Adenovirus Serotype 5 L4-22K and L4-33K Proteins Have Distinct Functions in Regulating Late Gene Expression[†]

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Adenoviruses express up to 20 distinct mRNAs from five major late transcription unit (MLTU) regions, L1 to L5, by differential splicing and polyadenylation of the primary transcript. MLTU expression is regulated at transcriptional and posttranscriptional levels. The L4-33K protein acts as a splicing factor to upregulate several MLTU splice acceptor sites as the late phase progresses. The L4 region also expresses a 22K protein whose sequence is related to the sequence of L4-33K. L4-22K is shown here also to have an important role in regulating the pattern of MLTU gene expression. An adenovirus genome containing a stop codon in the L4-22K open reading frame expressed low levels of both structural and nonstructural late proteins compared to the wild-type (wt) adenovirus genome; a decrease in intermediate proteins, IVa2 and IX, was also observed. However, early protein synthesis and replication were unaffected by the absence of L4-22K. Intermediate and late protein expression was restored to wt levels by L4-22K expressed in *trans* but not by L4-33K. Increased MLTU promoter activity, resulting from stabilization of the transcriptional activator IVa2 by L4-22K, made a small contribution to this restoration of late gene expression. However, the principal effect of L4-22K was on the processing of MLTU RNA into specific cytoplasmic mRNA. L4-22K selectively increased expression of penton mRNA and protein, whereas splicing to create penton mRNA is known not to be increased by L4-33K. These results indicate that L4-22K plays a key role in the early-late switch in MLTU expression, additional to and distinct from the role of L4-33K.

Human adenovirus serotype 5 (Ad5) is considered a candidate delivery vector for gene therapy and vaccination. However, despite the fact that it has already been used in a clinical setting, some key questions about the basic biology of Ad5 remain. One of these unsolved questions is how control is achieved of late protein expression from the major late promoter (MLP), which directs the production of an array of structural proteins from the major late transcription unit (MLTU) regions, L1 to L5, via differential splicing and polyadenylation of the primary transcript (Fig. 1A) (reviewed in reference 1). Understanding late gene expression is important for further Ad vector development, since Ad E1A[−] vectors that retain late genes show poor persistence of transgene expression due to immune responses to residual late gene expression products (27, 41–44).

Full expression from the Ad5 MLTU is the end result of a temporal pattern of regulated gene expression that has several phases (reviewed in reference 35). The earliest event is expression of the E1A transcriptional activator proteins. These proteins then upregulate transcription of the other early viral genes E1B, E2, E3, and E4. Despite its name, the MLP is active at a low level during this early phase, producing the abundant i-leader protein and L1-52/55K (38). Accumulation of E2A DNA binding protein (E2A-DBP), E2B precursor terminal protein, and DNA polymerase allows viral DNA replication to begin. Around the onset of DNA replication, MLTU expression expands to include L4 proteins (22). The interme-

diate gene products, IVa2 and IX, are also expressed at this time. IVa2 expression is regulated by a cellular repressor that is titrated out upon genome replication (19). IX and IVa2 are involved in upregulating transcriptional activity of the MLP during the early to late phase transition of MLTU expression (25, 26, 32, 40). However, it is only as viral DNA replication progresses that the MLP is activated to produce maximal expression of the late structural genes from all MLTU regions.

The temporal pattern of MLTU expression involves regulation at both the transcriptional and posttranscriptional levels. Early in infection, transcription does not proceed beyond the L3 polyadenylation site, and the L1 polyadenylation site is preferentially used, leading to the accumulation of L1-52/55K protein (30). After the initiation of viral DNA replication, MLP activity increases and transcription extends through L4 and L5 regions, allowing a full complement of structural proteins to be produced. Alongside these transcriptional changes, RNA processing is regulated to give altered patterns of mRNA. For example, in the L1 region, the proximal 52/55K splice acceptor site is used almost exclusively at early times, but during the late phase usage shifts to the distal IIIa acceptor site, due to the production of an infection-specific splicing factor (1). This has now been shown to be L4-33K (39).

L4 is now also known to encode a 22-kDa protein (L4-22K) (31), as predicted from bioinformatic analysis (11), that shares its N terminus with L4-33K but has a unique C-terminal domain (Fig. 1B). L4-22K has a role in genome packaging via its binding, in association with IVa2, to the A2 sequence within the packaging signal (31, 45, 46). L4-22K has also been shown to bind, with IVa2, to the downstream elements (downstream element 1 [DE1] and DE2) of the MLP (31), which have been shown previously to bind IVa2 as both a homodimer (DEF-B)

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FIG. 1. (A) Schematic diagram of the major late transcription unit showing the alternatively spliced mRNAs and encoded proteins. (B) Organization of the L4-22K and L4-33K ORFs. The position of the L4-22K truncation mutation in L4-22K⁻ is indicated (31). (C) 293TetOFF cells, which constitutively express the tetracycline transactivator protein, were transfected with pBiL1-3NheI (which expresses the MLTU region from L1 to L3 under the control of a tetracycline-regulated promoter), pCMV100KFLAG, and either empty vector (lane pBiL1-3NheI) or the L4-22KFLAG (+22KFLAG) or L4-33KFLAG (+33KFLAG) expression plasmid. Cell lysates were harvested 48 h posttransfection and separated on 10% polyacrylamide gels, and late proteins, as indicated to the right of the gel, were detected by Western blot analysis using AbJLB1 polyclonal anti-late protein serum. The positions to which proteins of known molecular mass migrated are shown to the left of the gel (in kilodaltons).

(25, 40) and in complex (DEF-A) with an unidentified viral protein (25, 28), suggesting that L4-22K, in conjunction with IVa2, may play a role in activating the MLP (31). However, DEF-A has also been shown to contain L4-33K, and IVa2 plus

L4-33K activated a MLP reporter (2). Thus, it is uncertain what the relative contributions of these two L4 proteins are to regulation of the MLP. The sequence relatedness between L4-22K and L4-33K also raises the possibility that they might have overlapping functions. We therefore sought to analyze the impact of L4-22K on the expression of Ad5 late proteins. Our data show that L4-22K is required for the transition to late phase MLTU expression and acts primarily at the level of RNA processing. However, the action of L4-22K is different from that of the splice factor L4-33K and the two proteins are not functionally redundant.

MATERIALS AND METHODS

Plasmids. pTG3602 (pWT), containing the wild-type (wt) Ad5 genome, and pTG3602-L4-22K⁻ (pL4-22K⁻), containing a premature stop codon within the C-terminal unique portion of the L4-22K open reading frame (ORF) that results in an additional AvrII restriction recognition site (Fig. 1B), have been described previously (8, 31). Expression plasmids for C-terminal FLAG-tagged L4-22K and L4-33K (pCMV-22KFLAG and pCMV-33KFLAG) were constructed by insertion of XbaI/PacI fragments, amplified, respectively, from viral genomic DNA or viral mRNA using primers containing the restriction enzyme recognition sites (primer sequences available on request) into pCMV-FLAG (12). pBiL1-3NheI, pCMV-100KFLAG, and pCMV-22/33KFLAG, which expresses both L4-22K (no epitope tag) and L4-33KFLAG (12), pCMV-IX (5), and pMEPCMV-IVa2 (4) have been described previously. pMLP+DELuc was generated by amplifying the MLP, including DE1 and DE2 (Ad5 positions 5939 to 6174) using primers containing restriction recognition sites for KpnI (5' primer) and NheI (3' primer) and cloning into pGL3-Basic luciferase reporter plasmid (Promega). pMLP-DELuc, containing the MLP without the downstream elements (Ad5 positions 5939 to 6133), was generated in a similar way. pcDNA3.1HisLacZ (Invitrogen) was used as a transfection control.

Cells and viruses. 293 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). 293-IVa2 cells constitutively express IVa2 from an Epstein-Barr virus replicon plasmid (4). 293TetOFF cells (Clontech) were maintained in DMEM supplemented with 10% tetracycline-free FBS and 100 µg/ml Geneticin (G418 sulfate; Melford Laboratories). An L4-22K-complementing, stable cell line (293-L4-22K) was generated by transfecting 293 cells with pCMV-22KFLAG and selecting clones in DMEM supplemented with 10% FBS and 500 µg/ml Geneticin. L4-22KFLAG expression was confirmed by immunoblotting and immunofluorescence using a mouse monoclonal antibody (MAb) to FLAG (M2; Sigma).

The wt virus was Ad5 strain 300 (wt300) (21). To generate an L4-22K⁻ virus, 293-L4-22K cells were seeded into a 12-well plate and transfected with L4-22K⁻ genome excised from pTG3602-L4-22K⁻ by PacI digestion, using Lipofectamine 2000 (Invitrogen). When 60 to 80% cytopathic effect was observed, cells were harvested, virus was released by three freeze-thaw cycles, and cellular debris was removed by centrifugation. This L4-22K⁻ virus stock was passaged five times to ensure removal of transfected genomic DNA. To confirm the presence of the mutated sequence in the P5 stock, virus particle DNA was isolated (16) and used as template for PCR to amplify the Ad5 L4 region from 26018 to 27086 bp. PCR products were then digested with AvrII, which is diagnostic for the 22K⁻ mutant (31). Virus titers were determined by fluorescent focus assays. Briefly, 293 cells were infected with a 10-fold dilution series of L4-22K⁻ virus or wt300, fixed at 20 h postinfection (p.i.) with 10% formalin in phosphate-buffered saline (PBS), and permeabilized with 0.5% NP-40 in PBS before staining with a mouse MAb to DBP (B6-8) (34) and Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen). Virus titers (focus-forming unit [FFU]/ml) were determined by the number of DBP-positive cells at a given dilution (33). For wt300, 1 FFU was equivalent to 7 PFU.

Transfection and immunoblotting. All transient transfections were carried out in 12-well plates at a density of 7×10^5 cells/well using either Lipofectamine 2000 (Invitrogen) or TransLT (Cambridge Bioscience) at a ratio of 3 µl/µg DNA following the manufacturer's protocols. Transfections used 1 µg L4-22K⁻ or wt genome, excised from pL4-22K⁻ or pWT, respectively, by PacI digestion, and 500 ng various expression plasmids or empty vector (pCMV-FLAG). Experiments using the pBi plasmid system were carried out as previously described (12). Transfected cells were harvested 48 h posttransfection directly into sample loading buffer (2% [wt/vol] sodium dodecyl sulfate, 50 mM dithiothreitol, 10% [vol/vol] glycerol, 25 mM Tris [pH 6.8], and 0.01% bromophenol blue), and from 10 to 33% cell lysate volume was resolved through either 10% or 15% sodium

dodecyl sulfate-polyacrylamide gels as appropriate. Proteins were transferred to ECL nitrocellulose membrane (GE Healthcare), and Western blot analysis was carried out as described previously (23). Proteins were detected using the following antibodies: FLAG, M2 mouse MAb (Sigma) at 1:10,000; Ad5 late proteins, AbJLB1 rabbit polyclonal serum at 1:10,000 (12); fiber, RI 89&99 rabbit polyclonal serum at 1:5,000 (obtained from V. Mautner, University of Birmingham, United Kingdom); E2A-DBP, mouse MAb B6-8 at 1:10,000; L1-52/55K, rabbit polyclonal serum raised against glutathione *S*-transferase-L1-52/55K fusion protein at 1:10,000 (3); L4-100K, mouse MAb at 1:10,000 (obtained from W. C. Russell, University of St. Andrews) (36); IVa2, rabbit polyclonal serum raised against glutathione *S*-transferase-IVa2 at 1:10,000 (4); and IX, rabbit polyclonal serum at 1:5,000 (5). Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse (Sigma) at 1:5,000 and HRP-conjugated goat anti-rabbit (Santa Cruz) at 1:100,000. HRP signal was detected using ECL-Advance Western blot detection kit (GE Healthcare) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR). Cells were scraped into PBS, pelleted by centrifugation, resuspended in isotonic buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.6], 1.5 mM MgCl₂) containing 0.6% (vol/vol) NP-40 and incubated on ice for 10 min before pelleting the nuclei by centrifugation at 16,000 × *g* for 3 min. The cytoplasmic fraction was reserved, and the nuclei were resuspended in lysis solution (isotonic buffer containing 1% NP-40 and 1% sodium deoxycholate). RNA was isolated from both the nuclear and cytoplasmic fraction using TRI reagent (Sigma) as described previously (29). Two micrograms of RNA and 100 U Superscript reverse transcriptase (RT; Invitrogen) were used with specific 3' primers to generate cDNA in a 25-μl volume reaction using the manufacturer's protocol. Equivalent RT-negative reaction mixtures lacked Superscript reverse transcriptase. A 2.5-μl portion of a RT-positive or RT-negative reaction mixture was added to a PCR mixture (1× PCR buffer, 0.5 mM [each] deoxynucleoside triphosphate, 0.5 μM 5' and 3' primer, 3 mM MgCl₂, and 1 to 2.5 U *Taq* polymerase [Fermentas]) in a total volume of 20 μl. Primers used were complementary to Ad5 tripartite leader 1 sequence 6049-6069 (sequence from positions 6049 to 6069) and Ad5 L2-penton 14889-14863, Ad5 L2-V 17445-17415, or Ad5 L3-hexon 19254-19228 sequence or to sequences flanking the Ad5 L3 poly(A) and E2A poly(A) sites, namely, the 22034-22061 and 22749-22722 sequences, or to human β-actin cDNA sequences 1430-1455 and 2383-2368. Reaction mixtures were incubated at 94°C for 5 min, followed by 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. PCR products were resolved through 1% agarose alongside size markers (1-kb GeneRuler; Fermentas).

Southern blot analysis. 293 cells were infected with L4-22K⁻ virus or wt300 at 2×10^{-3} FFU/cell and harvested 3 or 20 h p.i. Cells were processed either to purify packaged viral DNA, using spermine precipitation to remove unpackaged material (16), or for total low-molecular-weight DNA by Hirt extraction (18). DNA was digested with HindIII, resolved through 0.7% agarose, and transferred to a Hybond-N membrane (GE Healthcare), and viral DNA was detected using an L1 probe (10589 to 11565 bp from pGem4-L1 (37) labeled using the AlkPhos direct labeling kit (GE Healthcare).

Luciferase assay. 293 and 293-IVa2 cells were transfected with 500 ng reporter plasmid (pMLP+DEluc or pMLP-DEluc) and 100 ng pcDNA3.1HisLacZ, together with 500 ng pCMV-22KFLAG and/or 1 μg L4-22K⁻ genome. All transfections were equalized for DNA content by the addition of either salmon sperm DNA or pCMV-FLAG as appropriate. Cells were lysed 24 h posttransfection in 1× passive lysis buffer, and the luciferase assay was carried out according to the manufacturer's instructions (Promega). Cell lysates were also analyzed for β-galactosidase activity using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The ratio of firefly luciferase to β-galactosidase was calculated to correct for differences in transfection efficiency. Data are shown as changes in induction where luciferase expression from the reporter plasmids is set at 1. Statistical analysis was performed using Student's *t* test.

RESULTS

L4-22K is required for efficient late gene expression in 293 cells. Our previous studies of the early to late transition in MLTU expression utilized plasmid pBiL1-3NheI, in which MLTU genomic sequence encoding the tripartite leader and regions L1 to L3 was placed under the control of a heterologous promoter. Full late pattern expression could be induced from this plasmid by cytomegalovirus (CMV) expression vectors encoding L4-33K and L4-100K proteins (12). The "33K"

plasmid utilized was also capable of L4-22K expression, although L4-22K had not been proven to exist at that time. Subsequently, L4-33K was shown by Tormanen et al. to be a splicing factor capable of activating certain MLTU splice sites (39). To determine whether L4-22K made any contribution to the MLTU activation observed in this system, plasmids expressing either L4-22K or L4-33K with C-terminal FLAG tags were constructed. When cotransfected with pBiL1-3NheI and an L4-100K vector to permit efficient hexon protein accumulation, L4-22KFLAG increased levels of several late proteins independently of L4-33K (Fig. 1C). These effects were qualitatively distinct, with L4-22K increasing penton expression more than L4-33K did and L4-22K increasing hexon expression less than L4-33K did. These data suggested that L4-22K made a contribution to late protein expression from regions L1 to L3, although the possibility that this effect was due to expression of a truncated L4-33K-related protein from the L4-22K plasmid clone could not be excluded.

To investigate fully the role of L4-22K in regulating MLTU expression, given the prior evidence that L4-22K and/or L4-33K might act on the MLP, we focused further studies on a whole-genome system, in which the MLTU was controlled by the native MLP within the linear full-length genome excised from pTG3602 (8). This also provided the possibility of IVa2 production that was not present in the pBi plasmid system. The role and mechanism of action of L4-22K in late gene expression from this genome were assessed by comparison with an L4-22K⁻ genome that retained the ability to express full-length L4-33K (Fig. 1B) (31), kindly provided by P. Hearing.

293 cells transfected with L4-22K⁻ genome showed a decrease in late structural protein production (hexon, penton, IIIa, V, and fiber) compared to cells transfected with wt genome (Fig. 2A and B); while the absolute level of this defect varied between experiments, as exemplified here, it was always substantial. In addition, expression of the nonstructural late proteins L4-100K and L1-52/55K from L4-22K⁻ genome was reduced to virtually undetectable levels (Fig. 2B). The level of E2A-DBP detected was at least equivalent for L4-22K⁻ and the wt genome, and possibly somewhat increased in the mutant, demonstrating that the defect in late protein expression was not due to differences in transfection efficiency and that the absence of L4-22K does not cause a global downregulation of viral protein production. Expression of all late proteins from the L4-22K⁻ genome was restored to wt levels when cells were cotransfected with an expression plasmid for L4-22KFLAG (Fig. 2A), confirming that the reduced late protein expression seen from L4-22K⁻ genome was due solely to the lack of L4-22K expression. L4-22K has been shown previously to be involved in the packaging of Ad genomes into particles (31), so at 48 h posttransfection, it was possible that the observed differences in late gene expression resulted from reduced levels of second-round infection in L4-22K⁻ genome-transfected cultures consequent upon a failure to package genomes efficiently. However, this possibility was discounted, as similar results were observed at 20 h posttransfection, a time point when no progeny virus would have been able to reach the late phase of a secondary infection (data not shown). The late protein defect of L4-22K⁻ is kinetic rather than absolute, as when analysis was delayed to 60 h posttransfection, cells transfected with the L4-22K⁻ genome produced levels of late pro-

FIG. 2. Late protein expression from L4-22K⁻ genome. Cells were mock transfected or transfected with L4-22K⁻ genome and either empty vector or various L4 expression plasmids as indicated. The wt Ad5 genome was used as a control. Cell lysates were separated on 10% polyacrylamide gels, and the expressed proteins, as indicated to the right of each gel, were detected by Western blot analysis using either AbJLB1 polyclonal anti-late protein antiserum (top gels in panels A and B), RI polyclonal anti-late protein antiserum to detect L5 fiber (B), or monospecific/monoclonal antibodies as indicated. In the top gel of panel B, all lanes shown derive from the same exposure of a single blot with irrelevant lanes excised for clarity of presentation. Note that no FLAG-tagged 22K protein is detected from transfection of the single L4-22/33K plasmid, as the tag sequence is attached only to the 33K reading frame. However, the expected expression of the 22K protein from this plasmid can be inferred from its effect on late protein expression. The positions to which proteins of known molecular mass migrated are shown to the left of the gels (in kilodaltons).

teins similar to those seen at 20 h posttransfection for cells transfected with either wt genome or L4-22K⁻ genome co-transfected with L4-22KFLAG expression plasmid (data not shown). Thus, L4-22K upregulates late gene expression in 293 cells.

L4-22K acts via a mechanism different from that of L4-33K. The stop codon inserted into the L4-22K ORF in pL4-22K⁻ is located within the intron sequence of L4-33K ORF, and it was possible that this mutation might affect the expression of L4-33K, hence causing some part of the observed defect in late protein production. Although the defect was complemented by L4-22KFLAG, this plasmid also has the capacity to express a truncated L4-33K protein consisting of the N-terminal 129 amino acids that might contribute to this complementation. The truncated protein would lack the C-terminal RS motifs that have been shown to be essential for this activity (39), making this unlikely. However, to exclude this possibility, L4-22K⁻ was cotransfected with an expression plasmid for L4-33KFLAG. Expressing L4-33KFLAG in *trans* did not restore expression of either the structural proteins or L4-100K (Fig. 2B), although a small increase in L1-52/55K was observed. Late protein expression from L1 through to L5 was restored only when L4-22K was added in *trans* either from pCMV-22KFLAG or from a plasmid, such as pCMV-22/33KFLAG, that was capable of expressing both L4-22K and L4-33KFLAG from alternatively spliced transcripts (Fig. 2B). During complementation by pCMV-22K/33KFLAG, expression of the structural proteins and L4-100K was greater than that seen with L4-22K⁻ genome alone but was reduced compared to when L4-22KFLAG was expressed from pCMV-22KFLAG. This may reflect preferential expression of the spliced L4-33KFLAG

transcript from this plasmid, and consequently, a lower expression level of L4-22K protein was achieved than with pCMV-22KFLAG. These data show that the defect in late gene expression is not due to any unexpected aberrations in L4-33K expression occurring due to the mutation in the intronic sequence of L4-33K ORF. Furthermore, the evidence clearly shows that the mechanism of action of L4-22K is independent of L4-33K splicing function and thus indicates a direct role for L4-22K in the activation of the late phase of infection.

Reduced late gene expression in the absence of L4-22K is not due to a defect in replication. Full expression from the Ad5 MLTU is the end result of a temporal pattern of regulated gene expression. It is only once viral DNA replication has begun that the MLP is activated to produce maximal expression of the late structural genes from all MLTU regions. Thus, a defect in replication could account for the reduction in late protein production observed from L4-22K⁻ genome. To allow replication studies to be carried out without the concern of transfection efficiencies or the confusion of large quantities of input DNA, L4-22K⁻ virus was isolated by transfection of L4-22K⁻ genome into a stable L4-22K-complementing cell line, 293-L4-22K. Although this cell line expressed only a low level of L4-22KFLAG, it was sufficient to permit the growth of virus to a low titer.

The production of L4-22K⁻ virus was confirmed by AvrII digestion of PCR products amplified from DNA isolated from L4-22K⁻ virus particles (Fig. 3A). Using particle DNA ensured that only newly synthesized and packaged DNA was acting as a template in the PCR and not any remaining transfected genome. Incubation of the L4-22K⁻ virus product with AvrII resulted in the virtually complete cleavage to bands 532 and

FIG. 3. L4-22K has no effect on viral DNA replication. (A) Confirmation of L4-22K⁻ mutant status. L4-22K⁻ virus was generated and passaged five times in a stable L4-22K-complementing cell line, 293-L4-22K. The Ad5 region from bp 26018 to 27086 was amplified by PCR from DNA isolated from either L4-22K⁻ or wt virus particles. PCR products were either undigested or digested with AvrII as indicated. Lane M, 1-kb ladder, sizes marked on the left (in kilobase pairs); -ve, negative control. (B) L4-22K effects on packaging. 293 cells were either mock transfected or transfected with L4-22KFLAG and 24 h later infected with 2×10^{-3} FFU/cell L4-22K⁻ virus or wt Ad5. Packaged DNA isolated from infected cells at either 3 h p.i. or 20 h p.i. was digested with HindIII and analyzed by Southern blotting using an L1 probe. (C) L4-22K effects on replication. The experiment was performed as in panel B except DNA was obtained by Hirt extraction of cell cultures at 3 h or 20 h. Fifty nanograms of Ad5wt viral DNA was used as a positive control (+ve) (B and C).

536 bp in size, indicating the presence of the site diagnostic of the L4-22K⁻ mutation (31). The small amount of uncut DNA is likely to represent incomplete digestion. If any wt DNA were present, it would rapidly overgrow the slow-growing mutant, but no increase in the proportion of this uncut species was seen upon further passage; the presence of the inserted stop codon was also confirmed by sequencing (data not shown). The L4-22K⁻ virus had the packaging defect expected from previous work (Fig. 3B) (31). At 20 h p.i., viral DNA was not detected for L4-22K⁻ unless complemented by prior expression of L4-22KFLAG; when complemented, the amounts of L4-22K⁻ DNA were similar to those of wt virus. No viral DNA was detected at 3 h p.i., indicating that viral DNA detected at 20 h p.i. was due to newly synthesized, packaged genomic DNA and not virus input. These data confirm the L4-22K-deficient nature of the L4-22K⁻ virus generated in this study both genetically and biologically.

To investigate the growth properties of L4-22K⁻, the late

protein expression defect observed with transfected genome was first confirmed. Noncomplementing cells infected with L4-22K⁻ virus behaved identically to cells transfected with L4-22K⁻ genome in showing a substantial impairment of structural protein expression (data not shown). Genome replication was then assessed (Fig. 3C). At 3 h p.i., a low level of viral DNA was detected; this time point is too early for the synthesis of progeny viral DNA and thus shows the amount of input viral DNA. The increased level of viral DNA detected at 20 h p.i. was similar for uncomplemented and complemented L4-22K⁻ virus, showing that the absence of L4-22K has no effect on virus DNA replication. This result agrees with that reported previously in which replication of the L4-22K⁻ genome was investigated in HeLa cells (31). The block in MLTU gene expression must therefore occur after DNA replication.

A reduction in intermediate proteins IVa2 and IX is not fully responsible for the reduction in late protein expression from L4-22K⁻. The two intermediate proteins, IVa2 and IX, upregulate MLP activity during the transition between the early and late phases of infection (25, 26, 32, 40). IVa2 acts via binding, either as a homodimer (DEF-B) or heterodimer (DEF-A), to the DE1 and DE2 sequences within the MLP (20, 28). Furthermore, the pattern of differential protein expression observed here in the absence of L4-22K is similar to that reported previously for MLP DE1 and DE2 mutants (32), suggesting that L4-22K may be acting via IVa2, perhaps as a component of DEF-A, since enhanced binding of IVa2 to DE1 or DE2 in the presence of 22K has been reported (31).

Expression of IVa2 and IX from L4-22K⁻ genome alone was barely detectable, whereas the levels of both proteins were substantially increased when L4-22KFLAG was coexpressed (Fig. 4A). To determine whether this reduction in intermediate proteins was responsible for the limited expression of late proteins observed for L4-22K⁻ genome, these two proteins were expressed in *trans*. Cotransfection of L4-22K⁻ genome with IVa2 expression plasmid increased expression of hexon, penton, and the smaller isoform of protein V but failed to increase IIIa, the top isoform of V or pVI (Fig. 4B). In contrast, cotransfection with IX expression plasmid failed to increase the level of late gene expression significantly. When IX and IVa2 were expressed together, the levels of all late proteins tested increased a little compared to the level expressed by either protein alone. However, this late protein expression was very much lower than that seen when the L4-22K⁻ genome was directly complemented by L4-22KFLAG (Fig. 4B).

L4-22K clearly increases the levels of both IVa2 and IX proteins produced from transfected genome (Fig. 4A). In the case of IVa2, this may reflect a stabilization of the protein rather than a transcription or RNA processing event, since L4-22KFLAG also increases the level of IVa2 protein expressed from cDNA under the control of the heterologous CMV promoter (Fig. 4C); this effect was specific to L4-22K, as neither L4-33KFLAG nor L4-100KFLAG had any effect (Fig. 4C). This observation suggests that the increase in IVa2 seen when L4-22K⁻ genome is complemented is not mediated through IVa2's interaction with L1-52/55K, its known partner in viral DNA packaging (15), expression of which also increases upon complementation of L4-22K⁻ (Fig. 2B). The IVa2 sequence within pMEPCMV-IVa2 does not contain the binding site for the cellular repressor that blocks IVa2 tran-

FIG. 4. Restoring expression levels of the intermediate proteins IVa2 and IX does not fully complement L4-22K⁻. (A and B) 293 cells were mock transfected or transfected with L4-22K⁻ genome and either empty vector or various expression plasmids as indicated. The protein expressed by the plasmid is shown after a plus sign (e.g., +22K, L4-22K). The positions to which proteins of known molecular mass migrated are shown to the left of the gel (in kilodaltons). (C) 293 cells were mock transfected or transfected with the IVa2 expression plasmid with either empty vector or various L4 protein expression vectors as indicated. Cell lysates were separated on 10% or 15% polyacrylamide gels, and expressed proteins were detected by Western blot analysis using anti-IVa2, anti-IX, or AbJLB1 anti-late protein antisera.

scription prior to replication (9, 24), and thus, L4-22K cannot be acting by relieving this repression. In addition, no effect of L4-22K on expression of other proteins from the CMV promoter was observed (data not shown). Therefore, we hypothesize that L4-22K acts to stabilize IVa2 protein.

It is possible that lack of IVa2 stabilization by L4-22K is the reason why IVa2 expressed in *trans* can only partially complement the late protein expression defect of L4-22K⁻ genome. However, this seems unlikely, as the levels of IVa2 achieved were similar to those seen when L4-22K⁻ was fully complemented by L4-22KFLAG. Furthermore, similar results were obtained when a cell line constitutively expressing high levels of IVa2 was used (Fig. 5C), excluding the possibility that L4-22K somehow affects IVa2 transfection efficiency. Alternatively, the requirement for L4-22K may reflect a direct contribution of this protein to the DEF-A transcription factor that is required for maximal activation of the MLP (31).

L4-22K increases MLP activity in a DE-dependent manner. To determine whether L4-22K directly regulated transcription from the MLP and whether this was DE dependent, luciferase expression from MLP reporters either containing or lacking DE1 and DE2 was assessed in the presence of the L4-22K⁻ genome or complemented genome (Fig. 5A). The presence of

FIG. 5. L4-22K increases transcription activity of IVa2 at the MLP. (A) 293 cells were transfected with pMLP+DEluc or pMLP-DEluc alone or with L4-22K⁻ genome and various expression plasmids as indicated. (B) 293 cells or 293-IVa2 cells were transfected with pMLP+DEluc alone or with various expression plasmids as indicated. The protein expressed by the plasmid is shown after a plus sign (e.g., +22K, L4-22K). Firefly luciferase expression was detected using a luciferase assay, corrected for transfection efficiency using β -galactosidase expression from an independent control plasmid and expressed as the change in induction where expression from the reporter plasmids alone was set at 1. Error bars show standard deviations from three experiments. (C) 293 or 293-IVa2 cells were transfected with L4-22K⁻ genome and either empty vector or L4-22KFLAG expression plasmid. Cell lysates were separated on 10% polyacrylamide gels, and expressed proteins were detected by Western blot analysis using AbJLB1 anti-late protein or anti-IVa2 antisera. The positions to which proteins of known molecular mass migrated are shown to the left of the gel (in kilodaltons).

the genome ensured that all viral proteins other than L4-22K that might be required for transcriptional activation were present in the system. Luciferase expression was increased 45- to 60-fold by the presence of the L4-22K⁻ genome, regardless

FIG. 6. L4-22K increases the level of late mRNA. RT-PCR amplifications were performed on total cytoplasmic RNA from 293 cells that were either mock transfected or transfected with L4-22KFLAG and 24 h later infected with 2×10^{-3} FFU/cell L4-22K⁻ virus or wt Ad5 as indicated. Late mRNAs were detected by RT-PCR using a 5' primer specific for tripartite leader 1 and 3' primers directed to L2 penton (947-bp product), L2 V (1,138-bp product), or L3 hexon (662-bp product) 3' exons. Equivalent control reactions lacking reverse transcriptase (RT⁻) were performed; amplification of β -actin mRNA (511-bp product) served as a positive control. For V mRNA, both RT-PCR and RT⁻ results derive from the same experiment and are presented at the same exposure. The positions to which DNA size markers migrated are indicated to the left of the gel (in kilobase pairs). -ve, negative control.

of the presence or absence of the DE elements. This significant increase occurred despite the fact that the L4-22K⁻ genome displays a severe reduction in IVa2, IX, and late protein synthesis. Expression of L4-22KFLAG further increased the activity of pMLP+DEluc by twofold compared to L4-22K⁻ genome alone ($P < 0.005$), whereas the activity of pMLP-DEluc was not significantly altered ($P > 0.1$), demonstrating that the increase was DE dependent (Fig. 5A). In contrast, L4-22KFLAG expressed in the absence of viral genome had no effect on the activity of MLP containing the DEs (Fig. 5B), suggesting that another viral protein was required in association with L4-22K.

A likely candidate protein was IVa2, which was known to act via the DEs (40) and had been shown to be stabilized in the presence of L4-22K (Fig. 4). To test whether DE-dependent MLP activation by L4-22K was indirectly due to increased IVa2 levels or due to a direct interaction of L4-22K and IVa2 at the DEs, MLP reporter activity was analyzed in a cell line constitutively expressing high levels of IVa2 (293-IVa2) (4). Activity of the DE-containing MLP in 293-IVa2 cells was increased a modest 1.2-fold compared to its activity in 293 cells. When L4-22KFLAG was coexpressed in these cells, the increase was 1.6-fold compared to 293 cells, a 1.3-fold increase compared to 293-IVa2 cells (Fig. 5B). Therefore, both L4-22K and IVa2 contribute to MLP activation. However, the small increase in MLP activity observed when L4-22KFLAG is expressed in the presence of high levels of IVa2 does not explain the large differences in the expression of late proteins, notably penton and IIIa, from a complemented genome in the same cell line (Fig. 5C). This suggests that L4-22K may have a role in late gene expression that is posttranscriptional, in addition to its effects, with or via IVa2, at the MLP. This conclusion is

FIG. 7. L4-22K acts at the level of RNA processing. 293 cells were either mock transfected or transfected with L4-22KFLAG expression vector. Twenty-four hours posttransfection, cells were mock infected or infected with 2×10^{-3} FFU/cell L4-22K⁻ virus or wt Ad5, and nuclear RNA was isolated 20 h p.i. The amounts of L3 RNA unprocessed at the L3 poly(A) site (top left panel) and of E2A RNA unprocessed at its poly(A) site (top right panel) were determined by RT-PCR using specific primers. β -Actin mRNA served as a positive control (bottom left panel). To confirm that amplified products were derived from the RNA template, the same amount of RNA was added to reaction mixtures where the reverse transcriptase had been omitted (RT⁻) (bottom right panel). The positions to which DNA size markers migrated are indicated to the sides of the gels (in kilobase pairs).

supported by the observed increase in L1-3 gene expression from a heterologous promoter in the presence of L4-22KFLAG, when no IVa2 was present (Fig. 1C).

L4-22K acts at the level of late gene mRNA production/stability. To determine whether L4-22K was exerting its post-transcriptional effect at the level of mRNA or protein production, the amounts of penton, V, and hexon mRNA produced by L4-22K⁻ and wt virus were assessed. Penton and V mRNA production from L4-22K⁻ virus was barely detectable, compared to readily detectable expression from wt virus (Fig. 6); this defect was fully complemented when L4-22KFLAG was expressed in *trans* (Fig. 6). In contrast, the level of hexon mRNA produced by L4-22K⁻ virus was similar to that produced by wt virus and not further increased when complemented by L4-22K. PCR products were not a result of DNA contamination of the template, as equivalent reaction mixtures containing no reverse transcriptase were negative (Fig. 6). The quantity and quality of RNA used were confirmed by the equivalent amplification of β -actin mRNA (Fig. 6). Therefore, a major component of the reduction in late gene expression observed for L4-22K⁻ is at the level of late mRNA production and/or stability, and the action of L4-22K is selective for specific mRNAs.

L4-22K does not increase the level of unprocessed late mRNA. To further clarify the role of L4-22K in transcription and RNA processing, the level of unspliced nuclear MLTU RNA was determined by RT-PCR amplification of a region spanning the L3 polyadenylation signal sequence (Ad5 22034–22722); this region is present only in RNA unprocessed at the L3 polyadenylation site. The amounts of unprocessed L3 RNA in wt and L4-22K⁻ virus infections were comparable, and complementation of L4-22K⁻ virus with L4-22KFLAG had no effect (Fig. 7, top left panel). The level of unprocessed E2A RNA, tested as a control since its protein product was unaffected by L4-22K, was equal for all samples as expected (Fig. 7, top right panel), and control reactions lacking reverse trans-

criptase were negative (Fig. 7, bottom right panel), indicating that the PCR products were generated from RNA template. These data provide further evidence against a major role for L4-22K in transcriptional activation of the MLP and focus attention instead on MLTU mRNA processing as its principal site of action.

DISCUSSION

Ad5 late protein synthesis is regulated at the level of transcription, mRNA processing, and translation. Early in infection, MLTU expression is limited to L1-52/55K. However, after DNA replication begins, expression extends across MLTU regions L1 to L5, resulting in over 20 distinct mRNAs and thus proteins. The L4 region is expressed immediately after DNA replication (22), and two of its three nonstructural protein products have been described as factors involved in the regulation of late protein synthesis. L4-33K is required for late mRNA splicing (12, 39), whereas L4-100K is responsible for selective translation of late mRNAs (17) and for the stabilization and assembly of hexon trimers (6). The results presented here demonstrate that the third L4 nonstructural protein, L4-22K, is also involved in the regulation of late protein expression and appears to have distinct functions in transcription and RNA processing.

A L4-22K-deficient genome exhibited a reduction in both structural and nonstructural late protein expression from MLTU regions L1 to L5 (fiber, hexon, penton, IIIa, V, VI, L4-100K, and L1-52/55K) compared to the wt genome, and this defect was fully complemented by exogenous L4-22K expression. The first impact of L4-22K deficiency was in the late phase, as early gene expression, demonstrated by E2A-DBP levels, was not affected and newly synthesized DNA levels from L4-22K⁻ virus-infected cells were comparable to those when L4-22K⁻ was complemented with exogenous L4-22K. L4-22K has been shown previously to be required for viral genome packaging (31), and this same defect was also observed in this study. However, failure to package genome efficiently could not be the cause of the observed reduced late gene expression since the defect was seen at times postinfection when any secondary infections could not have reached the late phase of gene expression. Thus, defective late gene expression is a primary consequence of L4-22K deficiency.

L4-33K has been shown previously to upregulate late gene expression posttranscriptionally (12, 39), so it was possible that lack of L4-22K affected late protein production via a failure to express L4-33K correctly. No antibodies specific for L4-33K were available to test this directly. However, L4-33K expressed *in trans*, in contrast to L4-22K, had almost no impact on the defective late gene expression from L4-22K⁻ genome. Therefore, the reduction in late gene expression from this mutant genome is a direct effect of the absence of L4-22K and not a consequence of either unanticipated aberrant expression of L4-33K due to the L4-22K⁻ mutation that lies within the L4-33K intron or reduction in L4-33K levels that might result from the lack of effect of L4-22K on the MLP. Moreover, the action of L4-22K must be independent of the demonstrated effect of L4-33K on late mRNA splicing.

When high-level transcription from regions L1 to L3 was rendered independent of Ad5 IVa2 and other viral factors by

the use of a heterologous promoter, complementation by L4-22K upregulated penton levels disproportionately to hexon, the reverse of the effect of L4-33K. These observations fit with the work of Tormanen et al. who, when showing that L4-33K was the previously demonstrated Ad late splicing factor (39), found that L4-33K had no effect on penton mRNA splicing *in vitro*. These authors classified splice sites by the length of the polypyrimidine tract and showed that splicing stimulation by L4-33K correlated inversely with tract length. Our findings therefore raise the possibility that L4-22K might also be selective for a particular class of splice site. If so, it does not correspond to polypyrimidine tract strength; penton and V mRNA levels were both strongly responsive to L4-22K in our study, even though their 3' splice sites have very different polypyrimidine tracts and V mRNA splicing was reported to be strongly stimulated by L4-33K in contrast to the lack of effect on penton (39). Hexon mRNA accumulation did not require L4-22K in our study despite hexon protein production being strongly dependent on L4-22K. This discordance is explained by the dependence of hexon protein accumulation on the presence of L4-100K (12), which was itself found to be strongly dependent on L4-22K for its expression. Taken together, these data suggest that L4-33K and L4-22K each act posttranscriptionally to support the development of the full pattern of late viral gene expression in different ways.

In addition to the late proteins, the two intermediate proteins, IVa2 and IX, were reduced in the absence of L4-22K. These two proteins are expressed only after viral DNA replication has begun (10, 19), and both have been implicated in activation of the MLP. The data presented here show that L4-22K increases IVa2 protein levels, independent of the IVa2 promoter and known regulatory sequences. IVa2 has been shown previously to bind to L4-22K during Ad DNA packaging, and L4-22K also promotes the binding of IVa2 to the MLP DEs (31). Thus, we suggest that L4-22K increases IVa2 levels by binding and stabilizing it, and via this action L4-22K achieves a modest activation of the MLP that is dependent on the DEs. However, this effect is minor in comparison with the overall effect of L4-22K on late gene expression.

Previous studies have sought to address the function of L4 proteins through directed mutation of the L4 reading frames. A virus with a stop codon at position 20 of the shared L4-33K/22K reading frame (v33K.1) and expected to lack both proteins was viable, with normal early gene expression and viral DNA replication but a substantial defect in late protein synthesis (13), a phenotype that is similar to that found here for L4-22K⁻. However, a virus with its L4-33K reading frame truncated by stop codons and expected to lack the C-terminal 47 residues proved impossible to isolate, suggesting that it was a lethal mutation (14). This finding suggests that v33K.1 must be leaky for functional L4-33K expression and hence also for L4-22K, although since we have not been able to complement the $\Delta 47$ mutant genome (kindly provided by S. J. Flint) in a cell line expressing L4-33K (data not shown), it is also possible that the severe $\Delta 47$ phenotype includes pleiotropic effects not directly attributable to a lack of L4-33K. In this case, v33K.1 may represent the true null phenotype for both L4-22K and L4-33K. The L4-22K⁻ virus that was isolated here, using the mutated plasmid genome constructed by Ostapchuk and colleagues (31), was clearly viable and showed defects in late gene

expression and genome packaging. It retained the restriction site difference diagnostic of the original mutation, and its phenotype was identical to that of the transfected genome from which it was derived. Thus, although the possibility that its viability results from a second-site mutation(s) that compensates for aspects of the L4-22K⁻ phenotype cannot be excluded, it seems unlikely. Because of the shared L4-33K/22K exon, the L4-22K⁻ mutation truncates the reading frame only after 113 residues. It thus has the potential to express a substantive protein, albeit containing only eight residues of the 22K-unique sequence, which might have residual functions distinct from those of L4-33K. Although no shorter protein immunoreactive with anti-33K serum was detected from this genome (31), the possibility of a contribution of such a protein to the viability of L4-22K⁻ virus cannot be excluded. However, our data, taken together with previous studies, suggest that Ad5 can grow, albeit with low efficiency, without L4-22K and that impaired late gene expression and particle formation is the null phenotype for this protein.

Our results on late gene expression in the absence of L4-22K differ from those previously reported using the L4-22K⁻ genome. Ostapchuk and colleagues reported no differences from the wt in replication, DBP, L1-52/55K, hexon, or penton protein levels (31). In this study we also detected no differences in replication or DBP levels, but we did detect a clear reduction in the levels of L1-52/55K, penton, and to a lesser extent, hexon. We believe the previous data do in fact show some differences in L1-52/55K and penton levels but to a considerably lesser degree than our data indicate. Different cell types, 293 and HeLa, were used for the two studies, but this does not appear to explain this difference, since we consistently observed the same L4-22K⁻ late gene expression defect in infected HeLa cells as seen in 293 cells by either genome transfection or virus infection (data not shown). Possibly, either culture conditions or HeLa cell strain differences affect the expression of host cell factors with which L4-22K needs to interact to upregulate late gene expression and hence modulate the severity of the phenotype observed. Alternatively, differences between the two studies in the effective time postinfection/posttransfection that assays were conducted may provide an explanation, since the defect in late gene expression that we observed diminished as the time to assay was extended.

The MLP is activated by several factors, including the binding of DEF-A and DEF-B to the downstream elements DE1 and DE2. The importance of these factors is most apparent in the absence of activation via upstream promoter elements (32). DEF-A is a heterodimer of IVa2 and another protein, and there is published evidence for this protein being either L4-33K or L4-22K (2, 31). Our data do not bear directly on this issue, since no studies of protein interactions with the DEs were performed. However, MLP activity was shown to be only modestly stimulated by L4-22K, in cooperation with IVa2, suggesting that L4-22K is not the unknown component of DEF-A. This does not exclude the possibility that a complex of IVa2 and L4-22K can bind to the DEs, as previously reported (31); indeed, our data on IVa2 stabilization suggest that increased IVa2 complex formation on the DEs when L4-22K is present should be expected.

In contrast to the modest effects of IVa2 and L4-22K on MLP, the presence of the full viral genome in *trans* with the

MLP reporter caused a 60-fold increase in activity. This suggests that something other than IVa2 or L4-22K is required, either another factor expressed from the genome or the presence of replicating genome itself. One possible factor is E2A-DBP, which has previously been shown to increase MLP activity substantially (7) and is expressed from L4-22K⁻. Another potential activating factor coming from L4-22K⁻ is E1A. However, 293 cells already express E1A, and we have observed only a slight increase in luciferase expression from another E1A-responsive reporter in these cells when E1A is further overexpressed. Nonetheless, it is possible that the population of E1A isoforms expressed in 293 cells is functionally altered during viral infection and that the activation of the MLP in this context is a reflection of this. Further experiments are therefore required to determine the basis of MLP activation by the Ad genome.

This study, together with previous reports, shows that the L4 region of the MLTU provides two crucial regulators of the temporal transitions in MLTU RNA processing that are observed over the course of Ad infection. L4-22K and L4-33K have complementary effects that together provide the normal pattern of mRNA production during the late phase of infection. L4-33K was shown previously to be a splicing factor that activates splicing to specific late mRNA 3' splice sites. However, this activity did not extend to all splice sites whose activity is seen to increase during the early-late transition in MLTU expression. It is possible that L4-22K is also a splicing factor but that its specificity is different from that of L4-33K. Alternatively, it may act less directly, perhaps via changes in host proteins. Further experiments are needed to address these questions.

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