L4-33K, an Adenovirus-encoded Alternative RNA Splicing Factor*

Splicing of the adenovirus IIIa mRNA is subjected to a strict temporal regulation during virus infection such that efficient IIIa 3’ splice site usage is confined to the late phase of the infectious cycle. Here we show that the adenovirus L4-33K protein functions as a virus-encoded RNA splicing factor that preferentially activates splicing of transcripts with a weak 3’ splice site sequence context, a sequence configuration that is shared by many of the late adenovirus 3’ splice sites. Furthermore, we show that L4-33K activates IIIa splicing through the IIIa virus infection-dependent splicing enhancer element (3VDE). This element was previously shown to be the minimal element, both necessary and sufficient, for activation of IIIa splicing in the context of an adenovirus-infected cell. L4-33K stimulates an early step in spliceosome assembly and appears to be the only viral protein necessary to convert a nuclear extract prepared from uninfected HeLa cells to an extract with splicing properties very similar to a nuclear extract prepared from uninfected HeLa cells to an extract with splicing properties very similar to a nuclear extract prepared from adenovirus late-infected cells. Collectively, our results suggest that L4-33K is the key viral protein required to activate the early to late switch in adenovirus major late L1 alternative splicing.

The accumulation of mRNA from the MLTU is subjected to a temporal regulation at the levels of transcription elongation, poly(A) site choice and alternative 3’ splice site selection (reviewed in Ref. 1). Thus, during the early phase of infection the MLP is active at a level comparable with the other early transcription units, whereas the same promoter accounts for most of the transcriptional activity at late times of infection (2, 3). However, at early times transcription initiated at the MLP decreases gradually over a large region beginning after the L1 unit, with few RNA polymerases extending beyond the L3 polyadenylation sequence (4). At late times, this block in elongation is alleviated and transcripts initiated at the MLP continue to the right hand end of the genome. The control of MLTU transcription is further regulated by events taking place at the level of poly(A) and alternative 3’ splice site selection (2, 5, 6). Thus, although nuclear transcription proceeds across at least the L1, L2 and L3 poly(A) sites at early times of infection, only mRNAs from the L1 region accumulate in the cytoplasm. We have further shown that at a transient stage following initiation of DNA replication mRNAs from both the L1 and L4 regions are expressed (7). The significance of this initial burst of L4 mRNA expression has previously not been understood. However, a recent paper by Farley et al. (8) appears to provide an important clue to resolve this mystery. In this paper Leppard and colleagues (8) presented evidence suggesting that the L4-33K protein may play a decisive role in the early to late switch in mRNA expression from the MLTU by stimulating MLTU cytoplasmic mRNA accumulation. The authors suggested that L4-33K most likely exerted its effect at a post-transcriptional level (polyadenylation and/or splicing), although alternative mechanisms such as RNA export or RNA stability could not be excluded.

Here we conclusively show that the L4-33K protein functions as a virus-encoded alternative RNA splicing factor that both in vivo and in vitro regulates alternative splicing. To study its mode of action we used the L1 unit as a model substrate (Fig. 1). In the L1 unit a common 5’ splice site can be joined to two alternative 3’ splice sites, resulting in the formation of the so-called 52,55K (proximal 3’ splice site) or IIIa (distal 3’ splice site) mRNAs (Fig. 1). Early during infection the 52,55K 3’ splice site is exclusively used, whereas the IIIa 3’ splice site is activated in late virus-infected cells (reviewed in Ref. 9). Previous work has identified two cis-acting elements which appear to be critical for the temporal regulation of IIIa 3’ splice site usage (Fig. 1); the 49-nucleotide-long IIIa repressor element (the 3RE, Ref. 10), which binds the SR family of splicing factors, and the 28-nucleotide-long IIIa virus infection-dependent splicing enhancer (the 3VDE, Ref. 11), which up to this study functioned by an unknown mechanism. We have previously shown that the

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2 The abbreviations used are: MLTU, major late transcription unit; MLP, major late promoter; snRNA, small nuclear RNA.

3 The abbreviations used are: MLTU, major late transcription unit; MLP, major late promoter; snRNA, small nuclear RNA.
3VDE is the most critical element conferring the enhanced splicing phenotype of the IIIa pre-mRNA in nuclear extracts prepared from late virus infected cells (Ad-NE). The 3VDE consists of the IIIa branch point sequence, pyrimidine tract, and AG dinucleotide. Current results have suggested that the splicing enhancer activity of the 3VDE may operate through an U2AF-independent mechanism (11, 12). In our working hypothesis we have proposed that a novel factor, of viral and/or cellular origin, the 3VDE interacting factor (3VDF), replaces U2AF as the pyrimidine tract binding factor in late-infected cells (9). Here we show that L4-33K functions as a strong activator of constitutive IIIa splicing in vitro and of L1 alternative splicing both in vivo and in vitro. Most interestingly, this protein alone appears to be sufficient to convert a nuclear extract prepared from uninfected HeLa cells (HeLa-NE) to an extract with splicing properties almost identical to that observed in Ad-NE. Furthermore, we show that the 3VDE is the major cis-acting element responsible for this activation. Collectively, these results suggest that the L4-33K protein is the only viral component of 3VDF.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Transcript Synthesis**—All plasmids encoding for transcripts used in this study have previously been described (see supplemental data for details and supplemental references). Radiolabeled pre-mRNAs were generated by T7 transcription using linearized plasmid DNA or PCR amplified products (for protocols, see Refs. 12 and 13).

Cloning strategies and additional information about plasmid constructed for this project can be found in the supplementary section. Briefly, in pTripL1,L2 the MLP drives synthesis of a precursor RNA consisting of a complete tripartite leader cDNA followed by the genomic sequences encoding the L1 and L2 units. Plasmid pcDNA3-L4-33K contains the Ad5 genomic DNA fragment encoding the L4-33K gene. The L4-33K protein expressed will contain a carboxyl-terminal FLAG epitope tag.

For bacterial expression an Ad5 L4-33K cDNA was inserted into plasmid pET-24a (Novagen) resulting in the production of an L4-33K protein containing a carboxyl-terminal His-tag.

**Purification of Recombinant Histagged Proteins**—Proteins were expressed in Escherichia coli BL21(DE3) (Novagen) and purified under native conditions by standard nickel column chromatography essentially as described by the manufacturer (Novagen) with the following changes. The cell pellet was resuspended in 20 mM Hepes, pH 7.9, 300 mM KCl, 0.05% Triton X-100 with protease inhibitors (complete mini EDTA-free, Roche Applied Science). The packed column was washed in 20 mM Hepes pH 7.9, 300 mM KCl, 0.05% Triton X-100, and 40 mM Imidazole and proteins were eluted with a buffer containing 20 mM Hepes, pH 7.9, 100 mM KCl, and 500 mM imidazole. The eluted proteins were dialyzed against 20 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, pH 8, and 0.5 mM dithiothreitol and stored at −20 °C. The final protein concentration was determined by the Bradford method (14) with bovine serum albumin as a standard.

**In Vitro Splicing**—Nuclear extracts prepared from uninfected (HeLa-NE) or adenovirus infected (Ad-NE) HeLa spinner cells were made as described previously (13). Standard splicing reaction mixtures (12, 13) were incubated at 30 °C for 60–120 min in a total volume of 25 μl with or without 0.75–30 pmol of a recombinant His-tagged L4-33K protein. Splicing products were resolved on 8% denaturing polyacrylamide gels. All splicing reactions were performed multiple times with at least three different batches of HeLa-NE or Ad-NE.

**Spliceosome Assembly**—Standard splicing reactions were set up as described above. Reaction mixtures were incubated at 30 °C for 15–45 min and then mixed with heparin (final concentration: 0.5 μg/ml) and resolved on a native 3.5% (60:1 acrylamide:bisacrylamide) polyacrylamide gel.

**ATP Depletion**—Before addition of transcript to the in vitro splicing reactions 2 mM glucose and 10 units of hexokinase (Roche Applied Science) was added and preincubated for 10 min at 30 °C.

**U2 snRNA Depletion**—Oligonucleotide-directed RNase H cleavage of U2 snRNA in HeLa-NE was performed as described previously (15). The oligonucleotides used were E15 directed against the 5′-end of the U2 snRNA and R55 directed toward the 5′ flanking region.

**Transient Transfection**—Subconfluent monolayers of 293 cells grown on 60-mm plates in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% newborn calf serum, and 100 units/ml penicillin-streptomycin (Invitrogen) were transfected using the FuGENE 6 reagent (Roche Applied...
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Science) with plasmid pTripL1,L2 and increasing amounts of pcDNA3-L4-33K. Cells were harvested 40 h post-transfection and lysed in IsoB-Nonidet P-40 (10 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40) on ice. Cytoplasmic RNA was prepared by phenol/chloroform/isoamylalcohol (25: 24:1) extraction.

L1 Northern Blot Analysis—4 μg of total cytoplasmic RNA were separated on a 1% agarose gel containing 0.4M formaldehyde, transferred to a nitrocellulose filter, and hybridized with the Ad2 pHisD fragment DNA-probe ³²P-labeled by random priming (14).

RESULTS

L4-33K Activates L1 Alternative 3’ Splice Site Selection Both in Vivo and in Vitro—Farley et al. (8) showed that L4-33K activates many, if not all, of the alternative 3’ splice sites present in the MLTU. To investigate whether L4-33K, under our experimental conditions, reproduced the early to late switch in L1 mRNA accumulation we cotransfected 293 cells with plasmid pTripL1,L2 and increasing amounts of pcDNA3-L4-33K. Total cytoplasmic RNA was prepared 40 h post-transfection and L1 mRNA accumulation visualized by Northern blot analysis. As expected from previous results, transfection of the pTripL1,L2 plasmid alone resulted in an exclusive 52,55K mRNA accumulation (Fig. 2A, lane 1). Co-transfection of the L4-33K expressing plasmid, pcDNA3-L4-33K, resulted in a dose-dependent increase in IIIa mRNA accumulation (Fig. 2A, lanes 2–6). Note that this increase in IIIa mRNA accumulation does not occur at the expense of 52,55K mRNA accumulation. Thus, the activation of IIIa splicing does not necessarily result from a shift in L1 3’ splice site usage but rather an activation of IIIa 3’ splice site usage (see “Discussion”).

To directly demonstrate that L4-33K regulates gene expression at the level of alternative splicing we tested the activity of a recombinant L4-33K protein in an in vitro splicing assay. As a substrate we used a mini-52-3a transcript (10). In this transcript the 52,55K 3’ splice site has been brought in close proximity to the two regulatory elements controlling IIIa 3’ splice site usage (3RE and 3VDE; Fig. 1) by a deletion taking out almost the entire 52,55K open reading frame. As shown in Fig. 2B, incubation of the mini-52-3a transcript in HeLa cell nuclear extracts (HeLa-NE) resulted in an exclusive 52,55K 3’ splice site usage (lane 2). Complementing the HeLa-NE with the recombinant L4-33K protein resulted in a significant activation of distal IIIa 3’ splice site usage (lane 3). This activation was concentration-dependent and reproduced in multiple experiments (data not shown). It is interesting to note that the shift in L1 alternative splicing, although relatively weak, was essentially as efficient as that observed in nuclear extracts prepared from adenovirus late infected cells (Ad-NE) (lane 1). Based on this result, we conclude that L4-33K indeed functions at the level of alternative splicing we tested the activity of a recombinant L4-33K protein in an in vitro assay with

FIGURE 2. L4-33K is a virus-encoded alternative splicing factor. A, 293 cells were cotransfected with 2 μg of pTripL1,L2 and increasing amounts of plasmid pcDNA3-L4-33K. Total cytoplasmic RNA was isolated 40 h post-transfection and the L1 mRNAs visualized by Northern blotting. B, L4-33K activates mini-52,3a alternative splicing in vitro. HeLa-NE was supplemented with 12 pmol of a recombinant L4-33K protein, and the IIIa splicing activation profile is compared with Ad-NE.
transcripts with activation levels reaching up to 40-fold in many experiments whereas the weakly activated transcripts were stimulated between 2- and 5-fold.

In general, the results suggest a reasonably good correlation between the strength of the 3’/H11032 splice site and L4-33K responsiveness, with strong 3’/H11032 splice sites containing long pyrimidine tracts being non-responsive and weak 3’ splice sites with non-consensus type of pyrimidine tracts being highly or weakly activated by L4-33K (Fig. 3B; see also “Discussion”). The results further support our conclusion (Fig. 2) that L4-33K activates splicing through a mechanism that operates via the strength of the 3’ splice site. Thus, transcripts 52,55K, IIla, penton, pV, pVII, hexon, and 100K, which show a great variation in their responsiveness to L4-33K, all have the same first exon and 5’ splice site and differ from each other in the composition of the 3’ splice site region.

**The 3VDE Is the Major Element Mediating L4-33K Activation of Splicing**—Next, we analyzed the effect of L4-33K on IIla pre-mRNA splicing in more detail. As shown in Fig. 4B, addition of increasing amounts of the recombinant L4-33K protein to HeLa-NE resulted in a dose-dependent increase in IIla splicing with a maximum level of splicing reached around 6–12 pmol of L4-33K (lanes 5 and 6). Interestingly, at these concentrations the level of IIla splicing was similar to what we typically observe in Ad-NE (lane 1). To map the critical element(s) responsible for the splicing enhancer activity of L4-33K we added a fixed amount of recombinant L4-33K protein to HeLa-NE and resolved the splicing products by electrophoresis and visualized by autoradiography.

**FIGURE 3.** L4-33K activates splicing of pre-mRNAs with weak 3’ splice site content. A battery of viral and non-viral pre-mRNAs were tested in HeLa-NE supplemented with 12 pmol of recombinant L4-33K. The 3’ splice site sequences are schematically shown in A, and the effect of L4-33K on transcript splicing is shown in B. The position of spliced mRNAs are indicated by boxes.

**FIGURE 4.** The 3VDE is the critical element in the IIla pre-mRNA conferring the L4-33K splicing enhancer activity. A, schematic representation of the IIla and β-globin hybrid transcripts used. B, dose-dependent activation of IIla splicing by L4-33K in HeLa-NE. C, the indicated pre-mRNAs were incubated in HeLa-NE with or without 12 pmol of L4-33K and products resolved by electrophoresis and visualized by autoradiography. D, time course experiment to show that the non-responsiveness of transcript IIla (Δ3RE,3VDE) is not due to saturation of splicing.
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FIGURE 5. 3VDE is sufficient to convert the rabbit β-globin pre-mRNA to a splicing substrate activated by L4-33K. A, schematic representation of the IIIa and β-globin (glob) hybrid transcripts used to map the minimal L4-33K responsive element. B, the indicated pre-mRNAs were incubated in HeLa-NE with or without 12 pmol L4-33K and products resolved by electrophoresis and visualized by autoradiography.

represses IIIa splicing by binding the SR family of splicing factors (10). Transcript IIIa (Δ3RE) was activated to higher levels compared with the wild type IIIa transcript by L4-33K (lanes 2 and 4). In agreement with our previous results (11), replacing the 3VDE with the corresponding element from β-globin also resulted in a significant increase in basal IIIa (Δ3VDE) splicing (compare lanes 1 and 5). The IIIa (Δ3VDE) transcript was also activated by L4-33K (lane 6), although not to the same extent as IIIa (Δ3RE). A double mutant (IIIa (Δ3RE,3VDE), lanes 7 and 8) showed, as expected, an even higher basal splicing activity in HeLa-NE, an activity that was not stimulated by L4-33K. To examine whether the failure of the L4-33K protein to stimulate IIIa (Δ3RE,3VDE) splicing was due to a saturation of splicing we studied the IIIa (Δ3RE,3VDE) transcript in a time course experiment. As shown in Fig. 4D, the kinetic and efficiency of IIIa (Δ3RE,3VDE) splicing was very similar with (lanes 7–12) or without (lanes 1–6) optimal amounts of the recombinant L4-33K protein. Taken together, these results suggest that the 3VDE is the major element mediating L4-33K activation of splicing, with a smaller contribution made by the 3RE.

To test this hypothesis further we used chimeric transcripts where the 3RE and the 3VDE were used to replace the corresponding sequences in the β-globin pre-mRNA (Fig. 5A). As expected from our previous results transfer of the 3RE (Fig. 5B, lane 3) or the 3VDE (Fig. 5B, lane 5) to β-globin resulted in a dramatic reduction in basal β-globin splicing (10, 11). However, as shown in Fig. 5B, addition of L4-33K to either of these transcripts resulted in an activation of β-globin splicing (lanes 4 and 6). In agreement with the conclusion drawn from the IIIa hybrid constructs (Fig. 4) L4-33K activated glob (3VDE) (lane 6) more efficiently than glob (3RE) (lane 4), reinforcing the conclusion that the 3VDE is the major element mediating L4-33K activation of splicing in vitro. Transfer of both 3RE and 3VDE to β-globin (lane 7) resulted, as expected (11), in a strong repression of basal splicing activity. However, this transcript was activated by L4-33K (lane 8), although its induced activity was very low.

L4-33K Stimulates Spliceosome Assembly—To gain additional support that L4-33K activates splicing directly, we analyzed spliceosome assembly in HeLa-NE supplemented with the recombinant L4-33K protein. As shown in Fig. 6A, incubation of the IIIa pre-mRNA in HeLa-NE supplemented with the L4-33K protein resulted in a dramatic increase in pre-spliceosomal A complex formation (compare lanes 1 and 3). Slower migrating species corresponding to spliceosomal complexes B and C were not readily discernible except possibly in the sample supplemented with L4-33K (lane 3). This finding was not unexpected, since we have previously noted that the B and C complexes are very unstable on this transcript and difficult to resolve by native gel electrophoresis (18). The accumulation of the A complex was dependent on spliceosome assembly, since U2 snRNA depletion by oligonucleotide-directed RNase H cleavage abolished complex formation (lanes 2 and 4). Furthermore, depleting HeLa-NE of ATP also eliminated A complex formation (Fig. 6B, lane 2), while readdition of ATP to the depleted extract restored spliceosomal A complex formation (Fig. 6B, lane 3). Collectively, these results provide strong sup-

FIGURE 6. L4-33K stimulates pre-spliceosome formation. A, Pretreatment of HeLa-NE with an oligonucleotide directed against the 5′-end of U2 snRNA effectively blocked A-complex formation. B, formation of the A-complex is ATP-dependent. Complexes were resolved on a native polyacrylamide gel and visualized by autoradiography.
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port for the conclusion that L4-33K stimulates IIIa mRNA splicing by enhancing an early step in spliceosome assembly.

The RS Peptides Are Required for the L4-33K Splicing Enhancer Activity——To map functional regions of L4-33K deletion mutants were constructed in which different parts of the N terminus were deleted (Fig. 7A). As shown in Fig. 7B, a deletion removing the acidic amino terminus did not have any negative effects on the splicing enhancer activity of the protein (Δ1–55; lane 3). A further truncation removing the 106 amino acids encoded by the L4-33K first exon reduced the activity of the protein to ~30% of wild type (Δ1–106; lane 4). A further truncation preserving only the conserved carboxyl-terminus of the protein still retained ~30% of the splicing enhancer activity of the wild type protein (Δ1–157; lane 5). Collectively these results show that the 157 amino-terminal amino acids are not essential for the splicing enhancer activity of L4-33K. However, the region spanning amino acid 55–106 appears to encode a signal that augment the activity of the protein.

During the cloning of a cDNA for the L4-33K mRNA we found a novel mRNA generated by a double splicing event. The novel intron is flanked by authentic 5’ and 3’ splicing signals and appears to be a bona fide authentic intron. However, a quantitation of the expression levels of the double spliced L4-33K mRNA suggests that it is a rare mRNA that most likely is not crucial for virus growth, at least not during virus growth in tissue culture cells (supplemental Fig. 1). The novel splicing event causes an in-frame deletion of 27 amino acids in the carboxyl-terminal part of the L4-33K protein (Fig. 7A). We refer to this new protein variant as the L4-33Kds protein. As shown in Fig. 7C, L4-33Kds was completely unable to stimulate IIIa splicing (lane 9), suggesting that this protein domain is essential for the splicing enhancer activity of L4-33K. Interestingly, this protein domain encodes for three RS and one SR dipeptide (Fig. 7A). Such protein motifs have previously been characterized as signature modules for cellular splicing factors or splicing related factors (19, 20). However, L4-33K does not complement splicing in S100 extracts, suggesting that it is not a classical SR protein (data not shown).

To examine whether the RS motifs were required for the IIIa splicing enhancer activity of L4-33K we constructed a collection of serine to glycine substitution mutants of L4-33K (Fig. 7A). As shown in Fig. 7C, mutating serine 176, 189, or 196 individually did not have any adverse effect on the L4-33K splicing enhancer activity (lanes 3, 4, and 6). However, mutating serine 192 completely annulled the splicing enhancer phenotype of L4-33K (lane 5). Interestingly, combining serine mutations that individually did not show a phenotype created proteins with severe splicing defects. Thus, a triple mutant changing serines 176, 189, and 196 (lane 7) or a double mutant changing serines 176 and 189 (lane 8) were essentially inactive as IIIa splicing enhancer proteins. Taken together these results are compatible with the conclusion that the RS dipeptide motifs in L4-33K may be significant for activity with serine 192 being essential for activity and serines 176, 189, and 196 serving redundant functions.

DISCUSSION

Here we show that the adenovirus L4-33K protein functions as a virus-encoded alternative splicing factor, selectively enhancing splicing of transcripts containing weak 3’ splice sites. L4-33K activates IIIa splicing mainly through the 3VDE element, which we previously have shown is the minimal element, both necessary and sufficient, for activating splicing in Ad-NE (11). The results presented suggest that L4-33K is the only viral protein necessary to mediate the temporal activation of major late pre-mRNA splicing observed at late times of infection.

A comparison of the splicing efficiency of the different transcripts used in this study gives a clear indication that transcripts with a high basal activity in HeLa-NE (AdML, penton, 100K, globin, and Ftz) are not stimulated by L4-33K (Fig. 3B). This finding is also reproduced at the level of sequence features surrounding the 3’ splice site (Fig. 3A). Thus, the common feature of the highly and weakly activated 3’ splice sites is that they have a very low basal activity in HeLa-NE in the absence of L4-33K. Generally, these transcripts also have poor pyrimidine tracts that have been shown (IIIa and pV, Ref. 17) or are expected to bind U2AF inefficiently. In contrast, all the non-responsive transcripts have polypyrimidine tracts that would be predicted to bind U2AF efficiently. The notable exception is the 52,55K 3’ splice site, which has an extended pyrimidine tract that is interrupted by one G residue (Fig. 3A) and binds U2AF with an

FIGURE 7. Mutational analysis of L4-33K. A, schematic figure showing different motifs in the L4-33K protein with an expansion of the 27 carboxyl-terminal amino acids deleted in the L4-33Kds protein. The tiny RS repeat is boxed in the sequence. The complete amino acid sequence is shown in supplemental Fig. 2. B, the indicated L4-33K mutant proteins were incubated under splicing conditions in HeLa-NE programmed with the wild type IIIa transcript. Splicing products were resolved by gel electrophoresis and visualized by autoradiography.
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~10-fold higher efficiency compared with the IIIa 3’ splice site (17). This transcript has a moderate basal activity and is weakly stimulated by L4-33K (Fig. 3A).

Here we show that the major IIIa cis-acting element mediating L4-33K activation of splicing is the 3VDE (Figs. 4 and 5). This finding should be considered together with our previous observation that the critical sequence within the 3VDE-mediating enhanced IIIa splicing in Ad-NE is the IIIa pyrimidine tract (11), a finding that suggests that a factor must interact with the IIIa pyrimidine tract to stimulate IIIa splicing. Collectively, our results strongly argue that the L4-33K protein is 3VDF or at least the central viral component in a hypothetical multiprotein complex enhancing splicing through the 3VDE.

Our preliminary results suggest that L4-33K does not bind directly to the 3VDE (data not shown). Thus, it is possible that 3VDF is a complex consisting of an alternative cellular splicing factor that makes the direct contact with the 3VDE and indirectly recruits L4-33K to the 3VDE. Alternatively, L4-33K may function by a hit and run mechanism and transiently interact and post-translationally modify a cellular factor that stimulates IIIa splicing. The next step will be to identify L4-33K interacting partners and to determine whether L4-33K is a stable component of the early spliceosome. Irrespective of the outcome of these mechanistic studies it appears clear that L4-33K is the only viral protein needed to convert a HeLa-NE to an extract with essentially the same splicing properties as an extract prepared from adenovirus late-infected cells.

It may seem unexpected that activation of the late pattern of L1 alternative splicing does not result in a shift from 52,55K to IIIa mRNA splicing (Fig. 2, A and B). Thus, addition of L4-33K in vivo or in vitro results in a dose-dependent increase in IIIa mRNA production without a concomitant decrease in 52,55K splicing (Fig. 2). At a first glance this finding may seem strange. However, this result is not an artifact of our in vivo and in vitro splicing systems. The same result has also been observed in the context of an adenovirus infection. In an interesting study Gustin and Imperiale (21) constructed an adenovirus mutant defective in 52,55K protein expression. In this work they introduced point mutations in the 52,55K reading frame changing amino acid 18, 20, and 21 into stop codons. Clearly these mutations must have ablated an exonic splicing enhancer required for 52,55K mRNA splicing, since this mRNA was not expressed during infection. Despite the absence of 52,55K mRNA splicing this mutant virus exhibited a normal temporal activation of IIIa mRNA splicing. Thus, the temporal shift in L1 alternative splicing is not critically dependent on a cis competition between the 52,55K and IIIa 3’ splice sites. In support of such a conclusion we have previously shown that the regulated activation of IIIa splicing does not require cis competition in vitro (22). Also, our previous results have suggested that IIIa splicing is actively repressed in early infected cells by SR proteins binding to the 3RE (10), a repression that is relieved at the late phase of infection by a virus-induced dephosphorylation of SR proteins (23, 24). Furthermore, the 3VDE, which binds U2AF inefficiently (11, 12), adds to the low activity of IIIa splicing at early times of infection.

A reasonable explanation for a lack of a switch in 3’ splice site usage in L1 alternative splicing is that the 52,55K and IIIa 3’ splice sites appear to show a difference in U2AF requirement. Thus, we have shown that the 52,55K 3’ splice site, which has an extended pyrimidine tract, requires U2AF for activity (12). In contrast, the IIIa 3’ splice site, which binds U2AF inefficiently (17), appears to be spliced by an U2AF-independent pathway (11, 12). Thus, in the context of the L1 unit, two alternative pathways for 3’ splice site recognition coexist: 52,55K splicing being activated by the normal U2AF pathway and IIIa splicing activated by the L4-33K protein-induced 3VDF pathway.

As much as 157 amino-terminal amino acids of L4-33K could be deleted without a dramatic reduction in the splicing enhancer activity of the protein (reduced to ~30%; Fig. 7B). This part of the protein contains a conspicuous long stretch of acidic amino acids (Fig. 7A and supplemental Fig. 2). Although the precise amino acid sequence is not conserved between L4-33K proteins from different serotypes, the acidic nature of the amino terminus appears to be a conserved feature in human and animal L4-33K proteins. The L4-33K protein also contains a central region with two alanine stretches that could serve as hinge regions separating two protein domains that may have different functions. As we show here the carboxyl-terminus of the protein functions as a splicing enhancer domain. The amino terminus may theoretically function in other aspects of the viral life cycle (for an example, see Ref. 25).

L4-33K contains a tiny RS repeat (Fig. 7A). We show that mutating the four serines in this motif results in a severe impairment of the IIIa splicing enhancer activity of the protein. Mutating serine 192 abolished this motif in vivo and in vitro results in a severe impairment of the L4-33K splicing enhancer activity, whereas individual mutations affecting the other serines did not have any adverse effects on splicing. In contrast, a triple mutant changing serines 176, 189, and 196 was defective, suggesting that they may serve redundant functions in splicing. It has previously been shown that RS dipeptide repeats may serve redundant functions in SR proteins and SR-related splicing factors.

L4-33K has previously been shown to be one of the major phosphoproteins produced during a lytic infection (26–28). However, SR protein kinases CLK/Sty or SRPK1 did not phosphorylate L4-33K in vitro (data not shown). Thus, we need to establish that the RS motif in L4-33K is phosphorylated in vivo and also determine whether phosphorylation of this motif is of functional significance. Since L4-33K does not appear to bind RNA, and furthermore, the length and position of the RS motif is atypical for an SR protein, it did not come as a surprise that the protein did not complement splicing in S100 extracts (data not shown). However, it remains possible that L4-33K is an SR-related splicing factor. Such proteins have been shown to play an auxiliary role in splicing mediating important protein-protein interactions with other SR-related splicing factors (19, 20). Alternatively, the observed RS motifs may be haphazard. Since the carboxyl terminus of L4-33K is the most conserved region in the L4-33K protein (Fig. 7A), the positioning of serines may play an important function in L4-33K-mediated splicing enhancement unrelated to their location in RS motifs.

Collectively available evidence suggests that L4-33K, like many proteins encoded by viruses, is multifunctional and partake at several steps in the virus life cycle. Thus, the earliest studies suggested that L4-33K was required primarily for virus
assembly. In one study a stop codon was introduced converting amino acid 20 from tryptophan to an amber stop codon (29). This mutant showed no obvious defect in late mRNA and protein expression. The failure to detect a defect in mRNA expression in this study most likely resulted from a leaky reinitiation of translation resulting in the synthesis of an amino-terminal truncated L4-33K protein. Such a conclusion is supported by our observation that the amino terminus of L4-33K is not essential for L4-33K activity as a splicing enhancer protein (Fig. 7B). The second paper introduced two tandem translational stop codons in the conserved carboxy-terminal part of L4-33K (30). This mutation was lethal. The authors concluded that the mutation compromised new virus particle formation. However, it was not conclusively shown that the lack of L4-33K expression did not result in a defect in late viral mRNA or protein expression.

In summary, available data suggest that L4-33K, in addition to functioning as an alternative RNA splicing factor, also may serve a direct function in virus assembly and potentially additional steps in virus replication.

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