* 1988), but this ORF is absent from the B2 Ads (Flomenberg *et al.,* 1988; Mei and Wadell, 1992, 1993; Basler *et al.,* 1995). The structure of the subgroup B E3 mRNAs is also not known. Limited data suggest that there are splice acceptors upstream of each of the two 20K ORFs of the Ad3 E3 as well as splice acceptors upstream of the Ad3 ORFs homologous to the Ad2 14.5K and 14.7K ORFs (Signäs *et al.,* 1986). However, neither the overall structure nor the exact splice sites of these mRNAs have been determined. No data have been published regarding the subgroup B2 E3 mRNAs.

This paper presents the Ad35 E3 mRNA structures and quantities. These data should help in understanding which E3 ORFs encode proteins synthesized during Ad35 infection. Additionally, knowledge of the abundance and the sequence context of the AUGs of each mRNA should correlate with the amount of the E3 proteins translated from each mRNA. Comparative studies of the subgroup B2 Ad35 and the subgroup C Ad2 E3 mRNAs may elucidate molecular differences between these viruses which potentially influence viral pathogenesis. Since the Ad2 E3 has been used as a model for analyzing the regulation of splicing and polyadenylation in a complex transcription unit, the structure and abundance of the Ad2 and the Ad35 E3 mRNAs have been compared. This analysis may help refine our understanding of the *cis*-acting regulatory sequences which influence E3 pre-mRNA processing.

MATERIALS AND METHODS

Cells and virus

Initial reverse transcription–polymerase chain reaction (RT–PCR) experiments were performed using HeLa cells, while subsequent experiments utilized 293 cells. Cells were infected with Ad35 (10^3 to 10^4 particles/cell of Holden strain). Virus was added to $2–5 \times 10^7$ cells, at a concentration of 1×10^7 cells/ml, in suspension culture in the absence of serum, incubated with cells at 37° for 15 min before the cells were diluted to 1×10^6 cells/ml with Joklik's modified minimal essential medium containing 5% fetal bovine serum. To enhance accumulation of early viral mRNAs in Northern blotting experiments, cycloheximide (25 μg/ml) was added at 2 hr postinfection (p.i.) (Bhat *et al.,* 1986; Scaria and Wold, 1994). Addition of cycloheximide to Ad2-infected cells does not substantially affect the relative amounts of different Ad2 E3 mRNAs produced (Bhat and Wold, 1986b). For RT–PCR experiments and for RNase protection experiments, cytosine arabinoside (AraC, 20 μg/ml) was added one-half hour p.i. to prevent viral DNA synthesis, thus pre-

venting the switch to late viral gene expression. Infections were allowed to procede 9 hr in the presence of cycloheximide or 12 hr in the presence of AraC.

Isolation of RNA

Total RNA was isolated using TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer's instructions. Total RNA, used for RT–PCR and Northern blotting experiments, was treated with 10 units RNase-free DNase I for 15 min at 37° in 80 m*M* HEPES, pH 7.5, 12 m*M* $MgCl_2$, 10 m*M* NaCl, 10 m*M* DTT. Cytoplasmic RNA was prepared following the protocol for isolation of cytoplasmic RNA from mammalian cells outlined in Sambrook *et al.* (1989). Poly(A)$^+$ RNA was prepared using the Qiagen (Chatsworth, California) Oligotex-dT mRNA isolation kit. One hundred to three hundred micrograms of cytoplasmic RNA from uninfected 293 cells, from Ad35-infected 293 cells in the early phase of viral gene expression, or from Ad35-infected 293 cells in the late phase of viral gene expression was bound to Oligotex-dT suspension in binding buffer (10 m*M* Tris–HCl, pH 7.5, 300 m*M* NaCl, 1 m*M* EDTA), washed twice in wash buffer (10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA), and eluted in 80° elution buffer (5 m*M* Tris–HCl, pH 7.5).

RT–PCR analysis of Ad35 early RNAs

First-strand cDNA was synthesized using Gibco BRL Superscript Reverse Transcriptase, according to the supplier's directions, on oligo(dT)-primed, DNase-treated, total RNA from Ad35-infected HeLa or 293 cells. PCR reactions contained 10 m*M* Tris–HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.2 m*M* each dATP, dCTP, dGTP, dTTP, 2.5 units *Taq* DNA polymerase (Boehringer-Mannheim or Gibco BRL), 1 μl each primer, and 1 μl first-strand cDNA diluted 1:10 or 1 μl Ad35 genomic DNA. PCR primers used for RT–PCR analysis are shown in Fig. 1. Their sequence and location in the reported E3 sequence are indicated in the legend to Fig. 1. Reactions were performed in a Perkin–Elmer DNA Thermal Cycler. Reactions were incubated once at 94° for 7 min; at 94° for 1 min, 55° for 1 min, 72° for 1.5 min, for 25 cycles; and once at 72° for 8 min. Reactions were analyzed on 1% agarose/1× TAE gels.

RT–PCR reactions were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). Products were then cloned by ligating the extracted PCR reaction products to pGEM-T (Promega) or pT7Blue-T (Novagen) DNA. Possible recombinant plasmids were screened for by standard procedures. Plasmids containing the desired insert were purified according to manufacturer's instructions on Qiagen midi- or maxiprep columns prior to sequencing. Sequencing reactions were primed with T7 or SP6 promoter primers or with appropriate primers previously used to determine E3 sequence (Basler *et al.,* 1995).

RNase protection

Templates for RNase protection probes A, C, E, and F, the sequences of which are indicated in Fig. 1, were amplified by PCR, using custom synthesized oligonucleotide primers and Ad35 genomic DNA as PCR template, and the products cloned into pGEM-T or pT7Blue-T as described for the RT–PCR methods. The resulting plasmids were linearized with restriction endonucleases, and antisense probes were transcribed using the Ambion Maxiscript *In Vitro* Transcription kit with T7, T3, or SP6 RNA polymerase. Probes B and B′ were transcribed with T3 RNA polymerase using as template pBSAdC (Basler *et al.,* 1995) linearized with *Hpa*I or *Sma*I, respectively. Probe D was generated by removing the sequences between the *Bam*HI and *Xma*I sites from pBSAdPX (Basler *et al.,* 1995) and religating the plasmid, creating the plasmid p10.4RNAP. p10.4RNAP was linearized by digestion with *Eco*RI, and probe D transcribed with T7 RNA polymerase. RNA size marker was synthesized from Ambion RNA Century Marker template using T7 RNA polymerase. Transcription reactions were diluted 1:1 in gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, 2 m*M* EDTA). Two microliters was removed, diluted to 0.2 ml with ddH_2O, and 2 μl of this mixture was counted in a scintillation counter to determine total cpm/reaction. The remaining probe was then gel purified. The probe was heated to 95° for 3 min and run on a 5% acrylamide/8 *M* urea/1× TBE (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA) gel for 30 min at 300 V. The gel was subsequently exposed to Kodak XOMAT-AR film for 3–5 min until a band was visible on developed film. Using the film to determine band position within the gel, the full-length probe (usually the strongest, highest molecular weight band seen) was excised from the gel and eluted by soaking the gel slice in 0.35 ml 0.5 *M* ammonium acetate, 1 m*M* EDTA, 0.2% SDS for 2–5 hr at 37°. Two microliters of probe was counted in a scintillation counter to determine the amount of UTP incorporation.

RNase protection experiments were done using the Ambion RPAII RNase Protection Assay kit according to manufacturer's instructions using 1–3 μg of poly(A)$^+$ RNA or yeast total RNA per reaction. The entire RNase digestion sample was loaded on 5% acrylamide (19:1 acrylamide:bisacrylamide) gel except for "undigested probe" samples (probe hybridized to yeast RNA and not digested with RNase), of which only one-quarter of the sample was loaded on the gel.

3′ RACE reactions

3′ RACE (Rapid Amplification of cDNA 3′ Ends) (Gibco BRL) uses an adapter primer (AP) (5′GGCCACGCGTCGACTAGTAC$(dT)_{17}$3′), an oligo(dT) primer with a defined

(non-dT) sequence at its 5′ end, to prime first-strand cDNA synthesis. The oligo(dT) portion of AP hybridizes to the mRNA poly(A) tail to prime a reverse transcription reaction. The defined sequence at the 5′ end of AP is thus incorporated into all cDNA molecules synthesized. PCR can then be carried out on the first-strand cDNA; a universal amplification primer (UAP) (5′CUACUACUACUAGGCCACGCGTCGACTAGTAC3′), which is complementary to the defined sequence in AP, can then be used in conjunction with an upstream primer, which binds to known sequences, to specifically amplify from the upstream primer to sites of mRNA polyadenylation. Primers 1, 2, and 5 (Fig. 1) were used as upstream primers for 3′ RACE reactions reported in this paper.

Northern blotting

Ten micrograms of RNA size marker (Gibco BRL 0.24–9.5 kb RNA ladder) or approximately 15 μg, in 4.5 μl, of total RNA from Ad35-infected cells in the early phase of viral gene expression was added to 17.5 μl of RNA sample premix (6% formaldehyde, 46.5% formamide, 1× MOPS gel running buffer [20 m*M* MOPS, pH 7.0, 8 m*M* sodium acetate, 1 m*M* EDTA, pH 8.0, 1.4 mg/ml ethidium bromide, and 14% loading dye (50% glycerol, 1 m*M* EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol)]. Samples were heated to 65° for 5 min, chilled on ice, centrifuged briefly, and loaded onto an agarose/formaldehyde gel. The gel (1.5% agarose, 1× MOPS running buffer, 3% formaldehyde) was run in 1× MOPS running buffer at 120 V for 4 hr. After electrophoresis, the gel was rinsed several times in dH_2O, photographed with a ruler under ultraviolet light transillumination, and soaked for 45 min in 400 ml 10× SSC (20× SSC is 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0). The RNA was then transferred to Hybond N^+ (Amersham) nylon membranes by capillary action as described (Sambrook *et al.,* 1989). RNA was fixed on the membrane by soaking in 50 m*M* NaOH for 5 min followed by a brief wash in 2× SSC. Membranes were blocked by incubation in Amersham Rapid-Hyb buffer for at least 15 min at 65°. Heat-denatured probe ($0.5–1 \times 10^6$ cpm/ml hybridization buffer) was added and hybridization continued overnight at 65°. Hybridized membranes were then washed twice for 15 min in 250 ml 1× SSC, 0.1% SDS at room temperature and twice for 15 min in 250 ml 0.1× SSC, 0.1% SDS at 65°. Membranes were allowed to air dry and then exposed to film.

RESULTS

Initial experiments examining the structure of the Ad35 E3 mRNAs utilized a RT–PCR strategy. This strategy, placed in the context of the results, is presented in Fig. 1. A common 5′ primer and separate 3′ primers, each in a different E3 ORF, were generated based on the E3 sequence and used in reactions containing either first-strand cDNA generated from Ad35-infected cells in the early phase of viral gene expression as template or in control reactions containing Ad35 genomic DNA as template. It should be noted that in PCR reactions containing multiple templates with varying lengths of sequence between primer binding sites (i.e., cDNA from alternatively spliced mRNAs) the shorter-length templates will be preferentially amplified (Fejer *et al.,* 1994). However, when there are multiple templates of approximately equal size and amount, two bands have been observed (see below).

PCR reactions done using primer 1 and primer 6 (Fig. 1), complementary to sequences at the 3′ end of the 20.6-K ORF, resulted in the amplification of an approximately 2.56-kb product from Ad35 genomic DNA template and an approximately 1.3-kb product from cDNA (Fig. 2, lanes 1 and 2). The 1.3-kb product was cloned, sequenced, and shown to correspond to a spliced E3 mRNA with an excised intron spanning nucleotides 1187–2418 of the published Ad35 E3 sequence. The splice donor had the sequence AG/GT at the exon/intron boundry and the splice acceptor had the sequence AG/CC at the intron/exon boundry, suggesting genuine splice sites. In contrast, PCR reactions done with primer 1 and primer 3, complementary to sequences near the 3′ end of the 18.5K ORF, amplified approximately 1.3-kb bands from both genomic DNA and cDNA (Fig. 2, lanes 4 and 5), suggesting that no splice acceptor is present upstream of the 18.5K ORF in E3 mRNAs. Similarly, PCR done using primer 1 and primer 4, complementary to the 3′ end of the 20.3K ORF, amplified approximately 2.0 kb on both cDNA and Ad35 genomic DNA, suggesting that no splice acceptor lies upstream of the 20.3K ORF (Fig. 3, lanes 1 and 2).

PCR reactions done with common primer 1 and primer 7, complementary to sequences within the 15.2K ORF, amplified a 2.9-kb product from Ad35 genomic DNA (Fig. 4, lane 1) and two products of 1117 and 910 bp from cDNA (Fig. 4, lane 2). The products amplified from cDNA correspond to spliced mRNAs from which introns spanning nucleotides 1187–3008 (mRNA *c*) and nucleotides 1187–3215 (mRNA *d*) were removed. These two splice acceptors lie upstream of the 10.3K and 15.2K ORFs, respectively. The splice acceptor upstream of the 10.3K ORF had the sequence AG/GT at the intron/exon boundary, and the splice acceptor upstream of the 15.2K ORF had the sequence AG/AC at the intron/exon boundry, suggesting genuine splice sites. PCR reactions done with primer 1 and primer 8, complementary to sequence within the 15.3K ORF, produced a 3.5-kb product using Ad35 genomic DNA as template (Fig. 4, lane 3) and a 1.2-kb product using cDNA as template (Fig. 4, lane 4). The 1.2-kb product corresponded to an mRNA from which an intron spanning nucleotides 1187–3679 was removed. The splice acceptor had the sequence AG/TT at the intron/exon boundry, suggesting a genuine splice acceptor.

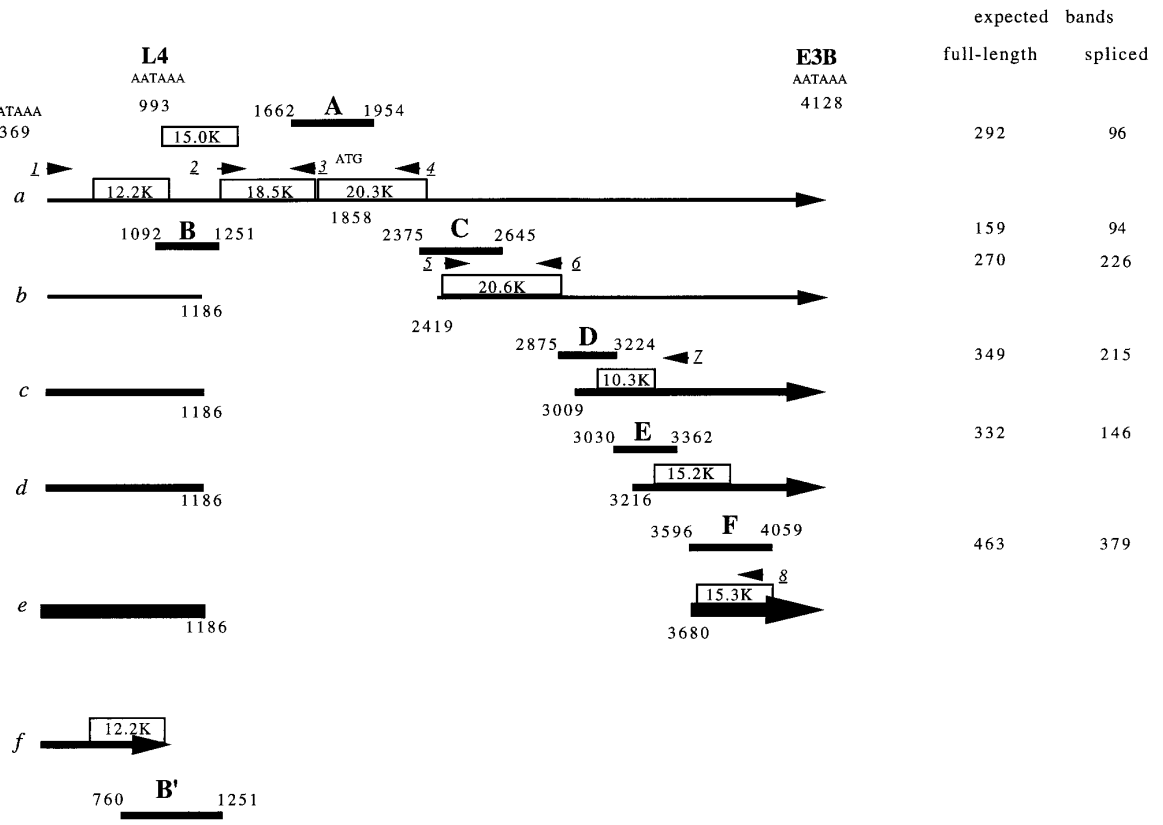

FIG. 1. The Ad35 E3 transcription unit and RNase protection strategy. The Ad35 mRNAs, the structures of which were determined by results presented in this report, are indicated by arrows and are labeled *a–f* in italics. The unbroken portions of the arrows represent exons, and the introns are indicated by breaks in the arrow. ORFs are shown as boxes above the mRNAs. An ORF is shown above the mRNA from which the encoded protein is most likely translated, and the coordinates for each ORF are as follows: 12.2K, 687–1004; 15.0K, 958–1353; 18.5K, 1338–1838; 20.3K, 1858–2403; 20.6K, 2421–2984; 10.3K, 3026–3301; 15.2K, 3306–3710; 15.3K, 3703–4110. The L4 and E3 polyadenylation signals are labeled at the top of the figure. The arrowheads above the mRNAs represent PCR primers used in RT–PCR and 3′ RACE reactions and are labeled 1–8 with underlined numbers. Primers correspond to the following coordinates in the reported Ad35 E3 sequence (Basler *et al.,* 1995; Genbank Accession No. U32664): primer 1, 433–452; primer 2, 1389–1405; primer 3, 1652–1680; primer 4, 2380–2403; primer 5, 2421–2444; primer 6, 2957–2984; primer 7, 3346–3362; primer 8, 3909–3927. RNase protection probes are indicated by the thick-lettered (A–F) bars above the mRNAs. Probe B′ was used in Northern blotting experiments. The nucleotide positions of the splice sites, the RNase protection probe termini, and the AUG of the 20.3-K ORF are also indicated. The table to the right indicates the anticipated RNase protection product sizes based on the RT–PCR data. "Full-length" refers to the length of probe colinear with unspliced segments of E3 mRNAs, the length of which is equivalent to the length of Ad35 genomic DNA. "Spliced" refers to the length of the probe expected to be protected by a spliced E3 mRNA.

RNase protection

RNase protection experiments were performed to confirm the use of the splice sites detected by RT–PCR and to quantify Ad35 E3 mRNA levels by directly probing mRNA. A series of [^{32}P]UTP-labeled, antisense RNA probes (Fig. 1, A–F) were generated. These were hybridized to poly(A)$^+$, cytoplasmic RNA from Ad35-infected cells in either the early phase or the late phase of viral gene expression. The relative abundance of each mRNA, as determined by RNase protection, is indicated by the thickness of the arrows in Fig. 1. The expected-sized products, predicted based on the RT–PCR data, are indicated to the right of each probe. Examples of the types of results obtained with these probes are shown below.

Probe A, complementary to nucleotides 1954–1662 (Fig. 1), overlapped the 20.3K ORF by 96 nucleotides. A splice acceptor just upstream of this ORF would be expected to yield a product of approximately 100 nucleotides using probe A in an RNase protection experiment. However, only fully protected probe A was detected using "early" mRNAs (Fig. 5) as well as after probing late phase mRNA (data not shown). Therefore, the RT–PCR data and RNase protection data are concordant; no splice acceptor upstream of the 20.3K ORF can be detected.

Probe B, complementary to nucleotides 1251–1092, overlapped the splice donor at nucleotide 1186. Cytoplasmic, poly(A)$^+$ RNA from both the early phase (Fig. 6, lane 2) and the late phase (Fig. 6, lane 3) of Ad35 gene expression protected a 94-nucleotide fragment of probe B, indicating the use of the splice donor early and late in infection. Complete protection of the adenovirus derived sequences in probe B resulted in the protection of a 159-nucleotide product indicating that the splice donor is not

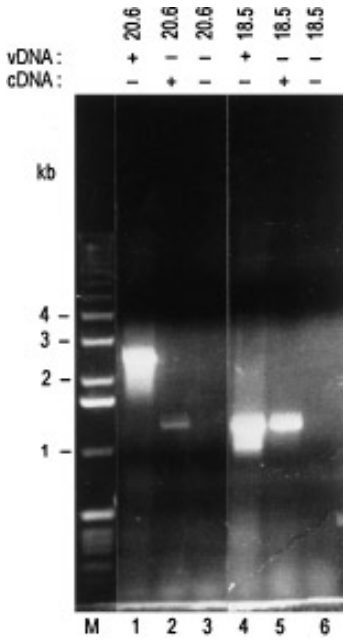

FIG. 2. RT–PCR demonstrating a spliced mRNA to the 20.6K ORF and unspliced mRNA to the 18.5K ORF. PCR amplification was carried out using primer 1, the E3 common 5′ primer, in conjunction with primer 6, to detect the 20.6K mRNA (lanes 1–3) or in conjunction with primer 3 to detect the 18.5K mRNA (lanes 4–6). Primer positions are indicated in Fig. 1. PCR template was Ad35 genomic DNA (vDNA) in lanes 1 and 4, and was oligo(dT)-primed, first-strand cDNA generated from Ad35-infected 293 cells in the early phase of viral gene expression (cDNA) in lanes 2 and 5. No template was added in lanes 3 and 6.

active in the formation of some of the E3 mRNAs either early or late in infection.

Probe C also detected two bands of the expected molecular sizes (data not shown) indicating the use of the splice acceptor at nucleotide 2419 as well as the presence of mRNA *a,* the formation of which requires that this splice acceptor not function. RNase protection with probe C demonstrated that mRNA *b* is the least abundant Ad35 E3 mRNA.

Probe D, complementary to nucleotides 3224–2875, overlapped the splice acceptor at nucleotide 3009. Two products were expected, a 349-nucleotide product, due to full protection of adenovirus sequences within the probe, and a 215-nucleotide product due to the use of the splice acceptor at nucleotide 3009. However, two doublets were seen when probe D was hybridized to mRNA from Ad35-infected cells in either the early phase (Fig. 7, lanes 2–4) or late phase (data not shown) of viral gene expression. A plus-sense, *in vitro* transcribed positive control RNA, corresponding to the sequences amplified by RT–PCR using primers 1 and 7, also yielded a doublet at the lower molecular weight (Fig. 7, lane 5). The doublets appear to arise due to the internal cleavage of some percentage of the RNA–probe hybrids. Since the positive control RNA also gives a doublet near the 215-nucleotide position, it is clear that the additional band is not due to the presence of additional, alternatively spliced mRNAs not detected by RT–PCR but rather arises due to some property of the RNA–probe hybrid. Therefore, the use of the splice acceptor at nucleotide 3009 has been demonstrated by RNase protection. The expected full protection of probe D by mRNAs *a* and *b* has also been demonstrated.

Probe E gave a result similar to probe D in that two sets of doublets were seen instead of two single bands. However, difficulties in producing full-length "positive control" RNA molecules for the probe E reactions have prevented an unequivocal demonstration that the doublets arise due to the properties of the RNA–probe E hybrids (data not shown). Although the splice acceptor at nucleotide 3216 as well as full protection of probe E by mRNAs *a–c* have been demonstrated, it cannot be absolutely excluded that the additional bands seen in reactions performed using probe E are due to the presence of alternatively spliced E3 mRNAs not previously detected by RT–PCR. Reactions performed with probe F clearly detected the use of the splice acceptor at nucleotide 3596 as well as mRNAs *a–d.* These reactions demonstrated that mRNA *e* is by far the most abundant Ad35 E3 mRNA, comprising approximately 50% of the total E3 mRNA.

Analysis of the 3′ ends of the E3 mRNAs

It was of interest to determine whether the mRNA which appeared to be unspliced from primer 1 to primer

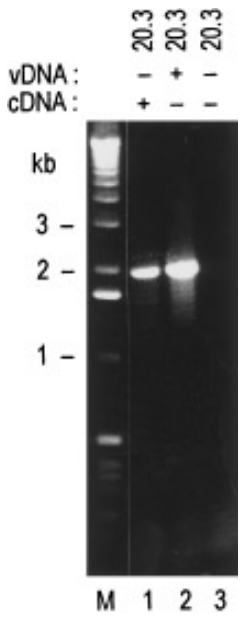

FIG. 3. RT–PCR detecting only unspliced mRNA to the 20.3K ORF. PCR was performed using primer 1, the common 5′ primer, in conjunction with primer 4 to detect the 20.3K mRNA. (See Fig. 1 for primer position.) Template for the reaction shown in lane 1 was first-strand cDNA prepared from Ad35-infected cells in the early phase of viral gene expression (cDNA). Template for the reaction shown in lane 2 was Ad35 genomic DNA (vDNA). A negative control reaction shown in lane 3 contained no added template.

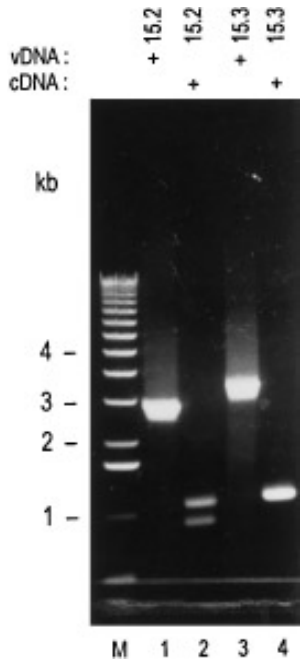

FIG. 4. RT–PCR of Ad35 E3 mRNAs for 10.3K, 15.2K, and 15.3K ORFs. PCR was performed using primer 1, the common 5′ primer, in conjunction with primer 7 to detect the 15.2K mRNA (lanes 1 and 2) or in conjunction with primer 8 to detect the 15.3K mRNA (lanes 3 and 4). (See Fig. 1 for primer position.) Template for the PCR reactions shown in lanes 2 and 4 was first-strand cDNA prepared from Ad35-infected 293 cells in the early phase of viral gene expression (cDNA). Template for the PCR reactions shown in lanes 1 and 3 was Ad35 genomic DNA (vDNA).

4 at the 3′ end of the 20.3K ORF (i.e., mRNA *a*) might contain a splice 3′ to the 20.3K ORF. In addition, it was important to identify the poly(A) sites used in formation of the Ad35 E3 mRNAs, as two poly(A) sites are used in the formation of the Ad2 and Ad5 E3 mRNAs. Therefore, 3′ RACE reactions were performed to examine the 3′ ends of the Ad35 E3 mRNAs and to define the location of the E3 poly(A) sites.

First-strand cDNA synthesis was performed using the "adapter primer" (AP) hybridized to template mRNA isolated from Ad35-infected cells in the early phase of viral gene expression. PCR was then performed on the first-strand cDNA using the "universal amplification primer" (UAP), complementary to the unique sequence in AP, in conjunction with either primer 2, complementary to sequences at the 5′ end of the 18.5K ORF, or primer 5, complementary to sequences at the 5′ end of the 20.6-kDa ORF (Fig. 1). In each reaction, only a single product was amplified, the size of which corresponded to the distance from the primer to the 3′ end of the Ad35 E3 region (Fig. 8, lanes 1 and 3). Therefore, only one poly(A) site downstream of the 18.5K ORF is used in the formation of Ad35 E3 mRNAs. Additionally, no splice sites downstream of the 18.5K ORF on mRNA *a* or the 20.6K ORF on mRNA *b* were detected (see Fig. 1).

The poly(A) site used for formation of the L4 family of mRNAs lies within the 12.2K ORF and includes the sequence AAUAAA at nucleotides 993–998. When 3′ RACE was performed using the UAP and primer 1, a 600-bp product was amplified, indicating the use of the L4 poly(A) site early during Ad35 infection (Fig. 9).

Northern blotting

In order to confirm the use of the L4 and "E3B" poly(A) sites and to confirm the absence of other E3 poly(A) sites, RNA blotting experiments were performed. RNA from Ad35-infected cells in the early phase of viral gene expression was probed with either probe B′; which is complementary to sequences at the 5′ end of the E3 and therefore should hybridize to all E3 mRNAs (Fig. 1), or with probe F, which should hybridize to all mRNAs which

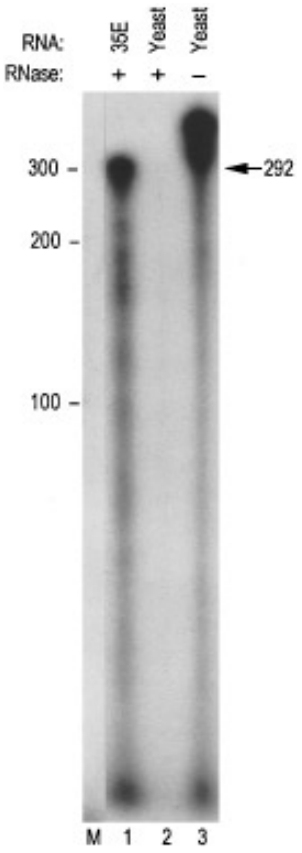

FIG. 5. RNase protection demonstrating no detectable spliced mRNA to the 20.3K ORF. [^{32}P]UTP-labeled, anti-sense probe A (see Fig. 1) was hybridized to 1 mg cytoplasmic, poly(A)$^+$ mRNA from Ad35-infected 293 cells in the early phase of viral gene expression (lane 1) or was hybridized to yeast RNA (lanes 2 and 3). Reactions 1 and 2 were digested with RNase A and T1 as described under Materials and Methods. Reaction 3 was not digested with RNase and only one-quarter of the total reaction was loaded on the gel. The arrow indicates the unspliced form of the mRNA detected by probe A. "Undigested" product, such as in lane 3, is of greater molecular size than "full-length" product, such as in lane 2, because both viral and plasmid sequences are transcribed during probe synthesis. The plasmid sequences are present in "undigested" product but not in "full-length" product. (See Fig. 1 for schematic of mRNAs and probes.)

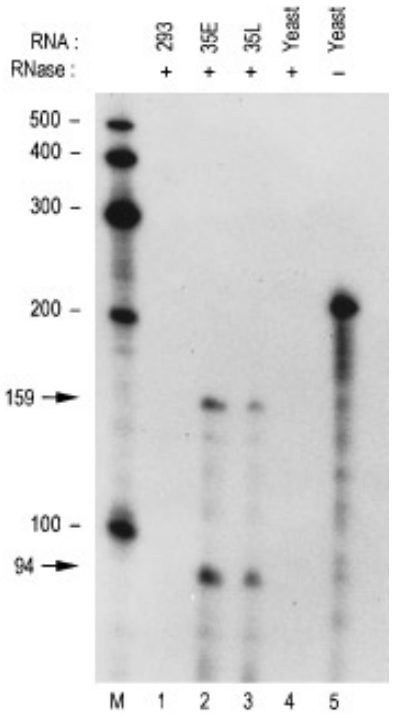

FIG. 6. RNase protection demonstrating the use of the common 5′ splice site. [^{32}P]UTP-labeled anti-sense probe B (lanes 1–5) was hybridized to 1 mg cytoplasmic, poly(A)$^+$ mRNA from uninfected 293 cells (lane 1), from Ad35-infected cells in the early phase of viral gene expression (lane 2), or from Ad35-infected 293 cells in the late phase of viral gene expression (lane 3). Probes were also hybridized to yeast RNA (lanes 4 and 5). All reactions were digested with RNase A and T1 except those shown in lane 5. One-quarter of samples from reaction 5 was loaded on the gel. The entire reaction was loaded on the gel for the remainder of the reactions. The upper arrow (159) on the left indicates the position of the unspliced E3 mRNA *a* which does not use the common 5′ splice site and is therefore colinear with probe B. The lower arrow indicates the product protected by spliced mRNAs *b*–*e* which use the 5′ splice site. (See Fig. 1 for schematic of mRNAs and probes.)

terminate at the "E3B" poly(A) site but not to mRNAs which hybridize to an "E3A" poly(A) site upstream of probe F (Fig. 1). Comparison of the bands produced after hybridization to probe B′ (Fig. 10, lane 1) to the bands produced after hybridization to probe F (Fig. 10, lane 2) indicates that only one mRNA terminates 5′ to probe F. This mRNA appears to be approximately 800 nucleotides long, roughly the size expected of a polyadenylated transcript which initiates at the E3 promoter and which terminates at the L4 poly(A) site. Therefore, in agreement with the 3′ RACE data, no "E3A" poly(A) site was found by blotting experiments. The same mRNA was also probed with probe E (Fig. 10, lane 3), which should hybridize to all mRNAs except *e* and *f* (Fig. 1). This experiment confirmed the identity of the lowest band as mRNA *f,* the next band as mRNA *e,* followed in increasing molecular size by mRNAs *d, c, b, b′*, and *a.* Of note, probes B′ and F hybridized to a *b* doublet (Fig. 10, lanes 1 and 2), while probe E hybridized to only the lower, mRNA *b* band. (Fig. 10, lane 3) The structure of the molecule represented by the upper *b′* band in the doublet is unknown.

DISCUSSION

This report presents the first map of the mRNAs produced by the immunoregulatory E3 region of a subgroup B Ad and presents the first data describing any aspect of the subgroup B2 Ad E3 mRNAs. There are several significant differences between Ad35 and the subgroup C Ad2 with regard to the abundance and structures of E3 mRNAs encoding homologous proteins (Fig. 11). The Ad35 10.3K, 15.2K, and 15.3K ORFs, encoding homologues of the Ad2 anti-TNFα proteins 10.4K, 14.5K, and 14.7K, respectively, are encoded by mRNAs *c, d,* and *e.* Data from the RNase protection and the Northern blots presented above indicate that mRNAs *d* and *e* are the two most abundant Ad35 E3 mRNAs comprising approximately 20 and 48%, respectively, of the total Ad35 E3 mRNAs. mRNA *c,* encoding the 20.6K protein comprises approximately 3–8% of the Ad35 E3 mRNAs. Additionally, mRNA *a,* which should primarily encode the Ad35 class I MHC binding protein, 18.5K, and perhaps also the 20.3K

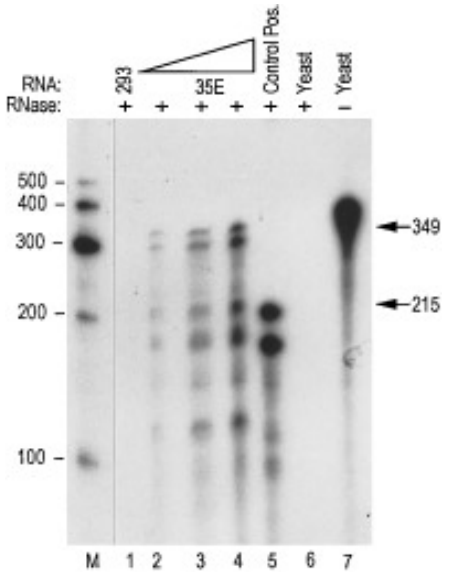

FIG. 7. RNase protection detecting a splice to the 10.3K ORF. [^{32}P]-UTP-labeled anti-sense probe D was hybridized to 1 mg of cytoplasmic, poly(A)$^+$ RNA from uninfected 293 cells (lane 1) or increasing concentrations of cytoplasmic, poly(A)$^+$ RNA from Ad35-infected 293 cells in the early phase of viral gene expression (lanes 2–4). Probe D was also hybridized to an *in vitro* synthesized positive-sense positive control RNA corresponding to the 10.3K mRNA encoding RT–PCR product amplified by primers 1 and 7 (see Fig. 4, lane 2) and to yeast RNA (lanes 6 and 7). All reactions were digested with RNase A and T1 except reaction 7 which was not digested with RNase. One-quarter of reaction 7 was loaded on the gel, while all of the remaining reactions were loaded on the gel. The upper arrow (349) indicates the product protected by mRNAs *a* and *b* which are colinear with probe D. The lower arrow (215) indicates the product protected by spliced mRNA *c* which corresponds to the 215-nucleotide product predicted by RT–PCR. (See Fig. 1 for schematic of mRNAs and probes.)

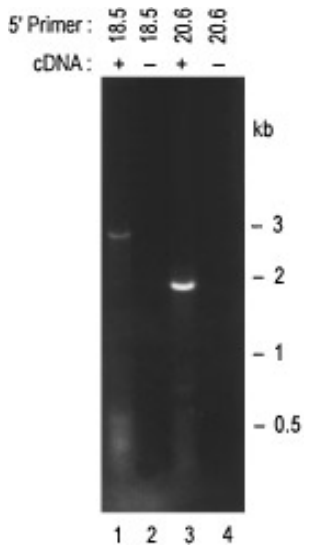

FIG. 8. 3′ RACE demonstrating usage of an "E3B" polyadenylation site but not an "E3A" site. PCR amplification of first-strand cDNA primed with the "adapter primer" (AP) was performed using the universal amplification primer (UAP), which is homologous to unique sequences at the 5′ end of AP, in conjunction with the indicated primers. Reactions used UAP and primer 2, the 18.5K primer (lanes 1 and 2) or primer 5, the 20.6K 5′ primer (lanes 3 and 4). Reactions contained cDNA template (lanes 1 or 3) or no template (lanes 2 and 4). See Materials and Methods for an explanation of the 3′ RACE reaction, and see Fig. 1 for position of 5′ primers.

protein (see below), comprises only 9–12% of the Ad35 E3 mRNA, calculated from results obtained with probes B and A and from Northern blotting data. For reasons which have not been resolved, Ad35 mRNA *a* appeared to be of lower abundance in experiments done using

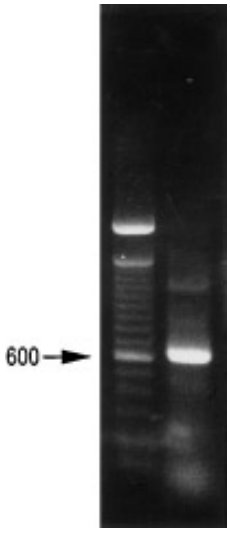

FIG. 9. 3′ RACE demonstrating early usage of the L4 poly(A) site in Ad35. PCR amplification of first-strand cDNA primed with the "adapter primer" (AP) was performed using the universal amplification primer (UAP), which is complementary to the 5′ end of AP, in conjunction with primer 1, the common 5′ primer. The left lane shows the size markers; the right lane is the 3′ RACE product.

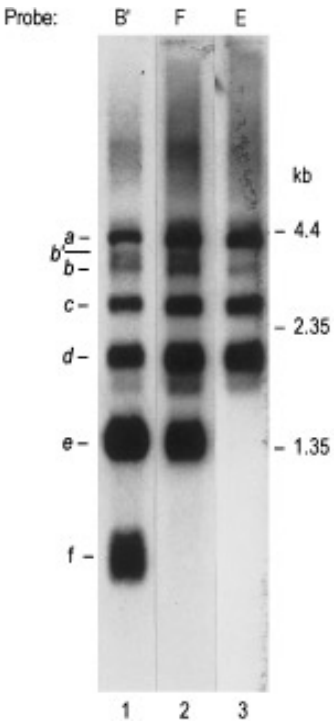

FIG. 10. Northern blot analysis of Ad35 early RNA. Total RNA isolated from Ad35-infected 293 cells in the early phase of viral gene expression was probed with [^{32}P]UTP-labeled, anti-sense RNA probe B′, which binds the 5′ end of the E3 (lane 1); probe F, which binds near the 3′ end of the E3 (lane 2); or probe E, which binds within the 15.2K ORF (lane 3). (See Fig. 1 for position of probes within the Ad35 E3.) RNA was electrophoresed on 1.5% agarose/formaldehyde/1× TBE gels, transferred to nitrocellulose, and probed as indicated above.

probe C. In contrast, the subgroup C Ads 2 and 5 devote nearly half of their E3 mRNAs to the production of mRNAs *a* and *c* which encode the class I MHC binding protein gp19K and relatively less mRNA to the production of the anti-TNFα 10.4K, 14.5K, and 14.7K proteins (Fig. 11, mRNAs *f* and *h*) (Wold, 1995).

The differences in levels of E3 mRNAs encoding Ad35 and Ad2 E3 protein homologues are likely a reflection of differences in the *cis*-acting sequences affecting pre-mRNA processing in the Ad35 and Ad2 E3 regions. It has previously been noted that Ad35 as well as other subgroup B2 Ads lack an E3 ORF homologous to the ORF encoding the 11.6K protein encoded by the Ad2 E3 (Flomenberg *et al.,* 1988; Mei and Wadell, 1992). A series of publications have demonstrated that *cis*-acting sequences within the Ad2 11.6-kDa ORF suppress splicing from the splice donor at nucleotide 951 to the splice acceptors which form the anti-TNFα protein encoding mRNAs (Deutscher *et al.,* 1985; Bhat *et al.,* 1986; Bhat and Wold, 1986; Scaria and Wold, 1994). When these *cis*-acting sequences are deleted from the Ad2 E3, the use of the splice donor at nucleotide 951 increases, and the abundance of mRNAs *a* and *c,* the formation of which require inactivity of this splice donor, decrease (Deutscher *et al.,* 1985; Bhat *et al.,* 1986; Bhat and Wold,

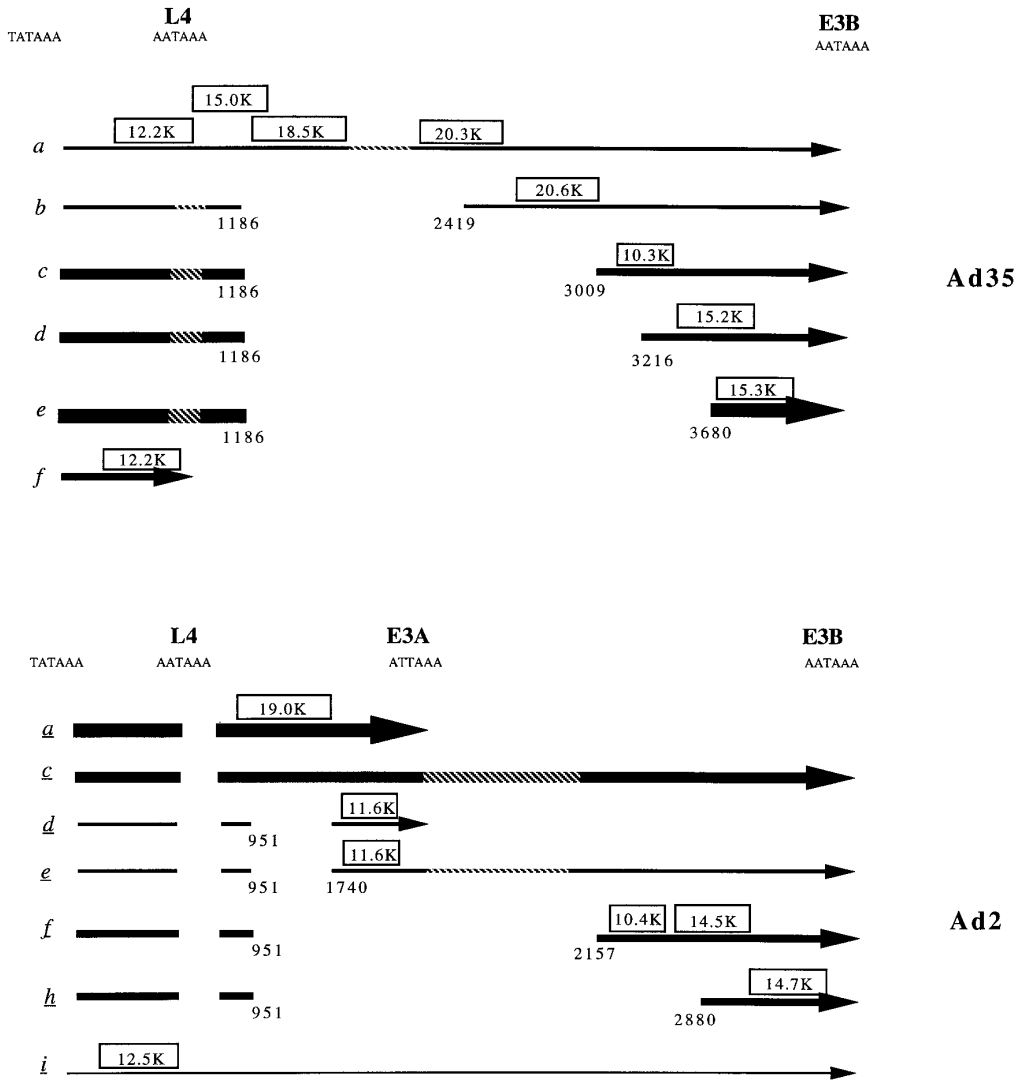

FIG. 11. Comparison of Ad35 and Ad2 E3 mRNAs. The structures of the Ad35 E3 and Ad2 E3 mRNAs are depicted below. Each arrow represents a separate mRNA molecule designated, at left, according to the letter by which it is referred in the text. Gaps in the arrows indicate introns. Hatched portion of the arrows indicate sequences which are deleted relative to the other virus. The boxes indicate the position of open reading frames expected to encode proteins, the molecular weights of which are indicated within the boxes. The numbers indicate the coordinates of splice donors and acceptors which are referred to under Results and/or Discussion. Polyadenylation sites are labeled with large, bold labels according to the designation (L4, E3A, or E3B) used to refer to the site in the text. Alignment of the Ad35 and Ad2 sequences indicates that the Ad35 splice donor at position 1186 and the Ad2 splice donor at position 951 lie within homologous sequences.

1986). The absence of such *cis*-acting sequences from the subgroup B2 Ad35 E3 may thus contribute to the relatively larger amount of splicing from the common splice donor at nucleotide 1186 to downstream acceptors and the relatively lesser amount of the mRNA in which the splice donor at nucleotide 1186 is not used (mRNA *a*). Additionally, it has been demonstrated that the presence of the E3A poly(A) site in the Ad2 E3 is essential for the accumulation of large amounts of mRNA *a.* This report demonstrates that the Ad35 E3 lacks an E3A poly(A) site. When the Ad2 E3A poly(A) site is deleted, an apparent competition between splicing and polyadenylation processes is relieved. This results in an increase in splicing to the splice acceptor at Ad2 E3 nucleotide 2157, resulting in increased mRNA *f* production and a decrease in mRNA *a* production (Deutscher *et al.,* 1985; Bhat *et al.,* 1986; Bhat and Wold, 1986). Since Ad35 appears to be a natural E3A "mutant," this may also contribute to this relative increase in splicing (compared to Ad2) to downstream splice sites which encode the anti-TNFα proteins.

The differences in abundance of various mRNAs likely leads to differences in the abundance of the Ad35 E3 proteins. Therefore, the Ad35 E3, compared with the corresponding Ad2 E3 proteins, may produce more of the anti-TNFα proteins 10.3K, 15.2K, and 15.3K and relatively less 18.5K. The RNA data, suggesting that Ad35 devotes only 10% of its E3 mRNA to 18.5K production while Ad2

devotes 50% of its E3 mRNA to gp19K production, is supported by observations of class I MHC binding protein production in Ad35- versus Ad2-infected cells. When HeLa cells were infected with equal multiplicities of infection with either Ad35 or Ad2, the Ad35-infected cells were found to produce approximately one-fifth as much virally encoded glycoprotein (Flomenberg *et al.,* 1987). This is of significance since gp19K (or 18.5K) acts stoichiometrically, binding directly to class I MHC and preventing its transit to the cells surface (Kvist *et al.,* 1978; Burgert *et al.,* 1985). If less gp19K (or 18.5K) is made, downregulation of class I MHC from the cell surface is likely to be less efficient. Such differences could potentially influence the ability of the viruses to cause acute disease or their ability to establish persistent infection. It is less clear what the significance of increased anti-TNFα protein levels might have on viral replication or pathogenesis; the amount of these proteins required for effective function is unknown.

The 20.3K and 20.6K ORFs of Ad35 do not have homologues in the E3 regions of the subgroup A (Ad12) (Sprengel *et al.,* 1994), C (Ads2 and 5) (Herisse *et al.,* 1980; Herisse and Galibert, 1981; Cladaras and Wold, 1985), or F (Ads 40 and 41) (Davison *et al.,* 1993). Homologues of these ORFs are, however, conserved among all of the B1 (Ads 3 and 7) (Signäs, 1986; Hong *et al.,* 1988) and B2 (Ads 11 and 35) (Flomenberg *et al.,* 1988; Mei and Wadell, 1992; Basler *et al.,* 1995) E3s sequenced thus far. No splice acceptor was found upstream of the Ad35 20.3K ORF. This contrasts with data from the Ad3 E3 region. An S1 nuclease mapping experiment on mRNA from Ad3-infected cells found a low abundance mRNA which appeared to be spliced immediately upstream of the 20.1K ORF. The exact splice site and structure of this mRNA, however, were not determined (Signäs, 1986). A similar mRNA was not found in Ad35-infected cells. Interestingly, the Ad3 contains the sequence Py_{10}ACAGCATG (Signäs, 1986) while the Ad35 contains the sequence Py_3ACAGACATG (Flomenberg *et al.,* 1988) near the start of the 20.3K ORF. Neither of these sequences conforms precisely to the mammalian splice acceptor consensus sequence $Py_nN\underline{PyAG}|G$, where the underlined sequence is highly conserved (reviewed in Green, 1991), but it is possible that the difference in the length of the polypyrimidine tract upstream of the 20.3K ORF contributes to the differences in splicing 5′ to the 20.3K ORF. The polypyrimidine tract binds the U2 snRNP auxiliary factor which facilitates U2 snRNP binding to the pre-mRNA (Green; 1991). Lengthening a polypyrimidine tract can improve splicing to an inefficient 3′ splice site (Bouk *et al.,* 1995). The sequence between the 18.5K ORF stop codon and the 20.3K ATG is identical in Ad35 and two subtypes of Ad11 (Mei and Wadell, 1992). It therefore seems likely that Ad11, like Ad35, will not utilize a splice site upstream of its 20.3K ORF to produce a 20.3K encoding mRNA early in infection. If the 20.3K protein is translated in Ad35-infected cells, it would appear to be encoded by mRNA *a.* Synthesis of the 20.3K would require either reinitiation of translation, where the 18.5K protein translation is initiated first, followed by translation termination and reinitiation, or by an internal initiation of translation. It has been suggested that the Ad2 E3 14.5K protein is produced from the same mRNA as the Ad2 10.4K protein by a reinitiation mechanism (Tollefson *et al.,* 1990).

The 20.6K encoding mRNA *b* is the least abundant Ad35 E3 mRNA. Low abundance of this mRNA is also reflected by the observation that the Ad35 20.6K protein is made at low levels in infected cells and visualization of the 20.6K by immunoprecipitation requires long exposures to film (unpublished observation). Recently, the Ad3 and Ad7 homologues of the 20.6K protein were shown to be produced during infection (Hawkins and Wold, 1995). A function for either the 20.3K or the 20.6K is not obvious based on sequence analysis. Presumably, these proteins are required in small amounts to carry out their function.

The 12.2K ORF, the ATG which likely initiates translation of the 15.0K protein, and a large portion of the 15.0K ORF are present on all the Ad35 E3 mRNAs (Fig. 1). However, neither the 12.2- nor the 15.0-kDa initiating ATG is in the sequence context (PyNNATGPy) which would promote efficient initiation of translation (reviewed in Kozak, 1992). Therefore, the scanning model of translation initiation may account for the read-through of these ATGs; the 12.2K and 15.0K ATGs could be bypassed by the 40S ribosomal subunit, permitting expression of downstream ORFs which are in better context for translation initiation. The presence of ATGs in an inefficient context for translation but which are known to function in translation initiation has precedent in the Ad2 E3. In Ad2, the 12.5K initiating ATG is present on all the Ad2 E3 mRNAs and the 6.7K initiating ATG is present on two of the Ad2 E3 mRNAs (Wold, 1995). Each of these proteins is produced in minor amounts compared with other E3 proteins produced from the same mRNAs (Hawkins and Wold, 1992; Wilson-Rawls *et al.,* 1990). The presence of an intron which is removed from all but one Ad2 E3 mRNA species results in the removal of much of the Ad2 12.5K ORF (Wold, 1995). Therefore, the Ad2 E3 mRNAs do not encode this protein in its entirety, and it can only be translated from minor E3 mRNA *i* and a minor late mRNA (Hawkins and Wold, 1992). In contrast, each of the Ad35 E3 mRNAs potentially encodes the entire 12.2K protein. Additionally, the existence of mRNA *f,* which originates at the E3 promoter and terminates at the L4 poly(A) site, potentially encodes the 12.2K protein as well. (It has not been determined, however, whether mRNA *f* is efficiently transported to the cytoplasm.) It is therefore possible that Ad35 will produce a relatively larger amount of its 12.2K protein compared with the Ad2 12.5K. In addition, the Ad35 E3 mRNAs potentially encode a family

of proteins which share common amino-terminal amino acids with the 15.0K protein. Each Ad35 E3 mRNA contains the 15.0K initiating AUG, but mRNAs *b–e* are spliced within the 15.0K ORF to downstream sequences. Translation initiation at the 15.0K AUG in these mRNAs would yield proteins with common amino termini but short, unique carboxy termini.

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