Nuclear Organization of Gene Expression in Adenovirus Infected Cells

BY

ANDERS ASPEGREN
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ABSTRACT


Adenovirus infected cells provide a good model system for studying nuclear organization during RNA production and transport. This thesis is focused on the dynamic organization of splicing factors during the late phase of Adenovirus infection in HeLa cells, the nuclear localization of viral RNA, and the pathway used for viral RNA transport to the cytoplasm.

Splicing factors are relocalized from interchromatin granule clusters to sites of transcription in Adenovirus infected cells at intermediate times of infection. Later, splicing factors and viral RNA accumulate posttranscriptionally in interchromatin granule clusters. The release of the splicing factors from transcription sites was energy dependent or preceded by energy requiring mechanisms. Our data indicated that phosphorylation events inhibited by staurosporine, and 3' cleavage of the transcript are two possible mechanisms involved prior to the release of the RNP complex from transcription sites.

A viral protein derived from orf6 of early region 4, 34K, is important for the nuclear stability and transport of late viral mRNA derived from the major late transcription unit. A viral mutant lacking this region is defective for posttranscriptional accumulation of viral mRNA in interchromatin granule clusters, and for the accumulation of viral RNA in the cytoplasm. These results suggest that posttranscriptional accumulation of viral RNA in interchromatin granule clusters may contribute to the maturation of the RNP complex or sorting of RNAs and proteins, to prepare the final RNP complex for transport to the cytoplasm.

A previous model suggested that adenoviral late mRNA is transported to the cytoplasm by utilizing the CRM-1 pathway. This pathway can be blocked by the drug leptomycin B. The data presented in paper IV suggests that this model might not be applicable, since leptomycin B did not inhibit adenoviral late gene expression.

Key words: Adenovirus, mRNA, transport, IGCs, E434kDa, E1B55kDa, NES, snRNP.

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"There must be a relationship between the events,  
like mother always said."

House of the spirits
LIST OF PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their roman numerals:


II. Aspegren, A., and Bridge, E. Release of snRNP and poly (A) RNA from transcription sites in adenovirus infected cells.

III. Mattson, K., Aspegren, A., and Bridge, E. Adenovirus early region 4 promotes the localization of splicing factors and viral RNA in nuclear interchromatin granules.


* Authors contributed equally to the work.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ActD</td>
<td>Actinomycin D, inhibitor of RNA polymerase II</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AraA</td>
<td>Arabinose-9-b-furanoside, inhibitor of 3' processing</td>
</tr>
<tr>
<td>Cluster cells</td>
<td>Ad infected cells at late phase of infection</td>
</tr>
<tr>
<td>dl1007</td>
<td>E4-deletion mutant</td>
</tr>
<tr>
<td>E2 72kDa</td>
<td>Ad early protein binding to DNA</td>
</tr>
<tr>
<td>E1B 55kDa</td>
<td>Ad early protein involved in RNA transport, 55K.</td>
</tr>
<tr>
<td>E4 orf3</td>
<td>Ad early protein involved in RNA transport, 11K</td>
</tr>
<tr>
<td>E4 orf6</td>
<td>Ad early protein involved in RNA transport, 34K</td>
</tr>
<tr>
<td>H5dlsma</td>
<td>E4-deletion mutant</td>
</tr>
<tr>
<td>hpi</td>
<td>hour post infection</td>
</tr>
<tr>
<td>IGC</td>
<td>interchromatin granule cluster</td>
</tr>
<tr>
<td>MLTU</td>
<td>major late transcription unit</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>oa</td>
<td>okadaic acid, inhibits protein phosphatases</td>
</tr>
<tr>
<td>PF</td>
<td>perichromatin fibril</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoproteins, splicing factors</td>
</tr>
<tr>
<td>SR proteins</td>
<td>serine-threonine proteins, splicing factors</td>
</tr>
<tr>
<td>Stp</td>
<td>staurosporine, inhibits kinases</td>
</tr>
<tr>
<td>Y12</td>
<td>antibody detecting a common epitope of snRNP</td>
</tr>
<tr>
<td>3dA</td>
<td>cordycepin, inhibitor of polyadenylation</td>
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INTRODUCTION

Introduction to the Adenovirus life cycle

Adenovirus (Ad) type 2 and the very similar type 5 are members of the group C human Ads and consist of an icosahedral capsid particle composed of 11 structural proteins, with a double-stranded DNA genome of about 36kbp. Ad enters the host cell by receptor-mediated endocytosis, penetrates the cytosol from endosomes, and delivers its DNA genome into the nucleus (Greber et al., 1993). The viral mRNAs transcribed during the early phase direct the synthesis of regulatory proteins that promote viral gene expression and replication (Challberg and Kelly, 1989; Horwitz, 1990), and influence cellular proteins involved in cell cycle control and apoptosis (reviewed by Moran, 1993).

Ad infection inhibits DNA replication of the host cell (Hodge and Scharf, 1969; Pina and Green, 1969). Cellular messages are transcribed at nearly normal levels but are not transported to the cytoplasm (Beltz and Flint, 1979), and translation of cytoplasmic cellular messages is inhibited (Dolph et al., 1988; Huang and Schneider, 1991). Thus, Ad causes marked changes in the characteristics of host cell gene expression as it recruits host components for its own expression. Ad has long been considered an invaluable model for understanding the mechanisms of mRNA transcription and processing in mammalian cells (reviewed by Ziff, 1980; Flint, 1986; Bridge and Pettersson, 1996). Ad infection progresses through an early and a late phase of gene expression. The late phase is preceded by the onset of viral DNA replication, which is crucial for the activation of late gene expression. An overview of the different stages of infection is presented below.

Early gene expression

Ad early proteins are synthesized in infected cells prior to viral DNA replication. The mRNAs that encode the early proteins are transcribed from seven different locations on the viral genome, designated early regions 1A (E1A), E1B, E2A, E2B, E3, E4, and late region 1 (L1). Many of these early products are necessary for proper regulation of viral gene expression. Region E1A is expressed first followed by E1B, E3 and E4 (Nevins et al., 1979; Lewis and Mathews, 1980). The E2 promoter is the last to be activated during the early phase of infection. The major late transcription unit (MLTU) is expressed at very low levels during the early phase of infection and is attenuated (Nevins and Wilson, 1981; Shaw and Ziff, 1980). Proteins expressed during the early phase interact with host cell proteins and other viral early proteins to optimize the synthesis of new virions. The E1A unit encodes proteins that induce the host to enter the S phase of the cell cycle and activate the expression of the other transcription units. E1B encodes two proteins, 19K and 55K, that cooperate with E1A products to induce cell growth and prevent apoptosis. E1B55K (55K) has an additional role in enhancing Ad late RNA transport (reviewed in Dobner
and Kzhyshkowska, 2001). E2 encodes three different proteins crucial for DNA replication. E3 encodes products that modulate the immune response of the host; these proteins are dispensable for the viral growth in cell culture. The E4 region encodes proteins with functions in viral DNA synthesis, cell growth, transcriptional regulation, and posttranscriptional accumulation of late RNA in the nucleus and the cytoplasm (Leppard, 1997). Of E4 products, only E4orf3 (11K) or E4orf6 (34K) is needed for essentially normal viral growth in cultured HeLa cells. The importance of two specific early genes, 55K and 34K, for late viral mRNA production, will be described under “RNA transport in Ad infected cells”, page 20.

DNA replication

Ad DNA replication requires terminal protein, Ad DNA polymerase, and a single stranded DNA binding protein of 72kDa (72K) (reviewed in Challberg and Kelly, 1989). DNA replication is initiated at either terminus of the double-stranded viral genome. At each replication fork only one of the two parental strands is replicated, producing a daughter duplex and a displaced single-stranded DNA (ssDNA). In a second stage of DNA replication the displaced single-strand serves as a template for synthesis of a complementary strand (Challberg and Kelly, 1989). Viral DNA replication occurs at discrete intranuclear locations that can be visualized with antiserum against the virally encoded 72K protein (Voelkerding and Klessig, 1986). At the ultrastructural level, 72K is predominantly detected in compact fibrillar structures with the shape of rings, crescents or spheres (Puvion-Dutilleul et al., 1984; II, fig. 1). These structures also accumulate viral ssDNA and have therefore been named ssDNA accumulation sites (Puvion-Dutilleul and Puvion, 1990 a, b).

Late gene expression

Following the onset of viral DNA replication, transcription is activated from three different promoters: IVa2, pIX, and the major late transcription unit (MLTU). DNA replication is mandatory for the activation of the MLTU during viral infection (Thomas and Mathews, 1980). Most Ad late messages are made from the viral MLTU (for review, see Sharp, 1984). The primary transcript produced from the MLTU is approximately 29,000 nucleotides in length, and it is processed to a minimum of 18 alternative mRNAs (reviewed in Shenk, 1996). These mRNAs are grouped into five families (L1 to L5) based on the location of their polyadenylation sites. Each viral transcript is differentially spliced and processed to only one mRNA. All MLTU mRNAs have a common leader sequence, the tripartite leader, which is spliced to the body of each mRNA sequence (reviewed in Flint, 1986). Most of the late proteins synthesized from these mRNAs are structural components of the Ad virion. Ad prevents the transport of newly synthesized cellular RNA to the cytoplasm during late stages of infection (Sarnow et al., 1984; Pilder et al, 1986; Cutt et al.,
and facilitates the late viral mRNA transport. Thus, the late phase provides a mechanism for the selective export of viral RNAs. Even though the virus manipulates its host cell for its own replication, it is absolutely dependent on host gene expression mechanisms for a productive infection. Ad-infected cells are an important model system for studying the molecular biology of gene expression (reviewed in Flint, 1986; Shenk, 1996). We are using Ad-infected cells as a model system for understanding how gene expression is organized relative to nuclear structures. The ability to study the cell biology of infected cells by microscopy will further enhance our knowledge of the spatial organization in the nucleus during mRNA transcription, processing and transport.

Nuclear organization of mRNA transcription and processing in eukaryotic cells

In situ hybridization, immunofluorescence microscopy, and electron microscopy have greatly contributed to our present understanding of how transcription and processing are spatially organized within the cell nucleus (Lawrence et al., 1989; Lewis and Tollervey, 2000; Dundr and Misteli, 2001). The interchromatin regions contain several characteristic structures which have been visualized by electron microscopy (reviewed in Fakan, 1994), including the transcriptionally active perichromatin fibrils (PFs) and transcriptionally inactive interchromatin granule clusters (IGCs). For a simplified overview, see figure 1. Several recent reports have led to the view of the transcription unit as an “RNA factory” where transcription, capping, splicing, polyadenylation and transport take place as coupled processes that can influence one another (for review see Bentley, 1999). The nuclear organization of transcription and posttranscriptional processing of pre-mRNA will be described below.

Figure 1. Schematic figure of a transcription site in uninfected cells. Transcripts are 5’ capped, spliced and 3’ processed at sites of transcription (perichromatin fibrils, PF). Splicing factors are recruited to transcription sites from the interchromatin granule clusters (IGCs), which function as a potential storage/preassembly site for splicing factors. The drawing is not to scale.
Spatial organization of transcription

RNA polymerase II (pol II) transcription sites and active pol II are widely distributed in the nucleus (Jiménez-Garcia and Spector, 1993; Zeng et al., 1997), suggesting that transcription occurs at multiple nuclear sites. PFs at the periphery of dense chromatin are strongly labeled after short pulses of $^3$H-uridine, which indicates that transcription occurs at these sites (Fakan and Puvion, 1980; Puvion and Puvion-Dutilleul, 1996). Transcription sites contain the active form of pol II and proteins required for processing (Iborra et al., 1998; Cmarko et al., 1999). Splicing and polyadenylation factors interact transiently with the hyperphosphorylated C-terminal domain (CTD) of pol II (Mortillaro et al., 1996; Yurvey et al., 1996; Cho et al., 1997; Du and Warren, 1997; McCracken et al., 1997a, b; Schul et al., 1998). It is now becoming clear that the CTD coordinates transcription with RNA processing to ensure that transcripts generated from pol II promoters are processed into mature messages with an optimal efficiency (reviewed in Bentley, 1999). Current evidence suggests that mRNA is incorporated into ribonucleoprotein complexes (RNPs) co-transcriptionally, that serves as substrates for both RNA processing (Krämer, 1996; Zhao et al., 1999) and transport (Mattaj and Englmeier, 1998). It is therefore likely that the formation and maturation of the RNP complex on nascent mRNA is an important step prior to release of RNA from transcription sites and transport to the cytoplasm.

Spatial organization of splicing

More than 50 proteins and five snRNAs come together to form a functional spliceosome (Will and Lührmann, 1997; Staley and Guthrie, 1998). A spliceosome is built from the five different snRNPs, U1, U2, U4, U5 and U6 (Hermann et al., 1995), a number of non-snRNP proteins such as U2AF (Zamore and Green, 1992), and members of the SR protein family (Zahler et al., 1992; Blencow et al., 1995; Screaton et al., 1995). Studies have shown that splicing factors undergo phosphorylation and dephosphorylation at different stages in the splicing process (Mermoud et al., 1992, 1994). SR proteins are highly phosphorylated in vivo (Colvill et al., 1996), and phosphorylated SR proteins are required for initiation of spliceosome assembly, while SR protein dephosphorylation appears to be required for splicing catalysis (Cao et al., 1997, reviewed in Misteli, 1999).

Several studies suggest that splicing can occur at sites of transcription (Beyer and Osheim 1988; Baurén and Wieslander, 1994). Wetterberg et al. (1996) have analyzed the excision of introns in the Balbiani ring 3 (BR3) gene pre-mRNA in the dipteran Chironomus tentans, and showed in vivo that intron excision initiates co-transcriptionally in an overall 5' to 3' order. The 5' proximal introns are excised co-transcriptionally, while the 3' distal introns most likely are excised posttranscriptionally, suggesting that splicing can occur both co- and posttranscriptionally, as previously suggested by other studies (Nevins and Darnell, 1978; Beyer and Osheim,
Although splicing factors localize in PFs at transcription sites throughout non-nucleolar regions of the nucleoplasm, both non-snRNP and snRNP splicing factors are also localized in distinct subnuclear structures including IGCs and coiled bodies (reviewed in Lamond and Carmo-Fonseca, 1993). It has been demonstrated that IGCs can serve as pools of splicing factors that are recruited to the transcription sites, and this recruitment is intron-dependent (Huang and Spector, 1996b; Misteli et al., 1997). Many active genes and pre-mRNAs are associated with nuclear speckles (Xing et al., 1993, 1995; Huang and Spector, 1996b; Ishov et al., 1997; Smith et al., 1999; Snaar et al., 1999; Johnson et al., 2000; Melcák et al., 2000). In cells producing a truncated pol II that lacks the CTD, the association between an active gene and IGCs is lost, suggesting that IGCs are close to active genes as a result of ongoing splicing (Misteli and Spector, 1999). Phosphorylation releases SR proteins from their storage sites (e.g. IGCs) and targets them to sites of transcription and splicing in the nucleus (Colwill et al., 1996; Caceres et al., 1998; Misteli et al., 1998).

Although splicing factors may be targeted to active transcription sites from IGCs, it has been reported that poly(A) RNA sequences (Carter et al., 1991) and injected RNA (Wang et al., 1991) are also associated with IGCs. Melcák et al. (2001) reported unspliced microinjected forms of RNA are in the IGCs, and Johnson et al. (2000) demonstrated the localization of intron-containing COL1A1 RNA in IGCs. These authors suggest that posttranscriptional splicing may occur in those structures. Berthold and Maldarelli (1996) suggest that the localization of RNA in IGCs may be an important aspect of the expression of at least some genes, since a construct producing HIV gag-RNA with an intron-insertion, targeted the gag-RNA to IGCs and increased the expression of the gag-protein several fold. Finally, Schul et al. (1998) have observed the presence of poly(A) polymerase in close association with IGCs and have postulated that there is a spatial and structural relationship of 3' processing to these structures.

**Spatial organization of 3' processing**

Poly(A) tails are generated in the nucleus in a two-step reaction that involves cleavage of the nascent transcript and subsequent polyadenylation of the upstream RNA fragment (Colgan and Manley, 1997). Proteins involved in 3' processing colocalize with nascent transcripts in vivo (Schul et al., 1998), and the spatial as well as physical connections between proteins involved in 3' processing and transcription has been established (reviewed in Zhao et al., 1999; Bentley, 1999). RNA pol II transcription continues past the site of cleavage and polyadenylation (Baurén et al., 1998). Polyadenylation factors (i.e. CstF and CPSF) are associated with the CTD of pol II and 3' cleavage and at least initiation of polyadenylation occurs on nascent transcripts (McCracken et al., 1997a, b; Baurén et al., 1998). Custódio et al. (1999) demonstrated that mutations in the 3' cleavage site of beta-globin RNA inhibit the release of the transcript from its gene,
indicating that 3' cleavage is important for the release of the pre-mRNA from its transcription site. By contrast, specific inhibition of polyadenylation with the drug cordycepin (3dA) does not block the cytoplasmic transport of RNA (Zeevi et al., 1982; Ioannidis et al., 1999), and intron-containing RNAs lacking the poly(A) tail could still accumulate in IGCs after microinjection into the nucleus (Melcák et al., 2001). This might indicate that the second step in 3' processing (i.e. polyadenylation) and the poly(A) tail itself may not be critical for release of transcripts from transcription sites, localization of RNA in IGCs, or transport to the cytoplasm.

In summary, pol II transcription occurs at the periphery of dense chromatin in PFs, and the CTD of pol II seems to be a key regulator in the coordination of transcription and processing. Splicing can occur both co-transcriptionally and posttranscriptionally, probably dependent upon the splicing efficiency of individual introns as well as on the location of the intron within the transcript. Introns located at the 5' end of the pre-mRNA are transcribed first and may be spliced co-transcriptionally more often than 3' introns (Wetterberg et al., 1996). Phosphorylation and dephosphorylation are important steps in regulating splicing, as well as for the subnuclear organization of splicing factors. The targeting of splicing factors to transcription sites is intron-dependent and phosphorylation of SR proteins activates and relocalizes splicing factors from IGCs to sites of splicing activity. Steps in 3' processing are also thought to occur at or near transcription sites, where at least 3' cleavage is an important step prior to release of the message from sites of transcription. The role of IGCs in gene expression is under debate. The prevalent view of IGCs as storage/preassembly sites for splicing factors (Misteli and Spector, 1998) does not explain why those structures also contain spliced, unspliced and polyadenylated RNAs expressed from at least some genes (Carter et al., 1991; Berthold and Maldarelli, 1996; Johnson et al., 2000). Although current research has greatly enhanced our knowledge of how gene expression is biochemically and spatially regulated, further studies with multiple different model systems are needed to fully understand how gene expression activities are related to the cell biology of the nucleus. The following section will describe some of the previous findings of nuclear organization during Ad infection and gene expression.
Nuclear organization during Ad gene expression

Nuclear organization during the early phase of infection

Ad viral DNA templates are delivered to the nucleus following infection (reviewed in Shenk, 1996), where they associate with the nuclear matrix (Schaack et al., 1990; Fredman and Engler, 1993). The expression of early mRNA from these templates produces the viral proteins that are needed for the onset of viral DNA replication. Cellular factors such as p53 (Yew and Berk, 1992; Yew et al., 1994), nuclear domain 10 (ND10) proteins (Carvalho et al., 1995) and nuclear factor NF-I and III (Challberg and Kelly, 1989) are used or modified by the virus to prepare the cell for viral transcription, replication, late gene expression and finally assembly of new virions. For example, the E1A proteins activate transcription through interactions with a variety of cellular regulatory proteins and transcription factors, like the retinoblastoma protein (pRB), a cellular transcription factor (E2F) and TATA-binding protein (TBP) (reviewed in Flint and Shenk, 1997). Both 34K and 55K interact with p53, which stimulates the rapid turnover of p53 in infected cells (Moore et al., 1996; Querido et al., 1997; Steegenga et al., 1998). These activities may facilitate the progression of cells from G1 to S-phase (Flint and Shenk, 1997).

ND10 structures appear as 8-10 round foci in the nuclei of many cell types. ND10 structures contain several cellular proteins including promyelocytic leukemia protein (PML), sp100, and small ubiquitin-related modifier 1 (SUMO-1) (reviewed in Maul, 1998). Ad infection causes a redistribution of PML from the ND10 domains into tracks within 4-6 hpi; the 11K protein of E4 orf3 is necessary and sufficient for this redistribution (Carvalho et al., 1995). Both E1A and E1B55K proteins are found in the 11K induced tracks (Carvalho et al., 1995; Doucas et al., 1996; Ishov and Maul, 1996). It has been suggested that Ad begins replication at ND10 domains (Maul, 1998). The biological significance for the 11K induced reorganization of ND10 is unclear since 34K can functionally replace the 11K in virus production without the redistribution of the proteins in ND10 domains. Further experiments are needed to address the significance of these changes in nuclear organization during the early phase.

Replication and transcription sites in viral late phase cells

Ad DNA replication requires three viral proteins, terminal protein, 72K, and Ad DNA polymerase, and three cellular factors, NF-I, NF-III and topoisomerase I (reviewed in Challberg and Kelly, 1989; Horwitz, 1990). During the early phase the 72K protein is localized diffusely throughout the nucleoplasm but excluded from the nucleolus. The onset of Ad DNA replication is followed by the formation of focal viral inclusions containing the 72K DNA binding protein. The 72K protein and viral single-stranded DNA (ssDNA) are partitioned in nuclear inclusions (Puvion-Dutilleul and
Pichard, 1992), and replication was found to occur at the edges of these 72K-containing centers. Several ND10 proteins are recruited to these viral replication centers (Ishov and Maul, 1996), as well as NF-I (Bosher et al., 1992). In their earliest forms (6-8 hpi), 72K protein-containing centers are active for both replication and transcription. These activities are then rapidly compartmentalized. Pulse-chase experiments using Br-dUTP have shown that ssDNA accumulates in 72K centers, while dsDNA is released to the surrounding nucleoplasm, where it is used for transcription (Pombo et al., 1994). Transcription overlaps with replication sites but extends further away from the 72K centers than newly synthesized DNA (Pombo et al., 1994). The region surrounding the 72K centers where transcription and replication occur is named the peripheral replicative zone (Puvion-Dutilleul and Puvion, 1990a,b, 1991; Puvion-Dutilleul et al., 1992; figure 2). Replication, transcription and processing of viral genomes may require a precise spatial organization of DNA templates and transcription (Pombo et al., 1994). The onset of replication and late transcription causes distinct changes in the nuclear organization of splicing factors, which will be described below.

![Figure 2](image.png)

**Figure 2.** Schematic figure of replication/transcription sites in adenovirus infected cells. The 72K centers contain single-stranded DNA (ssDNA). Replication and transcription occur at the peripheral replicative zone surrounding 72K centers. Splicing factors are recruited from interchromatin granule clusters (IGCs) to the peripheral replicative zone, which contains double-stranded DNA (dsDNA) and nascent transcripts (Ad RNA). Ad RNA from the major late transcription unit accumulates in IGCs together with snRNP. It is at present not known whether Ad RNA in IGCs is transported to the cytoplasm or directly transported to the cytoplasm following release from transcription sites. The drawing is not to scale.

**Changes in splicing factor organization during the viral late phase**

Two distinct snRNP splicing factor distribution phenotypes at late times of infection have been described in the literature: ring cells and cluster cells (Pombo et al., 1994; Bridge et al., 1993a, 1995, 1996). During
the onset of the late phase, splicing factors are recruited from their normal distribution in IGCs and coiled bodies and are localized at sites of transcription and viral RNA accumulation at the periphery of 72K containing centers in ring cells (Pombo et al., 1994). At later times of infection, splicing factors concentrate in IGCs in cluster cells. IGCs in late phase Ad-infected cells also contain spliced viral RNA (Bridge et al., 1996). Both ring and cluster cells are present at late times of infection, and one goal of this thesis was to correlate changes in viral gene expression to changes in splicing factor distribution. An overview of the dynamic reorganization of splicing factors in Ad-infected cells is given in figure 2.

The general view at present is that the IGCs are dynamic structures that respond to the gene expression activities of the cell (reviewed in Singer and Green, 1997; Misteli and Spector, 1998). IGCs are not sites of transcription in either infected or uninfected cells (Huang and Spector, 1996a), but transcription and transport of viral late RNA is ongoing during the formation of cluster cells (Bridge et al., 1996). Polyadenylated RNA and spliced tripartite leader exons from the MLTU are present in late phase IGCs (Puvion-Dutilleul et al., 1994; Bridge et al., 1996). The majority of the steady state viral RNA present in the nucleus is spliced at the tripartite leader exons. These RNAs are efficiently exported from the nucleus at a time when they could be detected in IGCs (Bridge et al., 1995, 1996). IGCs in infected cells contain many of the same factors present in IGCs in uninfected cells including the U1 70K protein, common snRNP proteins, snRNA, the B′′ protein from U2 snRNP (Bridge et al., 1993a), and SR proteins (Bridge et al., 1995).

In summary, virus infection causes marked changes in nuclear morphology as the infection enters the late phase. 72K centers are a useful marker for localization of sites containing viral ssDNA. Viral replication is believed to occur at the periphery of the 72K centers and replication is crucial for the transcription of the full length MLTU. Transcription of the MLTU triggers the relocalization of snRNP to sites of transcription where they colocalize with nascent transcripts in ring cells. However, at later times snRNP and spliced viral RNA localize in clusters corresponding to IGCs. The presence in IGCs of spliced RNA, poly(A) RNA, snRNP and non-snRNP splicing factors as well as other proteins involved in RNA maturation, suggest that IGCs have a posttranscriptional role in the production of RNA from at least some genes (Bridge and Pettersson, 1996; Berthold and Maldarelli, 1996; Johnson et al., 2000; Melcák et al., 2000). This idea is somewhat different from the prevalent view of IGCs as storage/preassembly sites for splicing factors (reviewed in Misteli and Spector, 1998). We think it is also possible that IGCs could have a role in splicing, sorting of splicing proteins from the fully processed mRNA, or in intranuclear RNA transport. The precise role of IGCs in mRNA metabolism is still unclear and more studies are needed to clarify its
function. In paper I of this thesis, we have correlated accumulation of snRNP and spliced RNAs in IGCs to steps in late gene expression. In paper II we have studied some mechanisms important for the release of nascent mRNA and snRNP from transcription sites, followed by accumulation in IGCs.

**RNA transport**

Eukaryotic cells use three different RNA polymerases, RNA pol I-III. Pol I transcribe rRNA genes in the nucleoli, pol II mainly transcribes mRNA, and pol III transcribes tRNA and 5S rRNA. Both pol II and III also synthesize snRNAs and snoRNAs (reviewed in Jackson et al., 2000). Export of RNAs involves movement of the RNP complex from sites of transcription to the nuclear pore complex (NPC), followed by translocation through the nuclear pore (reviewed in Daneholt, 2001). Microinjection of different classes of RNAs into the nucleus Xenopus oocytes in competition experiments, showed that the different classes of RNA are transported to the cytoplasm by different mechanisms (Jarmolowski et al., 1994).

The transport of mRNAs depends on interactions with specific transport receptors which are assumed to interact with specific nuclear export signals present on RNA binding proteins (reviewed in Nakielny et al., 1997; Stutz and Rosbash, 1998). Several proteins are also observed to follow the RNA from sites of transcription to the cytoplasm, while other proteins only associate with the RNPs at certain stages during pre-mRNA maturation (Daneholt, 2001). HnRNP A1 was the first RNA binding protein demonstrated to continuously shuttle between the nucleus and cytoplasm (Piñol-Roma and Dreyfuss, 1991), and since then several families of shuttle proteins with different nuclear export signals (NES) and nuclear localization signals (NLS) have been studied (reviewed in Mattaj and Englmeier, 1998). The exact role of hnRNPs and other mRNA binding proteins in mRNA transport has not yet been resolved. Recently several candidate proteins that may direct mRNA transport have been reported. RNPS1, Y14, and REF, interact directly with spliced mRNA (Le Hir et al., 2000), and accompany the spliced transcripts to the cytoplasm (Kataoka et al., 2000; Zhou et al., 2000; Kim et al., 2001). TAP is another protein implicated in RNA transport (Grüter et al., 1998; Katahira et al., 1999; Bachi et al., 2000). TAP may bind RNP complexes through protein-protein interactions with REF. TAP can also bind to the NPC (Bachi et al., 2000; Herold et al., 2000), and might therefore be important in the translocation through NPCs.

The asymmetric distribution of RanGTP (nuclear) and RanGDP (cytoplasmic) is important in nuclear-cytoplasmic transport of proteins that shuttle between the nucleus and the cytoplasm. RanGTP binds to transport receptor-cargo complexes in the nucleus followed by transport through the NPC and disassembly of the complex in the cytoplasm. (reviewed in
Interestingly, TAP interacts directly with the NPC but not with Ran (Bachi et al., 2000). If TAP is directly involved in mRNA transport, the role of Ran for mRNA export might be indirect by recycling proteins essential for mRNA export back to the nucleus (Clouse et al., 2001; reviewed in Zenklusen and Stutz, 2001). Although the mechanism of mRNA transport is not yet established, it is clear that two essential issues are the understanding of how maturation of mRNP is coupled to transport, and how RNA binding proteins direct export of the mature RNP to the cytoplasm.

Maturation of the RNP complex

Several protein factors accompany the RNA during intranuclear transport in Chironomus tentans. These include hrp36 (a sequence analog of the mammalian hnRNP A1), cap binding proteins, and SR proteins that remain associated with the RNA even after splicing is completed (Visa et al., 1996a, b; Alzhanova-Ericsson et al., 1996). Splicing is important for the formation of a mature RNP complex marked for export (Legrain and Rosbash, 1989; Chang and Sharp, 1990; reviewed in Stutz and Rosbash, 1998; Cullen, 2000). Some proteins specifically interact with spliced mRNA, including a coactivator of splicing, SRm160 (Blencowe et al., 1998), a splicing activator, RNPS1 (Mayeda et al., 1999), a novel RNA binding protein Y14 (Kataoka et al., 2000), and Aly (or REF) (Zhou et al., 2000). By contrast, the antibody Y12 detecting a common sm-core domain of snRNP, does not immunoprecipitate full-length spliced mRNAs but RNAs contained in spliceosomes (Le Hir et al., 2000). This suggests that some proteins, including snRNP splicing factors, are removed from the final RNP complex prior to transport while other proteins are loaded onto the complex either during or after splicing. Splicing and transport may be tightly coordinated in vivo just as previous data suggested close association of transcription and processing (Bentley, 1999). Thus the maturation of the pre-mRNA will not only assure a functional mRNA for translation, but will also result in the formation of an RNP complex that is competent for transport to the cytoplasm. Bennet et al. (1992) showed that each different mRNA species is associated with a unique combination of heteronuclear ribonucleoproteins (hnRNP) in vitro. The hnRNP compositions of individual mRNAs also differ in vivo, as demonstrated in both Drosophila (Matunis et al., 1993) and in Chironomus tentans (Wurtz et al., 1996). This suggests that each type of mRNA binds a specific subset of proteins that might influence different processes like splicing, transport, translation and degradation (Daneholt, 2001). Eukaryotic mRNA transport is only beginning to be understood. The mechanism of RNA export has been most extensively studied in retroviral systems. I will briefly summarize what is known about retroviral RNA transport, before I discuss Adenovirus RNA transport.
Retroviral RNA transport pathways

Retroviruses have been extensively used as model systems for the analysis of mRNA export. Human immunodeficiency virus (HIV) and simian type D retroviruses export intron-containing RNAs which contain specific secondary structures. Viral or cellular proteins bind to these structures, called the Rev-responsive element (RRE) in HIV RNA, and the constitutive transport element (CTE) in type D retrovirus RNA. These structures confer the ability to bypass the normal nuclear retention of intron-containing mRNAs (reviewed in Cullen, 1998; Hope, 1999). In the case of HIV, the viral Rev protein binds the RRE. Export of Rev and the RRE containing RNA to the cytoplasm is mediated by an NES within the Rev protein (Fischer et al., 1995; Wen et al., 1995). A cellular transport receptor, CRM-1, binds to Rev-like NESs (Stade et al., 1997; Fornerod et al., 1997). In the presence of Ran-GTP the complex is efficiently exported through the NPC to the cytoplasm. The drug leptomycin B (LMB) is a potent inhibitor of this pathway, since it has been demonstrated that LMB specifically binds to CRM-1 thereby blocking the Rev NES-mediated export (Fornerod et al., 1997; Wolff et al., 1997). By contrast, the CTE of type D retroviruses uses a different pathway for the export of its intron-containing mRNAs, since LMB has no effect on the CTE-mediated transport (Pasquinelli et al., 1997; Saavedra et al., 1997; Otero et al., 1998). Instead it has been shown that the CTE directly binds to the cellular protein TAP, which recently has been implicated in cellular mRNA export (Grüter et al., 1998; Bachi et al., 2000). These results suggest that HIV Rev mediated RNA export may target a different pathway than that used for the export of cellular mRNAs. In contrast, CTE containing RNAs may use the same export pathway as cellular mRNA.

RNA transport in Ad infected cells

RNA export in Ad infected cells is mediated by the viral 34K and 55K proteins. Interestingly, both 34K (Dobbelstein et al., 1997) and 55K (Dosch et al., 2000) were recently reported to have Rev-like NESs, and the authors have suggested that export of Ad late mRNA may rely on these export signals. To date, the only cellular RNAs transported by the CRM-1 mediated pathway are UsnRNAs and 5S RNAs (Hope, 1999). Since transcription and processing of Ad late mRNA is similar to that of host mRNA, it remains to be established whether these viral mRNAs, that are targeted for multiple splicing and contain no known secondary structure like RRE or CTE, actually use the CRM-1 mediated export pathway. For a schematic drawing of the proposed Ad mRNA transport model, see figure 3.
Figure 3. A simplified drawing of a proposed model for late viral mRNA transport. Both 34K and 55K contain a Rev-like nuclear export signal (NES) and both proteins have separately been suggested to carry the driving signal for RNA transport. (Dobbelstein et al., 1997 and Dosch et al., 2001, respectively). The cellular protein CRM-1 binds to the NES signal and is crucial for the translocation of Rev-like NES-containing proteins through the nuclear pore complex. 55K can bind unspecifically to RNA. X = cellular limiting factor(s) suggested to be involved in late viral mRNA transport. N = nucleus, C = cytoplasm.

Beltz and Flint (1979) have shown that although host RNAs are transcribed normally during the late phase, newly synthesized host RNA does not appear in the cytoplasm. This suggests that the late phase inhibits some aspect of the posttranscriptional maturation events of host RNAs, while selectively allowing cytoplasmic accumulation of newly synthesized viral messages. Inhibition of cellular transport does not appear to reflect inhibition of splicing of host mRNAs or to depend on the 5' tripartite leader sequence which is characteristic of late viral transcripts (Katze et al., 1984; Flint, 1986; Moore et al., 1987). Cellular mRNAs transcribed from recombinant viral chromosomes are transported to the cytoplasm late after infection, even at a time when the endogenous cellular transcript is restricted to the nucleus (Gaynor et al., 1984; Hearing and Shenk, 1985). This indicates that in contrast to the RRE element of HIV RNA, no specialized secondary structure is involved in facilitating Ad late mRNA transport. Instead it has been proposed that the efficient export and perhaps the processing of primary transcripts occurs by directing activated genes to specialized nuclear microenvironments (Pombo et al., 1994; Yang et al., 1996). Alternatively, Ad might direct a limiting cellular protein(s) involved in posttranscriptional maturation to sites of viral transcription and mRNA maturation (Ornelles and Shenk, 1991; fig. 3).

**E1B-55K and E4-34K in viral RNA transport**

Genetic studies show that 55K and 34K are important for the cytoplasmic accumulation of late viral RNA and inhibition of the cellular RNA transport during the late phase of infection (Beltz and Flint, 1979; Babiss et al., 1985; Halbert et al., 1985; Pilder et al., 1986; Weinberg and
Ketner, 1986; Bridge and Ketner, 1989; Huang and Hearing, 1989). Earlier work demonstrated that 55K exert its late effects after an RNA molecule is spliced and polyadenylated (Pilder et al., 1986; Leppard and Shenk, 1989). In the absence of 55K, late viral transcripts exit the nuclear matrix fraction inefficiently and fail to accumulate in a nuclear compartment where viral RNA normally accumulates posttranscriptionally (Leppard and Shenk, 1989; Leppard, 1993).

The 55K and 34K proteins can form a complex in Ad infected cells (Sarnow et al., 1984). Deletion mutants lacking the genes for either or both of the proteins show similar defects in late gene expression, consistent with the idea that the proteins exert their late viral RNA effects as a complex (Cutt et al 1987; Bridge and Ketner, 1990). It was proposed that the 55K/34K complex simultaneously inhibits cellular mRNA transport and activates viral mRNA transport by binding to and relocalizing a nuclear host factor required for mRNA export from the sites of host transcription and processing to the viral replication centers (Pilder et al., 1986; Ornelles and Shenk, 1991; fig. 2 and 3). A potential cellular limiting transport factor was reported by Gabler et al. (1998), who found that the 55K protein forms a complex with a nuclear RNA-binding protein, named E1b-AP5 (Gabler et al., 1998). Immunofluorescence and immunoelectron microscope studies show that 34K and 55K proteins are localized within and about the periphery of viral 72K centers (Ornelles and Shenk, 1991), that are believed to be associated with viral transcription and replication (Puvion-Dutilleul and Puvion, 1991). It is therefore possible that the 55K/34K complex is present at sites of transcription to participate in the transport of viral messages (Dix and Leppard, 1993; Leppard, 1993; Nordqvist et al., 1994). It is at present not clear how the 34K and 55K proteins promote the selective export of MLTU derived mRNA during the late phase. In the present investigation we have specifically studied the role of the CRM-1 mediated export pathway for Ad late gene expression (paper IV).
PRESENT INVESTIGATION

We are studying the cell biology of Ad gene expression. Our goal is to better understand how changes in the nuclear organization of splicing factors are related to steps in viral gene expression. We have also begun to study the mechanism of viral RNA export in infected cells. In paper I we characterized viral gene expression in late phase infected cells that have splicing factors organized in diffuse rings surrounding viral replication centers, or in enlarged IGCs. In paper II we have studied factors involved in the release of snRNP and poly(A) RNA from transcription sites in virus infected cells. In paper III we compare nuclear organization of splicing factors and RNA in wild type infected cells and in cells infected by an E4 deletion mutant that is defective for the accumulation of late viral mRNA in the nucleus and cytoplasm. In paper IV we investigate a proposed model for viral mRNA export at late times of infection.

Characterization of late viral RNA expression in ring and cluster cells (paper I)

Transcription of RNA in Ad-infected cells surrounds viral replication centers that contain the 72kDa viral DNA binding protein (Puvion-Dutilleul et al., 1992; Pombo et al., 1994). SnRNP splicing factors redistribute to transcription sites during the onset of the late phase. However, in later stages of infection splicing factors accumulate posttranscriptionally in enlarged IGCs along with late viral RNA. We therefore set out to further characterize viral gene expression in ring cells (Pombo et al., 1994; I fig. 1c) and cluster cells (Bridge et al., 1993a, 1995, 1996; I, fig 1d).

Both ring and cluster cells express late viral mRNA

Pombo et al., (1994) used a genomic probe to localize total viral RNA in ring cells. To distinguish late from early viral messages, we determined the localization of viral late RNA with biotinylated oligonucleotide probes complementary to RNA made from late region 2 (L2) and L5 (I, fig. 2). We found that both ring and cluster cells contained L2 and L5 RNA in the nucleus (I, fig. 2, and data not shown), indicating that late gene expression had been activated in both types of cells. We next used a probe complementary to the first spliced junction of the tripartite leader sequence present on MLTU derived mRNA, to determine if splicing of the transcribed late viral messages had occurred in ring cells. We used a fixing protocol to preserve the RNA in the cytoplasm so we could detect whether transport of spliced messages had occurred. Both ring and cluster cells contained at least partially spliced late viral mRNA, but the cluster cells had higher levels of RNA in the cytoplasm (I, fig. 3). In addition, the cluster cells had viral RNA in IGCs as earlier reported by Bridge et al. (1996). We conclude that both ring and cluster cells have expressed and at
least partially spliced late viral RNA in the nucleus, but that cluster cells have exported higher levels of viral late messages to the cytoplasm.

**Ring cells form transiently during the onset of viral late gene expression**

The redistribution of snRNP to the ring cell pattern or into enlarged IGCs is correlated with the expression of late viral messages. We next analyzed the relationship of these two snRNP distribution phenotypes relative to viral mRNA accumulation in the cytoplasm and late viral protein expression during a time course of viral infection. Infected cells were fixed and stained for snRNP and the viral late protein penton, at times indicated in figure 4 (I). Ring cells were first detected at 12 hour post infection (hpi), and the fraction of ring cells then remained constant between 12-24 hpi before declining to less than five per cent of the infected cell population by 30 hpi. Ring cells contain spliced viral RNA that is primarily nuclear, with low levels of spliced viral RNA in the cytoplasm (I, fig. 5), and low or undetectable levels of the late protein penton (I, fig. 6). By contrast, the cluster cells in the culture increase with time, have high levels of spliced RNA both in the nucleus and in the cytoplasm and have moderate to high levels of the late protein penton (I, fig. 4, 5 and 6). We think it is likely that ring cells form during the onset of viral late gene expression and that these cells convert to cluster cells as more late viral RNA is transcribed, processed and transported to the cytoplasm (I, fig. 5 and 6).

**Accumulation of late viral mRNA and snRNP in IGCs is correlated with late mRNA export to the cytoplasm and the production of late proteins**

The accumulation of snRNP in IGCs was well correlated with the accumulation of viral late RNA in the cytoplasm and the expression of viral late proteins (I, fig. 4-6). Cluster cells were first detected at 12 hpi and increased in number up to 30 hpi (last time point measured, fig. 4B). Similarly, the number of penton containing cells increased with number of cluster cells (I, fig. 4D), as well as the accumulation of transported viral RNA in the cytoplasm (I, fig 4C). In individual cells, the accumulation of late viral mRNA and snRNP in IGCs was first detected in cells with visible spliced RNA in the cytoplasm (I, fig. 5e-f). Staining of late viral proteins was first detected in cells with snRNP in small clusters (I, fig 6e-f). Finally, the concentration of cytoplasmic late viral mRNA and proteins increased with increasing accumulation of snRNP and viral RNA in the IGCs (I, fig. 5g-j and fig. 6g-h, respectively). These data show that cluster cells appear later than ring cells, and they have transported more spliced RNA to the cytoplasm and expressed more late viral proteins. We suggest that the accumulation of viral late mRNA in IGCs occurs at the same time as late RNAs are exported to the cytoplasm and that it reflects an important aspect of late viral mRNA metabolism in the nucleus (see discussion).
The release of snRNP and poly(A) RNA from transcription sites (paper II)

The ring cells described in paper I express MLTU derived transcripts. The approximately 29,000 nucleotide long primary transcript derived from the MLTU of Ad is alternatively spliced and 3' processed to one of at least 18 different mRNAs (reviewed in Shenk, 1996). The messages are transcribed and processed in the nucleus, but it is not known how much processing takes place at the site of transcription. In paper II we designed experiments to explore the parameters involved in release of RNA from transcription sites.

SnRNP and poly (A) RNA relocalize from transcription sites to IGCs when transcription is inhibited with actinomycin D

We first confirmed that transcription sites in ring cells surround viral replication centers containing the 72K protein (Pombo et al., 1994; II, fig. 1). SnRNP colocalizes with transcription in ring cells in about 25% of the infected population at intermediate times of infection (I, fig. 4A and II, fig. 2B and 5). We have studied the release of splicing factors and RNA from transcription sites in ring cells after transcription inhibition. When cells were treated for 30 minutes with Actinomycin D (ActD) to inhibit transcription, we detected a 5 to 7-fold decrease in the percent of ring cells in the culture (II, fig 2B and 5). These results indicate that snRNP is released from sites of transcription upon transcription inhibition with ActD. SnRNP accumulates in IGCs when cells are treated with ActD for 30 minutes (II, fig. 2A), suggesting that snRNP moves posttranscriptionally to IGCs after its release from sites of transcription.

We next studied the release of RNA from transcription sites following transcription inhibition using a biotinylated oligo d(T) probe to detect poly(A) RNA in infected cells. Poly(A) RNA could be detected in a diffuse pattern surrounding viral replication sites in cells not treated with ActD (II, fig 3). When transcription was inhibited with ActD, poly(A) RNA accumulated in enlarged IGCs together with snRNP (II, fig. 3), indicating that both RNA and splicing factors move from transcription sites to IGCs. Poly(A) RNA colocalized with snRNP both in rings and in IGCs (II, fig. 1 and 3 and data not shown). The localization of snRNP at transcription sites (i.e. ring pattern) and in IGCs was dependent on the presence of RNA in fixed cells (II, fig. 4). This indicates that snRNP may form a complex with RNA both at viral transcription sites and in IGCs (II, fig. 4). The data presented in figure 1 through 4 (II), are consistent with the possibility that snRNP and poly(A) RNA are released from sites of transcription and accumulate in IGCs as an RNP complex.

Factors controlling the release of snRNP and RNA from transcription sites

We next addressed the question of whether release of snRNP from transcription sites required energy, or if an RNP complex containing
snRNP could immediately diffuse away after transcription inhibition. If release is energy independent, then the population of ring cells would decrease when cells are treated with ActD at 4°C, since energy independent diffusion can occur at this temperature while energy dependent mechanisms are blocked (Michael et al., 1995). If the release of snRNP requires energy, then the population of ring cells should not be affected by ActD inhibition at 4°C. The population of ring cells was not reduced during a four hour ActD inhibition at 4°C, while the cell culture treated with ActD at 37°C contained no ring cells (II, fig. 5A). We conclude that the release of the snRNP from transcription sites requires energy.

It is possible that some energy requiring steps in RNA processing occur prior to the release of RNA from the site of transcription. We next studied whether the ActD triggered release of snRNP from transcription sites could be inhibited by drugs that affect 3' processing of RNA. The drugs 9-β-D-arabinofuranosyladenine (AraA) and cordycepin (3dA) were used to inhibit 3' processing of RNA. AraA is a stereoisomer of ATP reported to specifically inhibit both 3' cleavage and polyadenylation in vitro (Ghoshal and Jacob, 1991); it inhibits polyadenylation in vivo with only minor effects on RNA synthesis (Rose et al., 1982). 3dA functions as a chain terminator and affects polyadenylation without affecting 3' cleavage (Ghoshal and Jacob, 1991; Rose et al., 1982). Treatment with ActD or simultaneous treatment with ActD and 3dA for 30 minutes gave the same 5-fold reduction in ring cells, indicating that inhibition of polyadenylation does not prevent the release of snRNP from transcription sites (II, fig. 5B). By contrast, the reduction of ring cells was only 2-fold when cells were treated with ActD and AraA (II, fig. 5B). Thus, AraA inhibited the release of snRNP from transcription sites. This suggests that 3' cleavage may be important for efficient release of snRNP from sites of transcription. Our data does not indicate a critical role for poly(A) addition in snRNP release from transcription sites.

Phosphorylation and dephosphorylation are important regulatory processes involved in several cellular mechanisms, including splicing (Mermoud et al., 1992, 1994; reviewed in Misteli, 1999). We studied the effect of staurosporine (stp) and okadaic acid (oa) on the release of snRNP from transcription sites in Ad-infected cells. These drugs were previously shown to affect the movement of SR-protein splicing factors relative to IGCs in uninfected cells (Misteli et al., 1997). Stp is a non-specific kinase inhibitor (Gadbois et al., 1992), while oa inhibits protein phosphatases 1 and 2A, which are involved in dephosphorylation of SR-proteins (Mermoud et al., 1994). Oa did not inhibit the ActD induced reduction of ring cells, while a simultaneous treatment with stp and ActD showed only a 1.5-fold decrease of ring cells when compared to untreated cells (II, fig. 5C). This suggests that a stp sensitive phosphorylation step is important for efficient release of the RNP complex from transcription sites.
In summary, energy-requiring mechanisms precede the release of the RNP complex from sites of transcription. Our data suggest that 3' cleavage and phosphorylation are two energy dependent steps that may occur prior to efficient release of the RNP complex from transcription sites in Ad-infected cells.

**E4 products are needed for efficient localization of snRNP and late viral mRNA in IGCs (paper III)**

The transcription of mRNA from the MLTU is dependent on the onset of the viral DNA replication (Thomas and Mathews, 1980). E4 mutants make normal levels of viral DNA when cells are infected at high multiplicities and DNA levels are determined at late times of infection (20-24 hpi) (Bridge et al., 1993b). Despite the production of wild-type DNA levels, E4 mutants are still defective for both nuclear and cytoplasmic late mRNA accumulation (Halbert et al., 1985; Weinberg and Ketner, 1986; Bridge and Ketner, 1989). In this paper we studied splicing factor reorganization in HeLa cells infected with the E4 mutants H5dlSma (Hemström, 1991) and H5dl1007 (Bridge and Ketner, 1989) (III, fig. 1). E4 mutants are propagated on a complementing cell line, W162, which contains and expresses a stable integrated copy of the E4 region (Weinberg and Ketner; 1983). Large E4 deletion mutants have similar defects in late gene expression (Halbert et al., 1985; Bridge and Ketner, 1990; III, fig. 2).

**Ring cells are present at normal levels in E4 mutant infections**

We studied nuclear organization of splicing factors and viral late gene expression in HeLa and W162 cells infected by the E4 mutant viruses, H5dlSma and H5dl1007, relative to wild-type (wt) Ad5. The fraction of penton containing cells was similar in wt and H5dlSma infected W162 cells (III, fig. 2A), indicating that the mutant is not defective in the complementing cell line, as expected. In HeLa cells, the fraction of H5dlSma infected cells expressing late viral proteins was decreased by a factor of 10 at 18 hpi, compared to wt (III, fig. 2A c and i). Similarly, when late viral proteins were detected by western blotting, the E4 mutant was severely defective for the production of late proteins (III, fig. 2B) This is in agreement with previous studies showing that E4 deletion mutants are defective for late gene expression. We then studied the snRNP distribution patterns in E4 mutant infected cells by immunostaining and microscopy, to compare the fraction of ring and cluster cells with wt infections. Ring cells were present in E4 mutant infected cultures at normal levels (III, fig. 3A, a and b), and the transcription sites in E4 mutant infected cells revealed a similar staining pattern around viral replication sites as has been described for wild type (II, fig. 1 and III, fig. 4).

Experiments were next performed under infection conditions that resulted in equal levels of viral DNA in mutant and wt infected cells, to
better compare differences in late gene expression not derived from differences in DNA accumulation. Cells were harvested at 22 hpi and DNA accumulation, levels of cytoplasmic viral RNA, nuclear distribution of snRNPs and the fraction of penton containing cells were measured (III, table I). The fraction of ring cells was not reduced in E4 mutant infections. By contrast, cytoplasmic levels of viral RNA were decreased 10-fold, and the fraction of cluster cells and penton positive cells was decreased 4-fold. This suggests that E4 deletion mutants do not efficiently promote the accumulation of viral RNAs in IGCs, or in the cytoplasm.

Localization of RNA in cells infected by E4 mutants

The number of ring cells appeared normal at late times after infection by the E4 mutant, but the number of cluster cells and late protein containing cells was decreased (III, fig. 3, Table 1). During wt Ad5 infection, viral RNA is detected at transcription sites in ring cells and accumulates posttranscriptionally in IGCs. We next determined the localization of late viral RNA in cells infected with E4 mutants. In situ hybridization with the SJ1 probe revealed that the posttranscriptional accumulation of spliced late viral messages in IGCs (III, fig. 5A) and in the cytoplasm (III, fig. 5B) in E4 mutant infected cells was less efficient than in wt infected cells. We suggest that proteins from the E4 region promote posttranscriptional accumulation of spliced viral mRNA in IGCs and in the cytoplasm. The observation that the accumulation of viral RNA in IGCs and the cytoplasm is inefficient in E4 mutant infected cells, further supports a model wherein late viral mRNA accumulates in IGCs during a nuclear step in maturation or transport.

Ad late gene expression is not dependent on the CRM-1 mediated nuclear export pathway (paper IV)

The precise mechanism used by Ad to export its late viral mRNA is not known. Genetic studies have suggested a role for the 34K-55K complex in promoting viral RNA export while inhibiting cellular mRNA transport, and both proteins are reported to have a Rev-like NES (reviewed in Dobner and Kzhyshkowska, 2001). Dobbelstein et al. (1997) proposed that the export of Ad late mRNA relied upon the NES of 34K. In paper IV we set out to test this model in infected cells.

Efficient complementation of an E4 mutant virus with a plasmid construct expressing a 34K NES mutant

We first studied whether the NES of 34K is important for complementing late gene expression. Previous studies have shown that 34K can complement the late gene expression defect of a large E4 deletion mutant in trans, when the protein is expressed from a plasmid construct in E4 mutant infected cells (Ketner et al., 1989). We used this assay to
compare complementation by a wild type 34K (wt34K) construct to a construct bearing the same double mutant in the NES of 34K (34KNES) that was reported to abolish export of the protein (Dobbelstein et al., 1997). Transfected/infected cells were scored for the presence of late proteins to assay for late gene expression. Immunostained cells were first examined for the presence of 34K and then for the presence or absence of the late protein penton. We detected no difference in the number of cells expressing both 34K and late genes in complementation assays done with the wt34K and 34KNES mutant expression construct, indicating that the NES of 34K was not crucial for complementing late gene expression in trans (IV, fig. 1). This was confirmed by western blot analyses, showing that similar levels of 34K expressed from NES mutant and wt constructs, resulted in similar levels of late protein production in the transfected/infected cells (data not shown). This indicated that the NES of 34K is not crucial for the transport of late viral mRNA and the production of late proteins.

The 34K shuttling in infected cells is sensitive to leptomycin B

We next sought to establish whether 34K shuttles between the nucleus and the cytoplasm in infected cells. Previous investigators had only studied 34K shuttling in transfected cells (Goodrum et al., 1996; Dobbelstein et al., 1997). We developed a cell fusion assay to detect the movement of 34K from infected to uninfected nuclei within multinucleated cells. We reasoned that the number of stained cells/microscope field would increase in fused compared to unfused cells, if 34K was able to shuttle from one nucleus to another within the fused syncytia. With this assay we showed that 34K shuttles in infected cells (IV, fig. 3A). The shuttling was sensitive to the drug leptomycin B (LMB). LMB inhibits the formation of CRM-1 export complexes, and therefore inhibits the Rev-like NES mediated pathway (Fornerod et al., 1997; Wolff et al., 1997). Our data suggest that the CRM-1 export pathway at least in part mediates 34K shuttling.

Late gene expression is not dependent on the CRM-1 mediated nuclear export pathway

We did not observe a requirement for an intact NES for the ability of 34K to complement late gene expression of a defective E4 mutant. To further investigate the role of the Rev-like NES export pathway for viral late gene expression, we studied the effect of LMB on viral late gene expression. Infected cells were treated with LMB between 12-18 hpi to inhibit CRM-1 mediated export, or ActD to inhibit RNA transcription. Late gene expression was assayed by immunostaining for the viral protein penton. We measured a 40% increase in penton containing cells between 12-18 hpi, in both the presence or absence of LMB (IV, fig. 2A), which suggests that viral mRNA derived from the MLTU is not dependent by the CRM-1 mediated pathway. ActD treatment completely prevented the increase in the number of penton containing cell as expected. This shows
that no increase in the fraction of penton containing cells occurred when the production of new viral RNA was blocked by inhibiting transcription. This result further suggests that the increase in penton positive cells we observed in the presence of LMB was due to an increase in the number of cells that had exported newly synthesized viral RNA to the cytoplasm, rather than continued translation of pre-existing cytoplasmic RNA. Late gene expression was quantified by western blot analyses, and the amount of late viral proteins expressed in the absence or presence of LMB between 12 to 18 hpi revealed no differences (IV, fig. 2B). This indicates that the cytoplasmic accumulation of viral mRNA used for translation was not decreased in LMB treated cells.

In summary, we showed that 34K shuttles in infected cells. The shuttling is sensitive to LMB suggesting that the NES of 34K is responsible for its ability to shuttle, at least in part. However, our data do not support a model where the NES of 34K is required for transport of MLTU derived transcripts. Late gene expression was not sensitive to LMB, and a 34KNES mutant construct complemented late gene expression equally well as wt34K constructs.
DISCUSSION

This thesis investigates the nuclear organization of Ad late gene expression. Previous data have suggested that viral DNA synthesis, transcription and processing of transcripts may require a precise spatial organization (Pombo et al., 1994). I will first discuss the nuclear organization of replication and transcription in Ad infected cells, before discussing the potential role of IGCs in late gene expression, and what mRNA transport pathway is used during Ad late gene expression.

Sites of replication and transcription in Ad infected cells

Replication and transcription take place at the periphery of 72K centers in the peripheral replicative zone (Puvion-Dutilleul et al., 1992; Pombo et al., 1994). Double-stranded (ds)DNA is released from replication sites to the surrounding nucleoplasm, where it is used for transcription (Pombo et al., 1994). The 72K centers in ring cells appear as large rings and circles in a confocal or electron microscopy section (Puvion-Dutilleul and Pichard, 1992; II, fig.1, 2 and 3). In cluster cells, 72K centers are smaller, dense structures, that appear when the large amorphous 72K centers characteristic of intermediate phase cells break apart (Bridge et al., 1995; II, fig. 1 and 4).

We do not yet know if breaking up of the large 72K centers and release of viral dsDNA from 72K containing centers is important for subsequent steps in viral late gene expression. We have performed some preliminary experiments with aphidicolin treated infected cells, and noted that this drug affects the release of viral DNA from replication sites to the surrounding nucleoplasm, as well as the break-up of large amorphous 72K centers to the small dense centers characteristic of the late phase. Aphidicolin is reported to inhibit the cellular DNA polymerase α, but not the viral DNA polymerase at low concentrations (Kwant and van der Vliet, 1980). Indeed, large 72K centers are observed in aphidicolin treated cells, suggesting that substantial viral DNA replication has taken place. We do not know why aphidicolin inhibits viral DNA release and break-up of 72K centers. Aphidicolin treated cultures had a higher fraction of ring cells. At 14 hpi 75% of the cells in the culture were ring cells compared with only 25% in untreated cultures. The number of cluster cells containing the late protein penton was initially delayed, but was comparable to untreated cells at later times of infection, suggesting that interfering with the release of replicated DNA and/or the break-up of 72K centers might delay some aspect of late gene expression. Interestingly, similar observations are made in E4-deletion mutants. The 72K centers in infections with E4-deletion mutants grow extremely large and fail to break up into the smaller, condensed centers seen in wild type infected cells (II, fig. 4, and III, fig. 4, and data not shown). Preliminary data also indicate that the release of replicated dsDNA from 72K centers was inhibited in E4 mutant infected cells (Aspegren, unpublished results). The E4 mutants described in paper
III had two fold higher levels of ring cells than wt infected cells (III, fig. 3), and were reduced/delayed for the formation of cluster cells (III, fig. 2 and 3). E4-deletion mutants form concatemers of replicated DNA (Weiden and Ginsberg, 1994), and it was recently shown that the E4 products of 11K and 34K bind to and inhibit a kinase involved in dsDNA break repair (Boyer et al., 1999). If breaking up of the 72K centers is important for the efficient coordination of transcription, processing and export of late viral RNA, then this could at least partially explain why late viral transcripts fail to accumulate efficiently in the nucleus and cytoplasm of E4 mutant infected cells (Bridge and Ketner, 1989, 1990; III, fig. 5). Further experiments are needed to investigate the precise role of 72K and DNA organization in the nucleus for subsequent events in viral late transcription and processing. It would be interesting to study whether aphidicolin induces concatemer formation in wild type infections, since this could explain why release of dsDNA from replication sites was inhibited. A human glioma cell line used by Boyer et al. (1999) lacks a kinase involved in dsDNA break repair. It would be of interest to test if the defect in late gene expression of E4-deletion mutants is less severe in this cell line, which could indicate whether 72K break-up and/or release of dsDNA are indeed important events for efficient late gene expression.

The release of snRNP and poly(A) RNA from transcription sites

Splicing factors and poly(A) RNA are released from transcription sites when transcription is blocked by ActD (II, fig. 2 and 3). The release is blocked at 4°C, which shows that the release requires energy (II, fig. 5A). Since posttranscriptional processing events may initiate co-transcriptionally, it is likely that at least some steps in RNA processing occur prior to the release of nascent transcripts.

We find that the drug AraA (II, fig. 5B), inhibited release of snRNP from viral transcription sites. AraA inhibits both 3' cleavage and polyadenylation in vitro and in vivo (Rose et al., 1982; Zeevi et al., 1982; Ghoshal and Jacob, 1991; Ioannidis et al., 1999). By contrast, the drug cordycepin (3dA), which only inhibits polyadenylation (Rose et al., 1982; Ghoshal and Jacob, 1991), did not inhibit the release of snRNP from transcription sites (II, fig. 5B). We have suggested that snRNP leaves transcription sites in a complex with RNA in virus infected cells. Our results suggest that 3' cleavage, but not polyadenylation of the nascent transcript is required for release of snRNP and RNA. This is in agreement with Custódio et al. (1999) who find the efficient release of beta-globin RNA from its site of transcription also requires 3' cleavage.

Splicing is another energy requiring process that could begin prior to release of snRNP and RNA from transcription sites. Mutant beta-globin RNAs that fail to properly splice are inefficiently released from transcription sites (Custódio et al., 1999), and it was suggested that a fully functional spliceosome did not assemble at the mutated intron (Lamond et
al., 1987; Custódio et al., 1999). The assembly of spliceosomes is dependent on the phosphorylation of SR proteins, while the splicing reaction requires dephosphorylation of assembled SR proteins (reviewed in Misteli, 1999). The release of snRNP from transcription sites was inhibited in Ad-infected cells simultaneously treated with ActD and the broad-spectrum kinase inhibitor staurosporine (II, fig. 5C). By contrast, the release was not inhibited by the drug okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A (II, fig. 5C). Both staurosporine and okadaic acid were previously reported to influence the nuclear staining pattern of SR proteins (Misteli et al., 1997), indicating that the phosphorylation state of SR proteins can affect their localization in vivo. It is possible that staurosporine treated cells did not efficiently assemble spliceosomes, and that this in turn inhibited the release of nascent transcripts and snRNP (II, fig. 5C). This possibility has not yet been tested. It is also possible that staurosporine inhibits release by affecting a kinase that is not involved in splicing. Staurosporine inhibits several families of kinases (Gadbois et al., 1992), like the src and protein kinase C families (Weng et al., 1994; Schullery et al., 1997). These both kinase families phosphorylate hnRNP K, a protein recently suggested to function as a platform for the assembly of proteins involved in processing of pre-mRNAs (Ostrowski et al., 2000). The inhibition of snRNP release from transcription sites caused by staurosporine could be due to a general effect on the formation of the RNP complex. Clearly more experiments are needed to understand how release of RNA and snRNP from transcription sites might be regulated by phosphorylation.

**Role of IGCs during RNA maturation**

IGCs are thought to function as storage or preassembly sites of splicing factors (reviewed in Misteli and Spector, 1998). IGCs also contain members of several families of proteins implicated in mRNA production; these include SR protein kinases (Colwill et al., 1996; Wang et al., 1998), poly(A) binding protein 2 (PABP2), (Schul et al., 1998) and proteins that bind preferentially to spliced RNA and may be involved in RNA transport, RNPS1, Y14 and REF/Aly (Mayeda et al., 1999; Kataoka et al., 2000; Zhou et al., 2000). These observations indicate that IGCs are not just storage sites for splicing factors.

IGCs contain numerous proteins involved in RNA production but they also contain RNA. Several cellular gene transcripts localize adjacent to or in IGCs (Xing et al., 1993, 1995; Huang and Spector, 1996b; Ishov et al., 1997; Smith et al., 1999; Snaar et al., 1999; Johnson et al., 2000; Melcák et al., 2000). Polyadenylated RNA in uninfected cells also localizes in IGCs (Carter et al., 1991; Visa et al., 1993). In Ad-infected cells at least partially spliced late RNA accumulates in IGCs (Bridge et al., 1993a, 1995, 1996; paper I), as well as polyadenylated RNA released from transcription sites upon transcription inhibition with ActD (II, fig. 3). It is not yet clear why
some RNA transcripts accumulate in IGCs; several possibilities are discussed below.

Intron-containing transcripts (Johnson et al., 2000) and intron-containing RNAs injected into the nucleus (Melcák et al., 2001) localize in IGCs. Melcák et al. (2001) demonstrated that mutant intron containing RNAs that could not form a spliceosome complex, also failed to accumulate in IGCs. These results indicate that the presence of an intron can target RNA to IGCs and raise the possibility that introns may be spliced at IGCs. The data also suggests that spliceosome assembly is needed to target the RNAs to IGCs. Melcák et al. (2001) suggest that for some transcripts, the splicing process may not be completed by the time of release from transcription sites. For these transcripts, posttranscriptional splicing might occur at IGCs where there is a high splicing factor concentration. Other transcripts may mature at sites of transcription and then be directly transported to the cytoplasm without accumulating in IGCs. The presence of introns in polyadenylated Ad MLTU (Nevins and Darnell, 1978) suggest that at least some Ad viral transcripts are spliced after their release from sites of transcription; Ad late transcripts also accumulate in IGCs. Recently Mintz et al. (1999) developed a biochemical method to purify IGCs. It would be very interesting to study the population of RNAs in biochemically purified IGC fractions for the presence or absence of introns.

The fate of RNA present in IGCs is controversial. Huang et al. (1994) reported that poly(A) RNA is not chased out of IGCs after transcription inhibition, and suggested that this population of nuclear poly(A) RNA is not mRNA, but structural RNA involved in other nuclear functions. It was recently reported that PABP2 needs poly(A) RNA to localize in IGCs (Calado and Carmo-Fonseca, 2000), and it is possible that some structural poly(A) RNA is involved in nuclear organization. However, Visa et al. (1993) reported a significant decrease of poly(A) RNA staining in IGCs after inhibition of transcription, which is in contrast to Huang et al. (1994). Other groups have detected RNA from cellular or viral genes in IGCs and suggested that RNAs localized in IGCs are in transit to the cytoplasm (Xing et al., 1995; Smith et al., 1999; Johnson et al., 2000; Melcák et al., 2000). In particular, a heterozygous defect in splicing of intron 26 of COL1A1 RNA, allowed Johnson et al. (2000) to study both the intron-mutated and the wt transcript in the same cell. They detected both transcripts in IGCs, but the mutated form overaccumulated in IGCs and was not transported to the cytoplasm. This suggests that this particular transcript is in transit through the IGC, since overaccumulation only occurred when the transcript could not fully mature. We think that the Ad RNA in IGCs may eventually be exported for the following reasons: i) accumulation of late RNA in IGCs occurs at the same time as RNA export to the cytoplasm (I, fig. 4-6, and data not shown), ii) tripartite leader containing RNA is efficiently exported during the late phase and it
accumulates in IGCs (Bridge et al., 1996; II, fig. 5), and iii) a viral mutant that is defective for cytoplasmic RNA accumulation inefficiently accumulates viral RNA in IGCs (III, fig. 5B). Leppard and Shenk (1989) showed that late viral RNA accumulates in a biochemically defined nuclear fraction downstream of the transcription sites in the nuclear matrix fraction, as a natural step during late viral gene expression. Although this has to be verified experimentally, it is possible that this nuclear fraction corresponds to IGCs. Accumulation of viral RNA in this nuclear fraction was reduced in cells infected by a viral mutant lacking the E1B 55K protein (Leppard and Shenk, 1989). We also find that the accumulation of snRNP and late viral RNA in IGCs was reduced in E4-mutant infected cells (III, fig. 2 and 5). These data support the view that both E1B 55K and E4 34K proteins allow stable accumulation of viral RNA at posttranscriptional sites in the nucleus (Sandler and Ketner, 1989; Bridge and Ketner, 1990; reviewed in Dobner and Kzhyshkowska, 2001).

If Ad RNAs accumulate posttranscriptionally in IGCs prior to transport, this may reflect a “bottle-neck” in Ad RNA maturation resulting in the visible accumulation of mRNA and snRNP in IGCs. Possible activities resulting in the accumulation of RNA in IGCs could be splicing, recycling of splicing factors from spliced RNA, or the formation of export complexes. More studies are of importance to get a final view of the exact involvement of IGCs in RNA maturation. We support a view where IGCs not only play a part as splicing factor storage or preassembly sites, but might also be involved in posttranscriptional splicing and/or sorting of RNAs and proteins to prepare the final RNP complex for transport to the cytoplasm, at least for transcripts of some genes.

**Transport of Ad late RNA from the nucleus to the cytoplasm**

Efficient transport of Ad RNA from the nucleus to the cytoplasm requires at least two viral gene products, the E4 34K protein from ORF6 and the E1B 55K protein. The 34K and 55K proteins form a complex (Sarnow et al., 1984), which is thought to mediate both viral and cellular mRNA transport (Halbert et al., 1985; Cutt et al., 1987; Bridge and Ketner, 1990; Ornelles and Shenk, 1991). The precise mechanism through which these proteins mediate RNA export in infected cells is not known. 34K can bind to and inactivate the p53 tumor suppressor (Dobner et al., 1996), and reduces the half-life of p53 together with 55K (Querido et al., 1997; Steegenga et al., 1998). 34K and 11K also interact with DNA protein kinase involved in dsDNA break repair (Boyer et al., 1999), and 34K is involved in regulation of splicing of MLTU derived transcripts (Öhman et al., 1993; Nordqvist et al., 1994). The 34K protein plays a critical role in efficient late gene expression, and it is of great interest to understand how the activities of the 34K protein contribute to its role in RNA transport in infected cells (reviewed in Dobner and Kzhyshkowska, 2001).
Goodrum et al. (1996) demonstrated that 34K shuttles in transfected cells. Dobbelstein et al. (1997) showed that a Rev-like NES in the N-terminal part of the protein is important for this shuttling, and proposed a model where the NES of 34K mediates late viral mRNA transport as part of a complex with 55K. We have tested this model by a complementation assay, and by inhibiting the Rev-like transport pathway with LMB. We find that a 34KNES mutant protein could complement late gene expression equally well as a wt 34K protein (IV, fig. 1). LMB did not inhibit late gene expression in wild type infected cells (IV, fig. 2), even though shuttling of 34K was inhibited by the drug (IV, fig. 3). This indicates that 34K shuttles by the CRM-1 mediated pathway but the pathway is not critical for late gene expression. Our data suggests that the transport of MLTU derived transcripts is not mediated by the NES of 34K. By contrast, Weigel and Dobbelstein (2000) used a similar complementation assay to test the function of the same 34KNES mutant. Their results indicate that the 34KNES is important for viral DNA synthesis, late mRNA transport and protein synthesis. We think that the different results obtained by our laboratory may be explained by the fact that the rate of infection differed significantly between our complementation assay and the assay reported by Weigel and Dobbelstein (2000). Our transfected/infected cells had expressed late viral proteins at 24 hpi following complementation with wt 34K expression constructs, while Weigel and Dobbelstein did not even detect viral DNA until 48 hpi in similar complementation assays with a wt 34K construct. At 72hpi viral DNA levels attained in complementation assays with the 34KNES mutated construct were significantly less than the level attained with wt 34K. It has previously been reported that E4-deletion mutants are defective in the onset of DNA synthesis at low multiplicities of infection (MOIs) (Weinberg and Ketner, 1986). The onset of DNA synthesis is critical for late gene expression (Thomas and Mathews, 1980). It is possible that the NES of 34K is important for efficient DNA replication, especially at lower MOIs. We have preliminary data suggesting LMB inhibits the early phase of infection in a portion of infected cells. Only 50-70% of Ad infected cells were stained for 72K when cells were treated with LMB between 2-12 hpi, compared to more than 95% in cells not treated with LMB (Aspegren, unpublished observation). This suggests that CRM-1 mediated nuclear export may affect the onset of viral DNA replication, and is in agreement with the data presented by Weigel and Dobbelstein (2000). We do not yet know if this reflects a defect in NES mediated protein shuttling or in NES mediated RNA export. While these data are consistent with a role for CRM-1 mediated nuclear export during viral early gene expression we have no evidence indicating that this pathway is crucial for the export of Ad late RNA. It will now be important to construct viral mutants that express the 34KNES mutant to more precisely determine the role of the 34KNES during infection.
Recent data suggests that 55K also has an NES and shuttles from the nucleus to the cytoplasm in infected cells (Kratzer et al., 2000). Shuttling of 55K was sensitive to LMB suggesting that 55K shuttles via the CRM-1 mediated nuclear export pathway (Dosch et al., 2001). These results suggest that the NES of 55K could provide the export signal for Ad mRNA transport. Indeed, these authors find that the 34KNES does not support the export of a heterologous protein construct, and suggest that the 34KNES is not functional (Dosch et al., 2001). We have also studied 55K shuttling in infected cells. We find that shuttling of 55K in our cell fusion assay is inhibited by LMB (Aspegren, unpublished results) in agreement with Dosch et al. (2001). We also find that in LMB treated infected cells 55K is restricted to the nucleus, whereas in untreated cells it is detected in both the nucleus and the cytoplasm (Carter and Bridge, unpublished results). These results are also in agreement with Dosch et al. (2001), and suggest that LMB could prevent the accumulation of 55K in the cytoplasm by restricting its export from the nucleus. Although LMB treatment inhibits both 55K and 34K shuttling in our experiments we saw little effect of LMB on viral late gene expression. We observed the same increase in late protein levels between 12-18 hpi when cells were treated with 0, 10, 30 or 100 nM LMB (IV, fig. 3, and data not shown). Our results support the idea that these viral proteins use export and import signals to shuttle back and forth between the nucleus and the cytoplasm, but as yet there is no evidence to suggest that these shuttle proteins carry viral late RNA to the cytoplasm during their export.

In summary, 34K is important for late gene expression, but the role of its NES is currently under debate. Our data suggest that the involvement of 34K in late viral mRNA transport does not require its NES signal. Instead, it is possible that 34K might be important for the posttranscriptional accumulation of viral RNAs in IGCs, from where the RNA may be more efficiently transported to the cytoplasm. The accumulation of MLTU transcripts in a nuclear compartment downstream of the transcription sites seems to be rate limiting and requires 55K (Leppard and Shenk, 1989). This may also be true for 34K. The 34K and 55K proteins are multifunctional, and we do not yet know how their functions might contribute to viral RNA transport. A reasonable explanation could be that 34K inhibits concatenation of viral genomes during replication, which might facilitate release of DNA from replication centers and make it more accessible to the RNA transcription, processing, and export machinery. 34K also regulates splicing, and since splicing is important for the production of export competent RNPs, this activity could also contribute to the role of 34K in RNA transport. The 34K protein relocalizes 55K to transcription/replication sites, which in turn might relocalize a limiting cellular transport protein, like E1B-AP5, to transcription-/replication sites. The analysis of 34K and 55K mutants with specific defects in individual
functions will greatly clarify the role of these activities in viral RNA accumulation and transport.

In the past the nucleus has been viewed as just a storage space for the genome, with little obvious organization outside of the nucleolus. Recent studies suggest that gene expression is highly organized within the nucleus. The mechanisms by which the approximate 30,000 human genes are regulated in terms of cell specificity, alternative splicing, 3’ processing or mRNA transport are some crucial issues for future experiments. Greater knowledge will continue to accumulate from a variety model systems. Ad-infected cells have provided us with a good model system to study nuclear organization during RNA maturation and transport. It will now be interesting to see what future Ad infections will tell us about the nuclear archipelago of coiled bodies, ND10 domains, the nucleolus, PFs, IGCs, dots and spots.
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