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1   Title : Adenovirus serotype 5 L4-22K and L4-33K proteins have distinct functions in regulating late

2   gene expression

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13

Abstract

Adenoviruses express up to 20 distinct mRNAs from five major late transcription unit (MLTU) regions L1-L5 by differential splicing and polyadenylation of the primary transcript. MLTU expression is regulated at transcriptional and post-transcriptional levels. 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 that is sequence-related to 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 ORF expressed low levels of both structural and non-structural late proteins compared to a wt 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 stabilisation 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.

Introduction

Human adenovirus serotype 5 (Ad5) is considered as a candidate delivery vector for gene therapy and vaccination. However, despite already achieving use 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 – L5 via differential splicing and polyadenylation of the primary transcript (Fig. 1A, reviewed in (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 (35)). The earliest event is expression of the E1A transcriptional activator proteins. These 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 (DBP) and E2B- precursor terminal protein (pTP) & DNA polymerase (Pol) allows viral DNA replication to begin. Around the onset of DNA replication, MLTU expression expands to include L4 proteins (22). The intermediate 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 of MLTU regions.

61   The temporal pattern of MLTU expression involves regulation at both the transcriptional and

62   post-transcriptional levels. Early in infection, transcription does not proceed beyond the L3

63   polyadenylation site, and the L1 polyadenylation site is preferentially used leading to the

64   accumulation of L1-52/55K protein (30). After the initiation of viral DNA replication, MLP activity

65   increases and transcription extends through L4 and L5 regions, allowing a full complement of

66   structural proteins to be produced. Alongside these transcriptional changes, RNA processing is

67   regulated to give altered patterns of mRNA. For example, in the L1 region the proximal 52/55K

68   splice acceptor site is used almost exclusively at early times but during the late phase usage shifts to

69   the distal IIIa acceptor site, due to the production of an infection-specific splicing factor (1). This has

70   now been shown to be L4-33K (39).

71   L4 is now also known to encode a 22 kDa protein (L4-22K) (31), as predicted from

72   bioinformatic analysis (11), that shares its N-terminus with L4-33K but has a unique C-terminal

73   domain (Fig. 1B). L4-22K has a role in genome packaging via its binding, in association with IVa2,

74   to the A2 sequence within the packaging signal (31, 45, 46). L4-22K has also been shown to bind,

75   with IVa2, to the downstream elements (DE1, DE2) of the MLP (31), which have been shown

76   previously to bind IVa2 as both a homodimer (DEF-B) (25, 40) and in complex (DEF-A) with an

77   unidentified viral protein (25, 28) suggesting that L4-22K, in conjunction with IVa2, may play a role

78   in activating the MLP (31). However, DEF-A has also been shown to contain L4-33K, and IVa2 plus

79   L4-33K activated a MLP reporter (2). Thus it is uncertain what the relative contributions of these

80   two L4 proteins are to regulation of the MLP. The sequence-relatedness between L4-22K and L4-

81   33K also raises the possibility that they might have overlapping functions. We therefore sought to

82   analyse the impact of L4-22K on the expression of Ad5 late proteins. Our data show that L4-22K is

83   required for the transition to late phase MLTU expression and acts primarily at the level of RNA

84 processing. However, the action of L4-22K is different from that of the splice factor L4-33K and the

85 two proteins are not functionally redundant.

86

Materials and Methods

88 **Plasmids.** pTG3602 (pWT), containing wild type Ad5 genome, and pTG3602-L4-22K⁻ (pL4-22K⁻),

89 containing a premature stop codon within the C-terminal unique portion of the L4-22K ORF that

90 results in an additional A*vr*II restriction recognition site (Fig. 1B), have been described previously (8,

91 31). Expression plasmids for C-terminal FLAG-tagged L4-22K and L4-33K (pCMV-22KFLAG and

92 pCMV-33KFLAG) were constructed by insertion of X*ba*I/P*ac*I fragments, amplified respectively

93 from viral genomic DNA or viral mRNA using primers containing the restriction enzyme recognition

94 sites (primer sequences available on request), into pCMV-FLAG (12). pBiL1-3NheI, pCMV-

95 100KFLAG, and pCMV-22/33KFLAG, which expresses both L4-22K (no epitope tag) and L4-

96 33KFLAG (12), pCMV-IX (5) and pMEPCMV-IVa2 (4) have been described previously.

97 pMLP+DEluc was generated by amplifying the MLP including DE1 and DE2 (Ad5 5939-6174)

98 using primers containing restriction recognition sites for *Kpn*I (5' primer) and *Nhe*I (3' primer) and

99 cloning into pGL3-Basic luciferase reporter plasmid (Promega). pMLP-DEluc, containing the MLP

100 without the downstream elements (Ad5 5939-6133), was generated in a similar way.

101 pcDNA3.1HisLacZ (Invitrogen) was used as a transfection control.

102

103 **Cells and Viruses.** 293 cells were maintained in Dulbecco's MEM (DMEM) supplemented with 5%

104 fetal bovine serum (FBS). 293-IVa2 cells constitutively express IVa2 from an EBV replicon plasmid

105 (4). 293TetOFF cells (Clontech) were maintained in DMEM supplemented with 10% Tet-free FBS

106 and 100μg/ml geneticin (G418 sulphate, Melford Laboratories). An L4-22K complementing, stable

107   cell line (293-L4-22K) was generated by transfecting 293 cells with pCMV-22KFLAG and selecting

108   clones in DMEM supplemented with 10% FBS and 500μg/ml geneticin. L4-22KFLAG expression

109   was confirmed by immunoblotting and immunofluorescence using a mouse mAb to FLAG (M2,

110   Sigma).

111   Wild-type virus was Ad5 strain 300 (wt300) (21). To generate an L4-22K⁻ virus, 293-L4-22K

112   cells were seeded into a 12 well plate and transfected with L4-22K⁻ genome, excised from pTG3602-

113   L4-22K⁻ by P*ac*I digestion, using Lipofectamine2000 (Invitrogen). When 60-80% cpe was observed

114   cells were harvested, virus released by three freeze/thaw cycles and cellular debris removed by

115   centrifugation. This L4-22K⁻ virus stock was passaged 5 times to ensure removal of transfected

116   genomic DNA. To confirm the presence of the mutated sequence in the P5 stock, virus particle DNA

117   was isolated (16) and used as template for a PCR to amplify the Ad5 L4 region 26018-27086bp. PCR

118   products were then digested with A*vr*II, which is diagnostic for the 22K⁻ mutant (31). Virus titres

119   were determined by fluorescent focus assay. Briefly, 293 cells were infected with a 10-fold dilution

120   series of L4-22K⁻ virus or wt300, fixed at 20h.p.i. with 10% formalin in phosphate-buffered saline

121   (PBS) and permeablized with 0.5% NP40 in PBS before staining with a mouse mAb to DBP (B6-8,

122   (34)) and goat anti-mouse Alexafluor488 antibody (Invitrogen). Virus titres (FFU/ml) were

123   determined by the number of DBP positive cells at a given dilution (33). For wt300, 1 FFU was

124   equivalent to 7 pfu.

125

126   **Transfection and Immunoblotting.** All transient transfections were carried out in 12 well plates at

127   a density of 7×10^5 cells/well using either Lipofectamine2000 (Invitrogen) or TransLT (Cambridge

128   Bioscience) at a ratio of 3μl / μg DNA following the manufacturers' protocols. Transfections used

129   1μg L4-22K⁻ or wt genome, excised from pL4-22K⁻ or pWT respectively by *Pac*I digestion, and

130   500ng various expression plasmids or empty vector (pCMV-FLAG). Experiments using the pBi

131   plasmid system were carried out as previously described (12). Transfected cells were harvested 48h

132   post-transfection directly into sample loading buffer (2% (w/v) sodium dodecyl sulphate [SDS],

133   50mM dithiothreitol [DTT], 10 % (v/v) glycerol, 25mM Tris pH 6.8 and 0.01 % bromophenol blue)

134   and from 10 - 33% cell lysate volume resolved through either 10% or 15% SDS-polyacrylamide gels

135   as appropriate. Proteins were transferred to ECL nitrocellulose membrane (GE Healthcare) and

136   western blot analysis carried out as described (23). Proteins were detected using the following

137   antibodies: FLAG, M2 mouse mAb (Sigma) at 1:10,000; Ad5 late proteins, AbJLB1 rabbit

138   polyclonal serum at 1:10,000 (12); fibre, RI 89&99 rabbit polyclonal serum 1:5000 (obtained from

139   V. Mautner, University of Birmingham, U. K.); E2A-DBP, mouse mAb B6-8 at 1:10,000; L1-

140   52/55K, rabbit polyclonal serum raised to glutathione-S-transferase (GST) - L1-52/55K fusion

141   protein at 1: 10,000, (3); L4-100K, mouse mAb at 1:10,000 (obtained from W.C. Russell, University

142   of St. Andrews, (36)); IVa2, rabbit polyclonal serum raised to GST-IVa2 at 1:10,000, (4); IX, rabbit

143   polyclonal serum at 1:5000, (5). Secondary antibodies were goat-anti-mouse-horseradish peroxidise

144   (HRP; Sigma) at 1:5000 and goat-anti-rabbit-HRP (Santa Cruz) at 1:100,000. HRP signal was

145   detected using ECL-Advance western blot detection kit (GE Healthcare) according to the

146   manufacturer's instructions.

147

148   **RT-PCR.** Cells were scraped into PBS, pelleted by centrifugation, resuspended in isotonic buffer

149   (150 mM NaCl, 10 mM Tris.HCl pH 7.6, 1.5 mM $MgCl_2$) containing 0.6% v/v NP40 and incubated

150   on ice for 10 min. before pelleting the nuclei by centrifugation at 16000 x g for 3 min. The

151   cytoplasmic fraction was reserved and the nuclei resuspended in lysis solution (isotonic buffer

152   containing 1% NP40 and 1% sodium deoxycholate). RNA was isolated from both the nuclear and

153   cytoplasmic fraction using TRI-reagent (Sigma) as described (29). 2µg RNA and 100U Superscript

154   reverse transcriptase (RT; Invitrogen) were used with specific 3' primers to generate cDNA in a 25µl

155   volume reaction using manufacturer's protocol. Equivalent RT⁻ negative reactions omitted

156   Superscript. 2.5µl RT⁺ or RT⁻ reaction were added to a PCR (1x PCR buffer, 0.5mM each dNTP,

157   0.5µM 5' and 3' primer, 3mM $MgCl_2$ and 1-2.5U *Taq* polymerase (Fermentas) in a total volume of

158   20µl). Primers used were complementary to Ad5 tripartite leader 1 sequence 6049-6069 and Ad5 L2-

159   penton 14889-14863, Ad5 L2-V 17445-17415 or Ad5 L3-hexon 19254-19228, or to sequences

160   flanking the Ad5 L3 poly(A) and E2A poly(A) sites 22034-22061 and 22749-22722, or to human β-

161   actin cDNA positions 1430-1455 and 2383-2368. Reactions were incubated at 94°C for 5 min

162   followed by 30 cycles of 94°C, 1 min, 65°C, 1 min and 72°C, 1 min. PCR products were resolved

163   through 1% agarose alongside size markers (1kb Generuler, Fermentas).

164

165   **Southern Blot Analysis.** 293 cells were infected with 2 x 10^{-3} FFU/cell L4-22K⁻ virus or wt300, and

166   harvested 3 or 20h. p.i. Cells were processed either to purify packaged viral DNA, using spermine

167   precipitation to remove unpackaged material (16), or for total low molecular weight DNA by HIRT

168   extraction (18). DNA was digested with *Hind*III, resolved through 0.7% agarose and transferred to

169   Hybond-N membrane (GE Healthcare) and viral DNA detected using an L1 probe (10,589-11,565bp

170   from pGem4-L1 (37)) labelled using the AlkPhos direct labelling kit (GE Healthcare).

171

172   **Luciferase Assay.** 293 and 293-IVa2 cells were transfected with 500ng reporter plasmid

173   (pMLP+DEluc or pMLP-DEluc) and 100ng pcDNA3.1HisLacZ, together with 500ng pCMV-

174   22KFLAG and/or 1µg L4-22K⁻ genome. All transfections were equalised for DNA content by the

175   addition of either salmon sperm DNA or pCMV-FLAG as appropriate. Cells were lysed 24h post-

176 transfection in 1x passive lysis buffer and a luciferase assay carried out according to the

177 manufacturer's instructions (Promega). Cell lysates were also analysed for β-galactosidase activity

178 using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The ratio of firefly luciferase to β-

179 galactosidase was calculated to correct for differences in transfection efficiency. Data are shown as

180 fold induction where luciferase expression from the reporter plasmids is set as 1. Statistical analysis

181 was performed using Student's T-Test.

182

Results

184 **L4-22K is required for efficient late gene expression in 293 cells.** Our previous studies of the

185 early to late transition in MLTU expression utilised plasmid pBiL1-3NheI, in which MLTU genomic

186 sequence encoding the tripartite leader (TPL) and regions L1 – L3 was placed under the control of a

187 heterologous promoter. Full late pattern expression could be induced from this plasmid by CMV

188 expression vectors encoding L4-33K and -100K proteins (12). The "33K" plasmid utilised was also

189 capable of L4-22K expression, although L4-22K had not been proven to exist at that time.

190 Subsequently, L4-33K was shown by Tormanen et al. to be a splicing factor capable of activating

191 certain MLTU splice sites (39). To determine whether L4-22K made any contribution to the MLTU

192 activation observed in this system, plasmids expressing either L4-22K or L4-33K with C-terminal

193 FLAG tags were constructed. When co-transfected with pBiL1-3NheI and an L4-100K vector to

194 permit efficient hexon protein accumulation, L4-22KFLAG increased levels of several late proteins

195 independently of L4-33K (Fig. 1C). These effects were qualitatively distinct, with L4-22K increasing

196 penton expression more than, and hexon expression less than did L4-33K. These data suggested that

197 L4-22K made a contribution to late protein expression from L1-L3, although it could not be excluded

198   that this effect was due to expression of a truncated L4-33K-related protein from the L4-22K plasmid

199   clone.

200   To investigate fully the role of L4-22K in regulating MLTU expression, given the prior

201   evidence that L4-22K and/or L4-33K might act on the MLP, we focused further studies on a whole

202   genome system, in which the MLTU was controlled by the native MLP within linear full-length

203   genome excised from pTG3602 (8). This also provided the possibility of IVa2 production that was

204   not present in the pBi plasmid system. The role and mechanism of action of L4-22K in late gene

205   expression from this genome was assessed by comparison with an L4-22K$^-$ genome that retained the

206   ability to express full-length L4-33K (Fig. 1B, (31)), kindly provided by P. Hearing.

207   293 cells transfected with L4-22K$^-$ genome showed a decrease in late structural protein

208   production (hexon, penton, IIIa, V and fibre) compared to a wt genome (Fig. 2A & B); while the

209   absolute level of this defect varied between experiments, as exemplified here, it was always

210   substantial. In addition, expression of the non-structural late proteins L4-100K and L1-52/55K from

211   L4-22K$^-$ genome was reduced to virtually undetectable levels (Fig. 2B). The level of E2A-DBP

212   detected was at least equivalent for L4-22K$^-$ and the wt genome, and possibly somewhat increased in

213   the mutant, demonstrating that the defect in late protein expression was not due to differences in

214   transfection efficiency and that the absence of L4-22K does not cause a global downregulation of

215   viral protein production. Expression of all late proteins from the L4-22K$^-$ genome was restored to

216   wild-type levels when cells were co-transfected with an expression plasmid for L4-22KFLAG (Fig.

217   2A), confirming that the reduced late protein expression seen from L4-22K$^-$ genome was due solely

218   to the lack of L4-22K expression. L4-22K has been shown previously to be involved in the

219   packaging of Ad genomes into particles (31), so at 48 h post-transfection it was possible that the

220   observed differences in late gene expression resulted from reduced levels of second-round infection

221   in L4-22K⁻ genome-transfected cultures consequent upon a failure to package genomes efficiently.

222   However, this possibility was discounted as similar results were observed at 20 h post-transfection, a

223   time point when no progeny virus would have been able to reach the late phase of a secondary

224   infection (data not shown). The late protein defect of L4-22K⁻ is kinetic rather than absolute as, when

225   analysis was delayed to 60 h post-transfection, L4-22K⁻ genome produced similar levels of late

226   proteins to those seen at 20 h post-transfection for either wt genome or L4-22K⁻ genome co-

227   transfected with L4-22KFLAG expression plasmid (data not shown). Thus L4-22K upregulates late

228   gene expression in 293 cells.

229

230   **L4-22K acts via a different mechanism to L4-33K.** The stop codon inserted into the L4-22K ORF

231   in pL4-22K⁻ is located within the intron sequence of L4-33K ORF and it was possible that this

232   mutation might affect the expression of L4-33K, hence causing some part of the observed defect in

233   late protein production. Although the defect was complemented by L4-22KFLAG, this plasmid also

234   has the capacity to express a truncated L4-33K protein consisting of the N-terminal 129 amino acids

235   that might contribute to this complementation. The truncated protein would lack the C-terminal RS

236   motifs that have been show to be essential for this activity (39), making this unlikely. However, to

237   exclude the possibility, L4-22K⁻ was co-transfected with an expression plasmid for L4-33KFLAG.

238   Expressing L4-33KFLAG *in trans* did not restore expression of either the structural proteins or L4-

239   100K (Fig. 2B), although a small increase in L1-52/55K was observed. Late protein expression from

240   L1 through to L5 was only restored when L4-22K was added *in trans* either from pCMV-22KFLAG

241   or from a plasmid, pCMV-22/33KFLAG, capable of expressing both L4-22K and L4-33KFLAG

242   from alternatively spliced transcripts (Fig. 2B). During complementation by pCMV-22K/33KFLAG,

243   expression of the structural proteins and L4-100K was greater than that seen with L4-22K⁻ genome

244 alone but was reduced compared to when L4-22KFLAG was expressed from pCMV-22KFLAG.

245 This may reflect preferential expression of the spliced L4-33KFLAG transcript from this plasmid

246 and consequently a lower expression level of L4-22K protein than was achieved with pCMV-

247 22KFLAG. These data show that the defect in late gene expression is not due to any unexpected

248 aberrations in L4-33K expression occurring due to the mutation in the intronic sequence of L4-33K

249 ORF. Furthermore, the evidence clearly shows the mechanism of action of L4-22K is independent of

250 L4-33K splicing function and thus indicates a direct role for L4-22K in the activation of the late

251 phase of infection.

252

253 **Reduced late gene expression in the absence of L4-22K is not due to a defect in replication.** Full

254 expression from the Ad5 MLTU is the end result of a temporal pattern of regulated gene expression.

255 It is only once viral DNA replication has begun that the MLP is activated to produce maximal

256 expression of the late structural genes from all MLTU regions. Thus a defect in replication could

257 account for the reduction in late protein production observed from L4-22K$^-$ genome. To allow

258 replication studies to be carried out without the concern of transfection efficiencies or the confusion

259 of large quantities of input DNA, L4-22K$^-$ virus was isolated by transfection of L4-22K$^-$ genome into

260 a stable L4-22K complementing cell line, 293-L4-22K. Although this cell line only expressed a low

261 level of L4-22KFLAG, it was sufficient to permit the growth of virus to low titre.

262   The production of L4-22K$^-$ virus was confirmed by *Avr*II digest of PCR products amplified

263 from DNA isolated from L4-22K$^-$ virus particles (Fig. 3A). Using particle DNA ensured that only

264 newly synthesised and packaged DNA was acting as a template in the PCR and not any remaining

265 transfected genome. Incubation of the L4-22K$^-$ virus product with A*vr*II resulted in the virtually

266 complete cleavage to bands 532 and 536bp in size, indicating presence of the site diagnostic of the

267   L4-22K$^-$ mutation (31). The small amount of uncut DNA is likely to represent incomplete digestion.

268   If any wild-type DNA was present, it would rapidly overgrow the slow-growing mutant but no

269   increase in the proportion of this uncut species was seen upon further passage; the presence of the

270   inserted stop codon was also confirmed by sequencing (data not shown). The L4-22K$^-$ virus had the

271   packaging defect expected from previous work (Fig. 3B) (31). At 20 h. p.i. viral DNA was not

272   detected for L4-22K$^-$ unless complemented by prior expression of L4-22KFLAG; when

273   complemented, amounts of L4-22K$^-$ DNA were similar to wt virus. No viral DNA was detected at 3

274   h. p.i. indicating that viral DNA detected at 20 h. p.i. was due to newly synthesised, packaged

275   genomic DNA and not virus input. These data confirm the L4-22K deficient nature of the L4-22K$^-$

276   virus generated in this study both genetically and biologically.

277   To investigate the growth properties of L4-22K$^-$, the late protein expression defect observed

278   with transfected genome was first confirmed. Non-complementing cells infected with L4-22K$^-$ virus

279   behaved identically to cells transfected with L4-22K$^-$ genome in showing a substantial impairment of

280   structural protein expression (data not shown). Genome replication was then assessed (Fig. 3C). At

281   3h. p.i. a low level of viral DNA was detected; this time point is too early for the synthesis of

282   progeny viral DNA and thus shows the amount of input viral DNA. The increased level of viral DNA

283   detected at 20 h. p.i. was similar for uncomplemented and complemented L4-22K$^-$ virus, showing

284   that the absence of L4-22K has no effect on virus DNA replication. This result agrees with that

285   reported previously in which replication of the L4-22K$^-$ genome was investigated in HeLa cells (31).

286   The block in MLTU gene expression must therefore occur after DNA replication.

287

288   **A reduction in intermediate proteins IVa2 and IX is not fully responsible for the reduction in**

289   **late protein expression from L4-22K$^-$.** The two intermediate proteins, IVa2 and IX upregulate the

290   MLP activity during the transition between the early and late phases of infection (25, 26, 32, 40).

291   IVa2 acts via binding, either as a homodimer (DEF-B) or heterodimer (DEF-A), to sequences DE1

292   and DE2 within the MLP (20, 28). Furthermore, the pattern of differential protein expression

293   observed here in the absence of L4-22K is similar to that reported previously for MLP DE1 and DE2

294   mutants (32) suggesting that L4-22K may be acting via IVa2, perhaps as a component of DEF-A

295   since enhanced binding of IVa2 to DE1,2 in the presence of 22K has been reported (31).

296   Expression of IVa2 and IX from L4-22K$^-$ genome alone was barely detectable, whereas

297   levels of both proteins were substantially increased when L4-22KFLAG was co-expressed (Fig. 4A).

298   To determine whether this reduction in intermediate proteins was responsible for the limited

299   expression of late proteins observed for L4-22K$^-$ genome, these two proteins were expressed *in trans*.

300   Co-transfection of L4-22K$^-$ genome with IVa2 expression plasmid increased expression of hexon,

301   penton and the smaller isoform of protein V, but failed to increase IIIa, the upper isoform of V or

302   pVI (Fig. 4B). In contrast, co-transfection with IX expression plasmid failed to increase the level of

303   late gene expression significantly. When IX and IVa2 were expressed together, the level of all late

304   proteins tested increased a little as compared to either protein alone. However, this late protein

305   expression was very much lower than that seen when the L4-22K$^-$ genome was directly

306   complemented by L4-22KFLAG (Fig. 4B).

307   L4-22K clearly increases the level of both IVa2 and IX proteins produced from transfected

308   genome (Fig. 4A). In the case of IVa2 this may reflect a stabilisation of the protein rather than a

309   transcription or RNA processing event since L4-22KFLAG also increases the level of IVa2 protein

310   expressed from cDNA under the control of the heterologous CMV promoter (Fig. 4C); this effect

311   was specific to L4-22K as neither L4-33KFLAG nor L4-100KFLAG had any effect (Fig. 4C). This

312   observation suggests that the increase in IVa2 seen when L4-22K$^-$ genome is complemented is not

313   mediated through IVa2's interaction with L1-52/55K, its known partner in viral DNA packaging (15),

314   expression of which also increases upon complementation of L4-22K⁻ (Fig. 2B). The IVa2 sequence

315   within pMEPCMV-IVa2 does not contain the binding site for the cellular repressor that blocks IVa2

316   transcription prior to replication (9, 24) and thus L4-22K cannot be acting by relieving this

317   repression. In addition, no effect of L4-22K on expression of other proteins from the CMV promoter

318   was observed (data not shown). Therefore, we hypothesise that L4-22K acts to stabilise IVa2 protein.

319   It is possible that lack of IVa2 stabilisation by L4-22K is the reason why IVa2 expressed *in*

320   *trans* can only partially complement the late protein expression defect of L4-22K⁻ genome. However,

321   this seems unlikely as the levels of IVa2 achieved were similar to those seen when L4-22K⁻ was fully

322   complemented by L4-22KFLAG. Furthermore, similar results were obtained when a cell line

323   constitutively expressing high levels of IVa2 was used (Fig. 5C), excluding the possibility that L4-

324   22K somehow affects IVa2 transfection efficiency. Alternatively, the requirement for L4-22K may

325   reflect a direct contribution of this protein to the DEF-A transcription factor that is required for

326   maximal activation of the MLP (31).

327

328   **L4-22K increases MLP activity in a DE-dependent manner.** To determine if L4-22K directly

329   regulated transcription from the MLP, and whether this was DE-dependent, luciferase expression

330   from MLP reporters either containing or lacking DE1,2 was assessed in the presence of the L4-22K⁻

331   genome or complemented genome (Fig. 5A). The presence of the genome ensured all viral proteins,

332   other than L4-22K, that might be required for transcriptional activation were present in the system.

333   Luciferase expression was increased 45-60 fold by the presence of the L4-22K⁻ genome, regardless

334   of the presence or absence of the DE elements. This significant increase occurred despite the fact that

335   the L4-22K⁻ genome displays a severe reduction in IVa2, IX and late protein synthesis. Expression of

336   L4-22KFLAG further increased the activity of pMLP+DEluc by two-fold compared to L4-22K⁻

337   genome alone (p<0.005), whereas the activity of pMLP-DEluc was not significantly altered (p>0.1),

338   demonstrating that the increase was DE-dependent (Fig. 5A). In contrast, L4-22KFLAG expressed in

339   the absence of viral genome had no effect on the activity of MLP containing the DEs (Fig. 5B),

340   suggesting that another viral protein was required in association with L4-22K.

341   A likely candidate protein was IVa2, which was known to act via the DEs (40) and had been

342   shown to be stabilised in the presence of L4-22K (Fig. 4). To test whether DE-dependent MLP

343   activation by L4-22K was indirectly due to increased IVa2 levels or due to a direct interaction of L4-

344   22K and IVa2 at the DEs, MLP reporter activity was analysed in a cell line constitutively expressing

345   high levels of IVa2 (293-IVa2) (4). Activity of the DE-containing MLP in 293-IVa2 cells was

346   increased a modest 1.2 fold compared to its activity in 293 cells. When L4-22KFLAG was co-

347   expressed in these cells, the increase was 1.6 fold compared to 293 cells, a 1.3 fold increase

348   compared to 293-IVa2 cells (Fig. 5B). Therefore, both L4-22K and IVa2 contribute to MLP

349   activation. However, the small increase in MLP activity observed when L4-22KFLAG is expressed

350   in the presence of high levels of IVa2 does not explain the large differences in the expression of late

351   proteins, notably penton and IIIa, from complemented genome in the same cell line (Fig. 5C). This

352   suggests that L4-22K may have a role in late gene expression that is post-transcriptional, in addition

353   to its effects, with or via IVa2, at the MLP. This conclusion is supported by the observed increase in

354   L1-3 gene expression from a heterologous promoter in the presence of L4-22KFLAG, when no IVa2

355   was present (Fig. 1C).

356

357   **L4-22K acts at the level of late gene mRNA production/stability.** To determine whether L4-22K

358   was exerting its post-transcriptional effect at the level of mRNA or protein production, the amounts

359 of penton, V and hexon mRNA produced by L4-22K⁻ and wt virus were assessed. Penton and V

360 mRNA production from L4-22K⁻ virus was barely detectable, compared to readily detectable

361 expression from wt virus (Fig. 6); this defect was fully complemented when L4-22KFLAG was

362 expressed *in trans* (Fig. 6). In contrast, the level of hexon mRNA produced by L4-22K⁻ virus was

363 similar to that from wt and not further increased when complemented by L4-22K. PCR products

364 were not from DNA contamination of the template as equivalent reactions containing no reverse

365 transcriptase were negative (Fig. 6). The quantity and quality of RNA used was confirmed by the

366 equivalent amplification of β-actin mRNA (Fig. 6). Therefore, a major component of the reduction in

367 late gene expression observed for L4-22K⁻ is at the level of late mRNA production and/or stability,

368 and the action of L4-22K is selective for specific mRNAs.

369

370 **L4-22K does not increase the level of un-processed late mRNA.** To further clarify the role of L4-

371 22K in transcription and RNA processing, the level of unspliced nuclear MLTU RNA was

372 determined by RT-PCR amplification of a region spanning the L3 polyadenylation signal sequence

373 (Ad5 22034-22722); this region is only present in RNA unprocessed at the L3 polyadenylation site.

374 Amounts of unprocessed L3 RNA in wt and L4-22K⁻ virus infections were comparable, and

375 complementation of L4-22K⁻ virus with L4-22KFLAG had no effect (Fig. 7 left upper panel). The

376 level of unprocessed E2A RNA, tested as a control since its protein product was unaffected by L4-

377 22K, was equal for all samples as expected (Fig. 7 right upper panel) and control reactions lacking

378 reverse transcriptase were negative (Fig 7 right lower panel), indicating that the PCR products were

379 generated from RNA template. These data provide further evidence against a major role for L4-22K

380 in transcriptional activation of the MLP, and focus attention instead on MLTU mRNA processing as

381 its principal site of action.

382

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 through 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 non-structural 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 stabilisation and assembly of hexon trimers (6). The results presented here demonstrate that the third L4 non-structural 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 non-structural late protein expression from MLTU regions L1 through to L5 (fibre, hexon, penton, IIIa, V, VI, L4-100K and L1-52/55K) compared to a 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 synthesised 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 post-infection when any secondary infections could not have reached the late phase of

404   gene expression. Thus, defective late gene expression is a primary consequence of L4-22K

405   deficiency.

406   L4-33K has been shown previously to upregulate late gene expression post-transcriptionally

407   (12, 39), so it was possible that lack of L4-22K affected late protein production via a failure to

408   express L4-33K correctly. No antibodies specific for L4-33K were available to test this directly.

409   However, L4-33K expressed *in trans,* in constrast to L4-22K, had almost no impact on the defective

410   late gene expression from L4-22K⁻ genome. Therefore the reduction in late gene expression from this

411   mutant genome is a direct effect of the absence of L4-22K and a not a consequence of either

412   unanticipated aberrant expression of L4-33K due to the L4-22K⁻ mutation that lies within the L4-33K

413   intron or reduction in L4-33K levels that might result from the lack of L4-22K effects on the MLP.

414   Moreover, the action of L4-22K must be independent of the demonstrated effect of L4-33K on late

415   mRNA splicing.

416   When high level transcription from L1-L3 regions was rendered independent of Ad5 IVa2

417   and other viral factors by the use of a heterologous promoter, complementation by L4-22K

418   upregulated penton levels disproportionately to hexon, the reverse of the effect of L4-33K. These

419   observations fit with the work of Tormanen et al. who, when showing that L4-33K was the

420   previously demonstrated Ad late splicing factor (39), found that L4-33K had no effect on penton

421   mRNA splicing *in vitro*. These authors classified splice sites by length of polypyrimidine tract and

422   showed that splicing stimulation by L4-33K correlated inversely with tract length. Our findings

423   therefore raise the possibility that L4-22K might also be selective for a particular class of splice site.

424   If so, it does not correspond to polypyrimidine tract strength; penton and V mRNA levels were both

425   strongly L4-22K – responsive in our study even though their 3' splice sites have very different

426   polypyrimidine tracts and V mRNA splicing was reported to be strongly stimulated by L4-33K in

427 contrast to the lack of effect on penton (39). Hexon mRNA accumulation did not require L4-22K in

428 our study despite hexon protein production being strongly dependent on L4-22K. This discordance is

429 explained by the dependence of hexon protein accumulation on the presence of L4-100K (12), which

430 was itself found to be strongly dependent on L4-22K for its expression. Taken together, these data

431 suggest that L4-33K and L4-22K each act post-transcriptionally to support the development of the

432 full pattern of late viral gene expression in different ways.

433   As well as the late proteins, the two intermediate proteins, IVa2 and IX were reduced in the

434 absence of L4-22K. These two proteins are only expressed after viral DNA replication has begun (10,

435 19), and both have been implicated in activation of the MLP. The data presented here show that L4-

436 22K increases IVa2 protein levels, independent of the IVa2 promoter and known regulatory

437 sequences. IVa2 has been shown previously to bind to L4-22K during Ad DNA packaging, and L4-

438 22K also promotes the binding of IVa2 to the MLP DEs (31). Thus, we suggest that L4-22K

439 increases IVa2 levels by binding and stabilising it, and via this action L4-22K achieves a modest

440 activation of the MLP that is dependent on the DEs. However, this effect is minor in comparison

441 with the overall effect of L4-22K on late gene expression.

442   Previous studies have sought to address the function of L4 proteins through directed mutation

443 of the L4 reading frames. A virus with a stop codon at position 20 of the shared L4-33K/22K reading

444 frame (v33K.1) and expected to lack both proteins was viable, with normal early gene expression

445 and viral DNA replication but a substantial defect in late protein synthesis (13), a phenotype that is

446 similar to that found here for L4-22K⁻. However, a virus with its L4-33K reading frame truncated by

447 stop codons and expected to lack the C-terminal 47 residues proved impossible to isolate, suggesting

448 it was a lethal mutation (14). This finding suggests that v33K.1 must be leaky for functional L4-33K

449 expression and hence also for L4-22K, although since we have not been able to complement the Δ47

450   mutant genome (kindly provided by S. J. Flint) in a cell line expressing L4-33K (data not shown), it

451   is also possible that the severe Δ47 phenotype includes pleiotropic effects not directly attributable to

452   a lack of L4-33K. In this case, v33K.1 may represent the true null phenotype for both L4-22K and

453   L4-33K. The L4-22K⁻ virus that was isolated here, using the mutated plasmid genome constructed by

454   Ostapchuk and colleagues (31), was clearly viable and showed defects in late gene expression and

455   genome packaging. It retained the restriction site difference diagnostic of the original mutation and

456   its phenotype was identical to that of the transfected genome from which it was derived. Thus,

457   although it cannot be excluded that its viability results from second-site mutation(s) that compensate

458   for aspects of the L4-22K⁻ phenotype, this seems unlikely. Because of the shared L4-33K/22K exon,

459   the L4-22K⁻ mutation truncates the reading frame only after 113 residues. It thus has the potential to

460   express a substantive protein, albeit containing only 8 residues of the 22K-unique sequence, which

461   might have residual functions distinct from those of L4-33K. Although no shorter protein

462   immunoreactive with anti-33K serum was detected from this genome (31), a contribution of such a

463   protein to the viability of L4-22K⁻ virus cannot be excluded. However, our data, taken together with

464   previous studies, suggest that Ad5 can grow, albeit with low efficiency, without L4-22K and that

465   impaired late gene expression and particle formation is the null phenotype for this protein.

466   Our results on late gene expression in the absence of L4-22K differ from those previously

467   reported using the L4-22K⁻ genome. Ostapchuk and colleagues reported no differences from wt in

468   replication, DBP, L1-52/55K, hexon or penton protein levels (31). In this study we also detected no

469   differences in replication or DBP levels, but detected a clear reduction in the levels of L1-52/55K,

470   penton and to a lesser extent hexon. We believe the previous data do in fact show some differences

471   in L1-52/55K and penton levels but to a considerably lesser degree than our data indicate. Different

472   cell types, 293 and HeLa, were used for the two studies but this does not appear to explain this

473   difference since we consistently observed the same L4-22K⁻ late gene expression defect in infected

474   HeLa cells as seen in 293 cells by either genome transfection or virus infection (data not shown).

475   Possibly, either culture conditions or HeLa cell strain differences affect the expression of host cell

476   factors with which L4-22K needs to interact to upregulate late gene expression and hence modulate

477   the severity of the phenotype observed. Alternatively, differences between the two studies in the

478   effective time post-infection / transfection that assays were conducted may provide an explanation,

479   since the defect in late gene expression that we observed diminished as the time to assay was

480   extended.

481   The MLP is activated by several factors, including the binding of DEF-A and DEF-B to the

482   downstream elements DE1 and DE2. The importance of these factors is most apparent in the absence

483   of activation via upstream promoter elements (32). DEF-A is a heterodimer of IVa2 and another

484   protein, and there is published evidence for this protein being either L4-33K or L4-22K (2, 31). Our

485   data do not bear directly on this issue, since no studies of protein interactions with the DEs were

486   performed. However, MLP activity was shown to be only modestly stimulated by L4-22K, in

487   cooperation with IVa2, suggesting that L4-22K is not the unknown component of DEF-A. This does

488   not exclude that a complex of IVa2 and L4-22K can bind to the DEs, as previously reported (31);

489   indeed, our data on IVa2 stabilisation suggest that increased IVa2 complex formation on the DEs

490   when L4-22K is present should be expected.

491   In contrast to the modest effects on MLP of IVa2 and L4-22K, the presence of the full viral

492   genome *in trans* with the MLP reporter caused a 60-fold increase in activity. This suggests that

493   something other than IVa2 or L4-22K is required, either another factor expressed from the genome

494   or the presence of replicating genome itself. One possible factor is E2A-DBP, which has previously

495   been shown to increase MLP activity substantially (7) and is expressed from L4-22K⁻. Another

496 potential activating factor coming from L4-22K⁻ is E1A. However, 293 cells already express E1A,

497 and we have observed only a slight increase in luciferase expression from another E1A-responsive

498 reporter in these cells when E1A is further over-expressed, Nonetheless, it is possible that the

499 population of E1A isoforms expressed in 293 cells is functionally altered during viral infection and

500 that the activation of the MLP in this context is a reflection of this. Further experiments are therefore

501 required to determine the basis of MLP activation by Ad genome.

502 This study, together with previous reports, shows that the L4 region of the MLTU provides

503 two crucial regulators of the temporal transitions in MLTU RNA processing that are observed over

504 the course of Ad infection. L4-22K and L4-33K have complementary effects that together provide

505 the normal pattern of mRNA production during the late phase of infection. L4-33K was shown

506 previously to be a splicing factor that activates splicing to specific late mRNA 3' splice sites.

507 However, this activity did not extend to all splice sites whose activity is seen to increase during the

508 early-late transition in MLTU expression. It is possible that L4-22K is also a splicing factor, but with

509 a distinct specificity from L4-33K. Alternatively, it may act less directly, perhaps via changes in host

510 proteins. Further experiments are needed to address these questions.

511

Acknowledgements

518

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519

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633

634   **Figure legends**

635   **Figure 1.** (A) Schematic diagram of the major late transcription unit showing the alternatively

636   spliced mRNAs and encoded proteins. (B) Organisation of the L4-22K and L4-33K ORF. The

637   position of the L4-22K truncation mutation in L4-22K$^-$ is indicated (31). (C) 293TETOFF cells,

638   which constitutively express the tetracycline transactivator protein were transfected with pBiL1-

639   3NheI (which expresses the MLTU L1-3 region under the control of a tetracycline-regulated

640   promoter), pCMV100KFLAG and either empty vector or the L4-22KFLAG or L4-33KFLAG

641   expression plasmids. Cell lysates were harvested 48h post-transfection, separated on 10%

642   polyacrylamide gel and late proteins, as indicated on the right, detected by western blot analysis

643   using AbJLB1 polyclonal anti-late protein serum. The positions to which proteins of known

644   molecular mass migrated are shown on the left (kDa).

645

646   **Figure 2. Late protein expression from L4-22K$^-$ genome.** Cells were mock transfected or

647   transfected with L4-22K$^-$ genome and either empty vector or various L4 expression plasmids as

648   indicated. Ad5 wt genome was used as a control. Cell lysates were separated on 10% polyacrylamide

649   gels and expressed proteins, as indicated at the right of each panel, detected by western blot analysis

650   using either AbJLB1 polyclonal anti-late protein antiserum (top panels, A & B), RI polyclonal anti-

651   late protein antiserum to detect fibre (B) or monospecific / monoclonal antibodies as indicated. In top

652   panel B, all lanes shown derive from the same exposure of a single blot with irrelevant lanes excised

653   for clarity of presentation. Note that no FLAG-tagged 22K is detected from transfection of the single

654   L4-22/33K plasmid as the tag sequence is only attached to the 33K reading frame. However, the

655   expected expression of 22K from this plasmid can be inferred from its effect on late protein

656   expression. The positions to which proteins of known molecular mass migrated are shown on the left

657   (kDa).

658

659   **Figure 3. L4-22K has no effect on viral DNA replication.** (A) Confirmation of L4-22K⁻ mutant

660   status. L4-22K⁻ virus was generated and passaged five times in a stable L4-22K complementing cell

661   line, 293-L4-22K. Ad5 26018-27086 bp region was amplified by PCR from DNA isolated from

662   either L4 22K⁻ or wt virus particles. PCR products were either undigested or digested with *Avr*II as

663   indicated. Lane M, 1kb ladder, sizes marked on the left (kbp). (B) L4-22K effects on packaging. 293

664   cells were either mock transfected or transfected with L4-22KFLAG and 24 h. later infected with 2 x

665   10^{-3} FFU/cell L4-22K⁻ virus or Ad5 wt. Packaged DNA isolated from infected cells at either 3 h. p.i.

666   or 20 h. p.i. was digested with *Hind*III and analysed by southern blotting using an L1 probe. (C) L4-

667   22K effects on replication. As B. except DNA was obtained by HIRT extraction of cell cultures at 3

668   h. or 20 h. 50ng Ad5wt viral DNA was used as a positive control (B & C).

669

670   **Figure 4. Restoring expression levels of the intermediate proteins IVa2 and IX does not fully**

671   **complement L4-22K⁻.** (A&B) 293 cells were mock transfected, or transfected with L4-22K⁻

672   genome and either empty vector or various expression plasmids as indicated. (C) 293 cells were

673   mock transfected, or transfected with the IVa2 expression plasmid with either empty vector or

674   various L4-protein expression vectors as indicated. Cell lysates were separated on 10% or 15%

675   polyacrylamide gels and expressed proteins detected by western blot analysis using anti-IVa2, anti-

676 IX or AbJLB1 anti-late protein antisera. The positions to which proteins of known molecular mass

677 migrated are shown on the left (kDa).

678

679 **Figure 5. L4-22K increases transcription activity of IVa2 at the MLP.** (A) 293 cells were

680 transfected with pMLP+DEluc (black bars) or pMLP-DEluc (grey bars), alone or with L4-22K⁻

681 genome and various expression plasmids as indicated. (B) 293 cells (grey bars) or 293-IVa2 (black

682 bars) cells were transfected with pMLP+DEluc (black bars), alone or with L4-22K⁻ genome and

683 various expression plasmids as indicated. Firefly luciferase expression was detected using a

684 luciferase assay, corrected for transfection efficiency using β-galactosidase expression from an

685 independent control plasmid, and expressed as fold induction where expression from the reporter

686 plasmids alone was set as 1. Error bars show standard deviations where n = 3. (C) 293 or 293-IVa2

687 cells were transfected with L4-22K⁻ genome and either empty vector or L4-22KFLAG expression

688 plasmid. Cell lysates were separated on 10% polyacrylamide gels and expressed proteins detected by

689 western blot analysis using AbJLB1 anti-late protein or anti-IVa2 antisera. The positions to which

690 proteins of known molecular mass migrated are shown on the left (kDa).

691

692 **Figure 6. L4-22K increases the level of late mRNA.** RT-PCR amplifications were performed on

693 total cytoplasmic RNA from 293 cells that were either mock transfected or transfected with L4-

694 22KFLAG and 24 h. later infected with 2×10^{-3} FFU/cell L4-22K⁻ virus or Ad5 wt as indicated. Late

695 mRNAs were detected by RT-PCR using a 5' primer specific for tripartite leader 1 and 3' primers

696 directed to L2 penton (947bp product), L2 V (1138bp) or L3 hexon (662bp) 3'exons. Equivalent

697 control reactions were performed lacking reverse transcriptase (RT⁻); amplification of β-actin mRNA

698 (511bp product) served as a positive control. For V mRNA, both RT-PCR and RT⁻ results derive

699   from the same experiment and are presented at the same exposure. The positions to which DNA size

700   markers migrated are indicated (kbp).

701

702   **Figure 7. L4-22K acts at the level of RNA processing.** 293 cells were either mock transfected or

703   transfected with L4-22KFLAG expression vector. 24h post-transfection, cells were mock-infected, or

704   infected with 2 x 10^{-3} FFU/cell L4-22K^{-} virus or Ad5 wt and nuclear RNA isolated 20h p.i. Amounts

705   of L3 RNA unprocessed at the L3 polyA site (upper left panel), and of E2A RNA unprocessed at its

706   polyA site (upper right panel), were determined by RT-PCR using specific primers. β-actin mRNA

707   served as a positive control (lower left panel). To confirm that amplified products derived from

708   RNA template, the same amount of RNA was added to reactions where the reverse transcriptase had

709   been omitted (lower right panel). The positions to which DNA size markers migrated are indicated
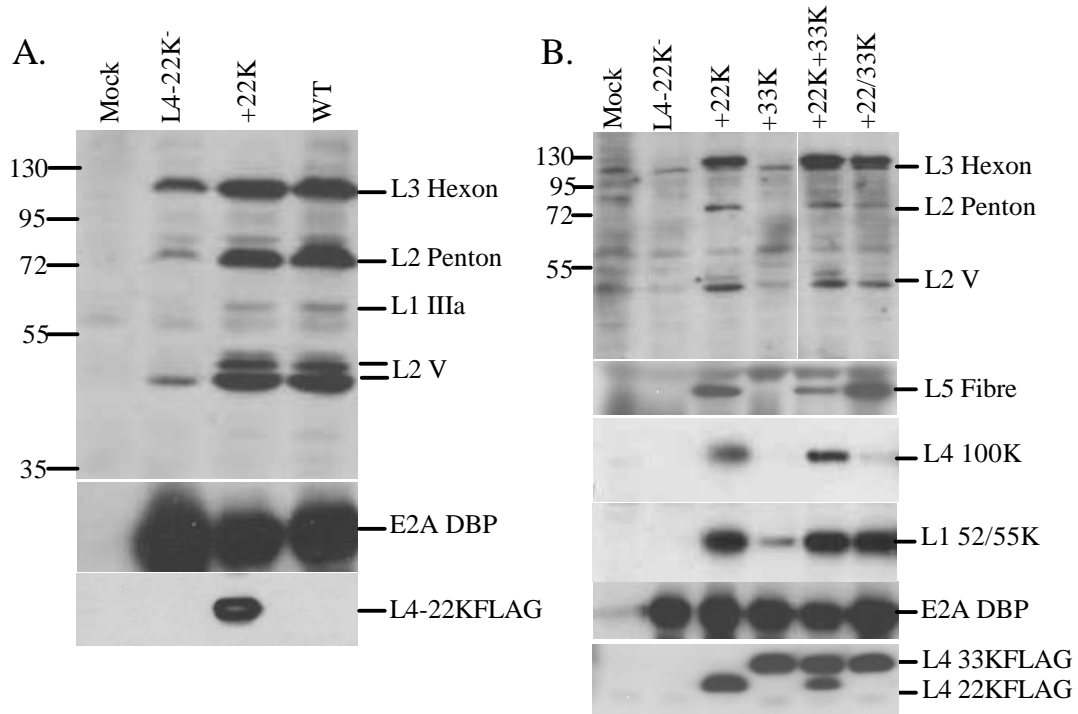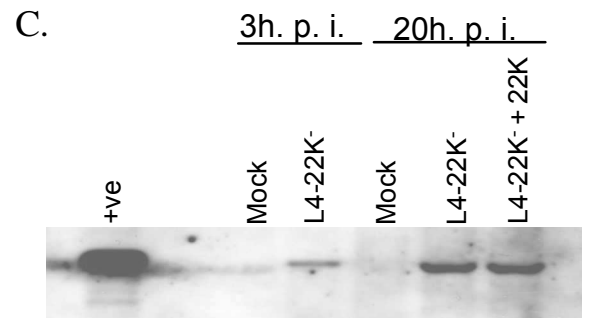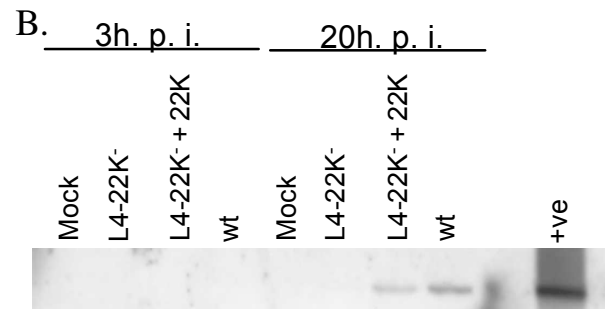
710   (kbp).

Figure 1

Figure 2

Figure 3

A.

Uncut   *Avr*II cut

M -ve L4-22K⁻ wt L4-22K⁻ wt

1.5—
1.0—
0.75—
0.5—

←uncut

←*Avr*II cut

B.   3h. p. i.   20h. p. i.

Mock L4-22K⁻ L4-22K⁻ + 22K wt Mock L4-22K⁻ L4-22K⁻ + 22K wt +ve

C.   3h. p. i.   20h. p. i.

+ve   Mock L4-22K⁻ Mock L4-22K⁻ L4-22K⁻ + 22K

Figure 4

Figure 5

Figure 6

Figure 7