Temporal regulation of adenovirus major late alternative RNA splicing

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1. ABSTRACT

Adenovirus makes extensive use of alternative RNA splicing to produce a complex set of spliced mRNAs during replication. The accumulation of viral mRNAs is subjected to a temporal regulation, a mechanism that ensures that proteins that are needed at certain stages of the virus life cycle are produced in a timely fashion. The complex interactions between the virus and the host cell RNA splicing machinery have been studied in detail during the last decade. These studies have resulted in the characterization of two viral proteins, E4-ORF4 and L4-33K, that adenovirus uses to remodel the host cell RNA splicing machinery. Here I will review the current knowledge of how mRNA expression from the adenovirus major late transcription unit is controlled with a particular emphasis on how cis-acting sequence element, trans-acting factors and mechanisms regulating adenovirus major late L1 alternative RNA splicing is controlled.

2. THE DISCOVERY OF SPLIT GENES AND RNA SPLICING

During spring 1977 several groups working at the Cold Spring Harbor Laboratory and Massachusetts Institute of Technology produced results that were difficult to explain using the accepted scientific models. The data they obtained suggested that mRNAs encoded from the adenovirus major late transcription unit (MLTU) were not collinear with the viral genome. Instead, the results showed that the mRNA sequences were derived from several discontinuous segments on the virus chromosome (1-5). The results were first presented to a larger scientific audience at the Cold Spring Harbor Symposium on “Chromatin” in May 1977. The description of the split gene concept represented a major turning point in scientific thinking. Clearly many scientists working in many model systems must have had similar hard to explain results that immediately became understandable once the new model
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Figure 1. The adenovirus type 2 transcription map. Arrows illustrate the structure of mRNAs expressed at different times of infection. Red arrows mRNAs expressed early; thin black arrows, mRNAs expressed at intermediate times; and yellow arrows, mRNAs expressed late during infection. Some of the more abundant polypeptides that have been assigned to the different transcription units are indicated. The two viral proteins discussed in more detail in this review are shown encircled. Figure adapted from references (7, 66).

had been presented. Therefore it did not take long before the split gene and RNA splicing concept was shown to be the rule rather than an exception. In fact, when the symposium book was printed several research groups had been able to supplement their chapter with data showing the existence of introns in their experimental systems. Today we know that essentially all human genes contain introns. The average human gene contains around 7 introns, which make up approximately 90% of the precursor RNA (pre-mRNA). Introns typically interrupt the protein-coding portion in our genes thereby creating small protein modules that are encoded by individual exons. In contrast, adenovirus genes contain few introns, typically one to three. Further, in adenovirus the vast majority of introns are positioned in the 5' non-coding portion of the pre-mRNA. There are only a handful of adenovirus genes that have an intron interrupting the gene itself: E1A, IVa2, L4-33K, E4-ORF6/7 plus a few minor species of unknown function.

After the initial discovery of split genes the progress was rapid. For example, it did not take long until it was shown that not only are adenovirus genes spliced, but the majority of the transcription units produces multiple alternatively spliced mRNAs in a temporally regulated process (6, 7) (Figure 1), requiring late viral protein synthesis (8, 9). The potential to produce multiple alternatively spliced mRNAs in a regulated process is of great importance since it allows the virus to control the synthesis of proteins that are needed at the different stages of the virus life cycle (Figure 1).

3. THE SCOPE OF THIS ARTICLE

Previous review articles have summarized what is known about the post transcriptional control of adenovirus gene expression (10) and the mechanisms controlling adenovirus E1A and L1 alternative splicing (11). Here I will focus on the advances in our understanding of factors and mechanisms controlling adenovirus major late gene expression.

4. THE ADENOVIRUS MAJOR LATE TRANSCRIPTION UNIT

Late after infection transcription initiates predominantly from the major late promoter (MLP), which is located at map unit 16.8. (Figure 1). This promoter accounts for approximately 30% of total RNA synthesis at late times of infection. This does not necessarily mean that the MLP is a strong promoter since maximal transcription occurs under conditions when infected cells contain more than 100 000 copies of viral DNA. In fact, electron microscopic studies have suggested that each DNA template that is actively transcribed only has four nascent RNA polymerases distributed over the entire transcription unit (12). The MLTU generates a primary transcript that is
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**Figure 2.** Pathway showing the temporal activation of the MLTU during a lytic virus infection. The thickness of lines and boxes indicate increase in activity. See text for further details. The figure is adapted from (20).

around 28,000 nucleotides long. Approximately 80% of the RNA polymerases that initiate transcription at the MLP terminate prematurely generating short promoter proximal transcripts less than 800 nucleotides in length (13). The primary transcript becomes polyadenylated at one of five positions along the MLTU, generating five families of mRNAs with coterminous 3' ends (L1 to L5, Figure 1). Poly (A) site selection usually precedes splicing but is not an absolute requirement (14). Following selection of the poly (A) site the transcript is spliced in such a way that around 20 mature mRNAs are produced, each containing a common 201-nucleotide tripartite leader sequence at their 5' end (Figure 1). The tripartite leader does not contain a translational start codon and all mRNAs generated from the MLTU use the first AUG present in the mRNA body for translation initiation. The tripartite leader has been shown to have a crucial function as a translational enhancer late after infection (15). This leader comes in two variants, either with or without the 440-nucleotide i-leader exon (Figure 1). Inclusion of the i-leader exon is temporally regulated. Thus, splicing of MLTU mRNAs at early times of infection usually leads to the inclusion of the i-leader exon whereas the mRNAs expressed at late times contain the classical tripartite leader (for a review see reference 10). Efficient removal of the i-leader requires late viral protein synthesis (16). Two viral proteins, E4-ORF3 and E4-ORF6 have been shown control i-leader exon inclusion/skipping in transient transfection assays (17). The E4-ORF3 protein promotes i-leader inclusion whereas the E4-ORF6 protein functions as an exon skipping factor.

4.1. The temporal shift in major late L1 to L5 mRNA expression

Although the MLTU is maximally expressed at the late stage of the infectious cycle the MLP is also active at early times of infection. During the early phase this promoter is active at comparable levels to other early viral promoters (18). However, at early times transcription terminates gradually over a large distance (40 to 75 map units) of the MLTU (19). The L1 poly (A) site is preferentially used at this stage (reviewed in reference 10). Thus, although transcription proceeds across the L1, L2 and L3 poly (A) sites only mRNAs from region L1 accumulate as cytoplasmic mRNA (Figs. 1 and 2) (7-9). At a transient stage soon after the start of viral DNA replication mRNAs from regions L1 and L4 are selectively expressed (Figure 2) (20). This RNA phenotype appears to reflect the activity of the MLTU at an intermediate stage where viral DNA synthesis has commenced without late viral protein synthesis. The activation of the full set of L2 to L5 families of mRNAs requires late viral protein synthesis. The results were interpreted to indicate that the L4 region encodes for regulatory proteins that may control RNA splicing and mRNA accumulation from the MLTU (see section 5.4.).

5. REGULATION OF L1 ALTERNATIVE RNA SPlicing

The L1 unit represents an example of an alternatively spliced gene where the last intron is spliced using a common 5' splice site and two alternative 3' splice sites, generating two predominant cytoplasmic mRNAs; the 52.55K (proximal 3' splice site) and the IIIa (distal 3' splice site) mRNAs, respectively (Figure 3). The pattern of L1 pre-mRNA splicing is regulated during the infectious cycle with the 52.55K mRNA produced both early and late, and the IIIa mRNA produced exclusively at late times of infection (7-9).

Although IIIa mRNA splicing is a predominantly late event, the IIIa 3' splice site is also active in transient transfection experiments (21), in vitro in nuclear extracts.
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Figure 3. Schematic drawing showing the splicing pattern of the L1 pre-mRNA at early and late times of infection. Below, the nucleotide sequence of the IIIa 3' splice site, with the position of the 3RE and 3VDE indicated. Asterisks indicate the two A residues used as branch sites in IIIa splicing. Figure adapted from (11).

It is interesting to note that although L1 alternative splicing is subjected to a tight temporal control during infection the absence of 52,55K expression at early times (23) or expression of the IIIa protein from the start of infection (32) does not have a detrimental effect on virus growth in tissue culture cells. The absence of 52,55K protein expression resulted in a 30 to 40% reduction in total late mRNA expression at 18 hours post infection, a difference that was not apparent at later times. The 52,55K protein appears to function as a protein stabilizing association of viral DNA with empty capsids (23). Overexpression of the IIIa protein, from the start of infection, results in a slight (2 to 5-fold) decrease in late protein expression (32). This study also showed that the IIIa protein may partake in an auto-regulatory loop that amplifies IIIa mRNA production by inhibiting 52,55K mRNA splicing, late after infection. It is conceivable that the lack of 52,55K expression, or overexpressing the IIIa protein may exert a more profound effect in vivo when adenovirus has to grow in humans.

5.1. Two cis-acting element regulates IIIa splicing

IIIa splicing is tightly regulated by two cis-acting viral elements (Figure 3): the IIIa repressor element (3RE), and the IIIa virus-infection dependent splicing enhancer (3VDE). In the following sections the current views on how the 3RE and the 3VDE contribute to the regulated expression of the IIIa mRNA in adenovirus-infected cells will be summarized.

5.1.1. Function of the 3RE

The 3RE functions as a IIIa splicing repressor element, by binding the SR family of cellular splicing factors (33). SR proteins are essential splicing factors required at multiple steps in constitutive splicing and functioning as regulators of alternative splicing (reviewed in reference 34). Typically, SR proteins bind to exonic splicing enhancer elements and help to initiate spliceosome
assembly, by recruiting U1 snRNP and U2AF to the 5' and 3' splice sites, respectively. The 3RE is a 49 nucleotide long RNA segment that binds all tested members of the SR family of splicing factors (33). It is located immediately upstream of the IIIa branch site (Figure 3), and SR protein binding to the 3RE inhibits IIIa splicing by blocking U2 snRNP recruitment to the IIIa branch site (33). The absence of IIIa splicing in early virus-infected cells can therefore, in part be explained by a direct inhibitory effect of the cellular SR proteins on IIIa 3' splice site usage. Interestingly, moving the 3RE from its natural intronic position to the IIIa second exon splicing enhancer element located around 50-60 nucleotides downstream of the 3' splice site (23). Collectively, our results have suggested that the activity of SR proteins is position dependent. If they bind to an intron they repress splicing whereas whey activate splicing from an exonic position.

One SR protein, ASF/SF2, has been studied in more detail. The splicing enhancer and splicing repressor functions of ASF/SF2 can be separated and attributed to distinct domains in the protein. The RS domain functions as the splicing enhancer domain (reviewed in reference 35), whereas the second RNA recognition motif (RRM2) encodes for the splicing repressor function (36). The repressor activity of RRM2 appears to be a general effect since tethering ASF/SF2-RRM2 at an intronic position in the rabbit b-globin pre-mRNA similarly resulted in splicing repression.

SR proteins binding to the 3RE does not block IIIa splicing by stericly hindering U2 snRNP recruitment to the IIIa branch site. Thus, tethering a bulky protein, exceeding the size of ASF/SF2, at the position of the 3RE did not result in splicing repression, whereas tethering of ASF/SF2-RRM2 alone was sufficient to reproduce the splicing repressor phenotype (36). Further, a heptapeptide motif, SWQDLKD, which is completely conserved in SR proteins with two RRM2s was shown to be essential for activity of the repressor domain (36).

In the context of mini-gene constructs where the distance between the 52,55K and the IIIa 3' splice sites has been shortened to 80 nucleotides the 3RE functions simultaneously as a splicing repressor element for IIIa splicing and a classical second exon splicing enhancer for 52,55K splicing (33). However, in the authentic L1 unit the 3RE is too far away (approximately 1200 nucleotides) and does not function as a 52,55K splicing enhancer element. Instead, it appears that the 52,55K 3' splice site has its own second exon splicing enhancer element located around 50-60 nucleotides downstream of the 3' splice site (23).

### 5.1.2. Function of the 3VDE

Although the 3RE plays a defined role in IIIa 3' splice site activation several lines of evidence has suggested that the 3RE is not the primary element controlling IIIa pre-mRNA splicing. For example, a limited mutational analysis of the weak IIIa 3' splice site showed that the short IIIa pyrimidine tract is the critical element required for enhanced splicing in Ad-NE (25, 37). The IIIa 3' splice site does not bind the general splicing factor U2AF efficiently (24, 25, 37). U2AF bind with a strong preference to long pyrimidine rich sequences (38, 39). In general, splicing of pre-mRNAs that contain long prototypical polypyrimidine tracts, and bind U2AF efficiently, is repressed in Ad-NE, while splicing of 3' splice sites, which have a weak sequence context, are enhanced in Ad-NE (24, 37).

In vitro, IIIa pre-mRNA splicing is activated dramatically in Ad-NE, whereas b-globin splicing is slightly repressed (25). By analysis of chimeric transcripts between IIIa and b-globin, the IIIa branch site/polypyrimidine tract (Figure 3) was shown to be the key element controlling IIIa splicing. This element functions as a “Janus element”; it blocks splicing in HeLa-NE and functions as a splicing enhancer selectively in Ad-NE. Because of these properties this sequence element was named the IIIa virus-infection dependent splicing enhancer (3VDE). The 3VDE is essential for regulated IIIa pre-mRNA splicing and sufficient to convert a heterologous transcript from a pre-mRNA that is repressed to a pre-mRNA that is activated in Ad-NE (25).

### 5.2. Relaxed requirement for U2AF in adenovirus infected cells

U2AF has been characterized as an essential splicing factor (reviewed in reference 40). U2AF is a heterodimer consisting of a 65K and 35K subunit. The U2AF binds specifically to the pyrimidine tract whereas U2AF makes contact with the 3' splice site AG dinucleotide (41, 42). Introns with weak pyrimidine tracts that bind U2AF inefficiently require the U2AF subunit interaction with the 3' splice site AG to be functional, so-called AG-dependent introns. In contrast, 3' splice sites with strong pyrimidine tracts can splice without the contribution of U2AF interaction with the 3' splice site AG, so-called AG-independent introns. Recent results have shown that U2AF appears to be completely dispensable for splicing in Ad-NE. Thus, the IIIa intron, which is an AG-dependent intron in HeLa-NE become AG-independent in Ad-NE (24). In fact, splicing of the IgM pre-mRNA, which like the IIIa 3' splice site is characterized as weak with a short polypyrimidine tract, undergoes the first catalytic step of splicing in U2AF depleted Ad-NE (24). Collectively, these results indicate that a novel U2AF-independent pathway of splicesome assembly is operational in late adenovirus-infected cells. This pathway selectively favor splicing of 3' splice sites with a weak sequence context, a feature typical of many of the late adenoviral introns.

The mechanism by which the 3VDE activates splicing is still not entirely resolved. Current experimental evidence is compatible with the model that splicing activation by the 3VDE operates through a novel mechanism that may be U2AF independent, or alternatively does not require stable binding of U2AF to the IIIa pyrimidine tract. However, U2 snRNP is required for 3VDE function (25). In our working hypothesis we predict that a yet to be characterized factor, the 3VDE interacting factor, 3VDF, replaces U2AF as the pyrimidine tract recognition factor, and aids in the recruitment of U2 snRNP to the IIIa branch site. New data from our laboratory suggests that the viral L4-33K protein may be the viral component of 3VDF (see section 5.4.).
5.3. E4-ORF4 and SR protein dephosphorylation

SR proteins exist in cells as highly phosphorylated proteins that are subjected to regulated reversible phosphorylation (reviewed in reference 43). Thus, phosphorylated SR proteins are needed for spliceosome assembly whereas SR protein dephosphorylation appears to be required during splicing catalysis (44). Activation of IIIa splicing in Ad-NE is accompanied by a virus-induced partial dephosphorylation of SR proteins (45). This SR protein dephosphorylation renders them non-functional as splicing enhancer, or splicing repressor proteins. Importantly, re-phosphorylation of SR proteins isolated from late adenovirus-infected cells restores their activity as splicing repressor proteins (46). Since SR proteins are essential for generic pre-mRNA splicing (47, 48), this virus-induced dephosphorylation results in an inhibition of splicing of pre-mRNAs with consensus-type of splicing signals (45). As such this mechanism may contribute to the virus-induced inhibition of host cell gene expression seen late after infection.

The adenovirus E4-ORF4 protein is a multifunctional viral regulator that has been shown to block E1A induced transcription activation (49-51), induce hypo-phosphorylation of various viral and cellular proteins (45, 49), and regulate adenovirus alternative RNA splicing (45). It also induces p53-independent apoptosis selectively in transformed cells (52, 53), and G2/M arrest in yeast and mammalian cells (54). Current data suggests that E4-ORF4 exerts these multiple activities by binding to the cellular serine/threonine specific protein phosphatase 2A (PP2A) (reviewed in reference 55). The E4-ORF4 protein activates IIIa splicing by inducing SR protein dephosphorylation (45). E4-ORF4 activation of splicing requires the 3RE. Thus, deletion of the 3RE from the IIIa pre-mRNA increases basal IIIa splicing and eliminates the stimulatory effect of E4-ORF4 on splicing (45, 56). Conversely, transfer of the 3RE to the β-globin pre-mRNA reduces basal β-globin splicing but makes this transcript sensitive to E4-ORF4 activation of splicing (56).

E4-ORF4 interacts specifically with ASF/SF2 and SRp30c (56). However, the interaction with SRp30c did not correlate with E4-ORF4 induced activation of IIIa splicing, suggesting that ASF/SF2 is the primary target for E4-ORF4. Further, E4-ORF4 selectively interacts with the highly phosphorylated form of the SR protein, the form that is present at the early stage of virus infection. However, since all SR proteins, except SRp20, appear to be hypo-phosphorylated in Ad-NE (45), these results suggest that the E4-ORF4 induced SR protein dephosphorylation cannot be the only mechanism controlling SR protein phosphorylation during infection. In a more recent study the de novo synthesis of the sphingolipid ceramide, at the late stage of an adenovirus infection, has been suggested to cause accumulation of hypo-phosphorylated SR proteins (57). Previous work has shown that ceramide through activation of the PP1 pathway causes SR protein dephosphorylation (58). Collectively, available results therefore may suggest that adenovirus uses both the E4-ORF4/PP2A and the ceramide/PP1 pathways to control SR protein function during infection.

Although it has not been experimentally tested it appears likely that the dramatic effect of adenovirus on SR protein phosphorylation also will have effects on other virus-specific splicing events. Thus, the temporal activation of the multiple 3’ splice site within the MLTU most likely are similarly to the IIIa 3’ splice site activated in response to the reduction in the level of SR protein phosphorylation. Such a speculation is in agreement with the observation that most of the 3’ splice sites that are activated at late times of infection have a surprisingly weak sequence context.

5.4. L4-33K is a virus-encoded alternative splicing factor

As described in section 4.1., at an intermediate stage in MLTU expression mRNAs from region L1 and L4 are preferentially expressed (20). The significance of this burst in of L4 mRNA accumulation has previously not been understood. However, a recent study by Leppard and colleagues (59) provided an important piece of evidence that has increased the interest in the function of L4 proteins. They presented results suggesting that the L4-33K protein stimulated MLTU cytoplasmic mRNA accumulation, most likely at a posttranscriptional level.

Several previous reports have suggested that L4-33K might function as a virus assembly factor (60-62). Thus, mutations introduced into the L4-33K protein-coding region resulted in viruses that produced surprisingly little virus or viruses that were impossible to grow. L4-33K is translated from a spliced L4 mRNA. In infected cells both the spliced and unspliced L4 mRNAs are expressed. More recent data has shown that translation of the unspliced L4-33K mRNA generates another protein, the L4-22K, which binds to the viral packaging sequence and provide the virus assembly function (63). The L4-33K and L4-22K proteins share a common 105 amino acid amino-terminus but have distinct carboxy-terminal amino acid sequences. We have shown that L4-33K functions as a strong activator of IIIa pre-mRNA splicing in vitro and L1 alternative splicing in transient transfection experiments (31). In fact, L4-33K appears to be the only viral factor needed to convert a nuclear extract prepared from uninfected HeLa cells to an extract with splicing properties very similar to that of Ad-NE. Further, L4-33K activates IIIa splicing primarily through the 3VDE making this protein the prime candidate of being 3VDF, or at least the viral component of 3VDF. In agreement with such a speculation, L4-33K preferentially activates transcripts with weak 3’ splice sites, a sequence context that is typical for many of the viral 3’ splice sites activated at the late stage of infection (31). It is therefore important to note that L4-33K most likely is required for the activation of several of the 3’ splice sites within the MLTU and is not specific for IIIa 3’ splice site activation.

Recently L4-33K has been suggested also to function as a transcription factor stimulating RNA synthesis from the MLP (64). However, this function has been questioned since another group has presented evidence suggesting that the L4-22K protein functions as the L4 protein stimulating MLP transcription (63). The amino terminus, common to L4-33K and L4-22K is not conserved at the primary amino acid sequence level. However, it is
Conservation of feature. Thus, the amino-terminus of the L4 proteins are very charged with a great excess of acidic amino acids. The unique carboxy-terminus of L4-33K is highly conserved at the amino acid level and has been shown to be the region functioning as the IIIa splicing enhancer domain in vitro (31). Interestingly, this splicing domain contains a tiny RS repeat, which we have found is important for L4-33K function. Such RS dipeptide repeats are hallmarks for splicing factors of the SR protein and SR-related splicing factor families (reviewed in reference 65). However, whether L4-33K is a virus-encoded SR-related splicing factor remains to be tested.

6. REGULATION OF IIIA 3' SPLICE SITE ACTIVITY: A MODEL

Figure 4 shows a model for how IIIa 3' splice site activity might be regulated during an adenovirus infection. Early during infection IIIa splicing is low, or absent, because highly phosphorylated SR proteins bind to the 3RE and block U2 snRNP recruitment to the IIIa branch site. Further, the IIIa pyrimidine tract is weak and does not efficiently bind U2AF. Late after infection, a virus-induced partial SR protein dephosphorylation relieves the inhibitory effect of SR proteins on IIIa splicing by reducing the capacity of SR proteins to bind to 3RE. IIIa splicing is further augmented by the 3VDF factor which binds to the 3VDE and stimulates U2 snRNP recruitment to the IIIa 3' splice site. The composition of 3VDF is currently not known. However, our results suggest that the L4-33K protein is the key viral component of 3VDF. So far we have not been able to demonstrate that L4-33K binds directly to the 3VDE. We therefore speculate that 3VDF also contain a cellular RNA binding factor that makes the direct contact with 3VDE (31). We have no evidence indicating that U2AF is this cellular component. This model is compatible with available data but contain several speculative elements that are currently experimentally tested.

7. PERSPECTIVE

Viruses typically inhibit host cell gene expression at multiple levels to gain exclusive access to the biosynthetic machineries in the cell. We have characterized two adenovirus proteins, E4-ORF4 and L4-33K, which appears to be key proteins in the viral remodeling of the host cell RNA splicing machinery. So far our work has focused on the temporal regulation of L1 alternative splicing. However, since essentially all viral transcription units undergo changes in alternative splicing our future work need to address the significance of these two proteins in the global transformation of viral alternative splicing. Are these two proteins solely responsible for the temporal switch in splicing or do we have additional factors participating? It should be remembered that we have previously shown that the E4-ORF3 and E4-ORF6 proteins, at least in transient transfection experiments, regulates i-leader inclusion (17). A further interesting question concerns the potential effect of these powerful viral proteins on splicing of host cell mRNAs. It is clear from model experiments that both E4-ORF4 and L4-33K can regulate splicing of cellular transcripts. Do they regulate splicing of cellular transcripts during infection and if so is this important for the virus life cycle? It seems likely that the adenovirus system will continue to be a productive tool that teaches us new things about the gene expression machineries in our cells.
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