Selective effects on adenovirus late gene expression of deleting the E1b 55K protein

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The E1b gene of human adenovirus 5 (Ad5) encodes a 55K product previously shown to be required for the efficient accumulation of mRNAs derived from the major late primary transcript in the cytoplasm of infected cells. This finding is extended here to include the transcripts from other viral promoters activated during the late phase of infection. Conversely, accumulation of mRNA derived from the major late promoter at early times is not dependent on this E1b function.

Introduction

Human adenovirus serotype 2 or 5 gene expression during lytic infection of cultured cells has been widely studied as a model system for the control of gene expression in eukaryotes (for review, see Horwitz, 1990). Viral transcription has two phases, early and late, separated by the onset of DNA replication. Primary transcripts from all but one of the viral promoters are spliced; those from the major late promoter (MLP) in particular show extensive differential splicing and also differential polyadenylation. These events are clearly regulated, since the pattern of processed mRNAs deriving from a particular transcript varies with time post-infection (p.i.) (for review, see Flint, 1986), although the basis for this control is not understood.

A further post-transcriptional control is exerted on the movement of mRNA from the site of processing in the nucleus to the cytoplasmic pool of translatable mRNA and/or on its stability. Studies of adenovirus 5 (Ad5) mutant viruses have implicated the E1b 55K protein (Babiss et al., 1985; Pilder et al., 1986; Williams et al., 1986) and the products of E4 open reading frames (ORFs) 3 and 6 (Halbert et al., 1985; Sandler & Ketner, 1989; Bridge & Ketner, 1989; Huang & Hearing, 1989) in this process. Genetic and biochemical evidence suggests that E1b 55K and E4 ORF6 function as a molecular complex (Sarnow et al., 1984; Cutt et al., 1987). This complex is located in the nucleus of infected HeLa cells in association with virus-specific inclusions that are believed to be sites of DNA replication and transcription within the late-infected cell (Ornelles & Shenk, 1991). No association of E1b 55K with nuclear pore complexes or with the nuclear periphery has been observed (Smiley et al., 1990; Ornelles & Shenk, 1991).

Ad5 mutant dl338, which is unable to synthesize an intact E1b 55K protein, shows a post-transcriptional defect in late viral gene expression at the level of RNA transport (Pilder et al., 1986). This defect is manifest early in the pathway of gene expression in the nucleus, with susceptible RNAs showing impaired release from the site of transcription and processing in the nucleus and a concomitant increase in intranuclear turnover (Leppard & Shenk, 1989). However, E1b 55K function is not required for the cytoplasmic accumulation of all mRNAs in the infected cell; accumulation of viral MLP-derived mRNA is E1b-dependent whereas mRNA transcribed from early promoters accumulates equally well in wild-type and in dl338-infected cells (Pilder et al., 1986). This E1b function also affects cellular gene expression since some cell mRNAs whose accumulation is blocked during the late phase of a wild-type infection show continued accumulation in its absence (Pilder et al., 1986). Accumulation of other cell mRNAs whose transcription is induced by Ad5 infection is unaffected (Moore et al., 1987). Explanations for the selective action of the E1b/E4 complex have focused either on RNA sequence-specific effects or on differences in the functional environment of early and late viral and cellular transcription complexes within the nucleus (Moore et al., 1987; Leppard & Shenk, 1989) with the balance of evidence favouring the
latter model. Results presented here show that no viral RNA, even from the MLP, is Elb-dependent at early times but RNAs from all the late-active promoters show some measure of dependence at late times, further arguing against the sequence-specific model. Differences in the degree of dependence on Elb 55K function displayed by individual late mRNAs suggest that RNA processing, as well as the subsequent handling of mature mRNA, might play a role in the manifestation of the Elb 55K-deficient phenotype.

**Methods**

*Isolation of RNA from infected cells.* The origins of Ad5 dl309 and dl338 and the methods for their growth in cell culture have been described previously (Leppard & Shenk, 1989). All experiments were carried out in HeLa cells at a multiplicity of infection of 10. Cytoplasmic and nuclear fractions of infected cells were prepared by Nonidet P40 lysis and subsequent low-speed centrifugation, and total RNA was prepared from each fraction by phenol/chloroform extraction in the presence of SDS and EDTA, all as described (Leppard & Shenk, 1989).

Quantitative RNase protection assays were carried out according to Melton et al. (1984) using 5 μg of unselected cytoplasmic or nuclear RNA and an excess of [α-32P]UTP-labelled antisense probe RNA transcribed in vitro from clones of appropriate fragments of Ad5 genomic DNA in pGEM vectors (Promega). Protected fragments were separated on 5% polyacrylamide–7 M-urea gels and detected by exposure to preflashed Fuji RX film at −70 °C. Protected fragments were quantified by laser scanning densitometry (Molecular Dynamics) using suitable film exposures.

*Measurement of transcription rates.* Transcription rates were measured by run-on assay in isolated nuclei (Hofer & Darnell, 1981; Groudine et al., 1981) as previously described (Leppard & Shenk, 1989). M13 antisense clones for MLP segments, L1, L2 and L3, were as described by Pilder et al. (1986). The IVa2 antisense ssDNA clone was a BstEII (5186) to XhoI (5788) fragment of Ad5 genomic DNA cloned between Smal and SalI sites of M13 mp18.

**Results**

*mRNAs from minor late promoters are Elb-dependent*

A simplified transcription map of Ad5, indicating the positions of the various promoters of transcription, is shown in Fig. 1. The RNA transport defect for late viral mRNAs displayed by dl338 was defined previously by assaying mRNAs derived from the Ad5 MLP (Pilder et al., 1986). However, three other Ad5 promoters, for polypeptides IX and IVa2, and the late E2 (E2-L) promoter, are active only during the late phase of infection (Chow et al., 1979; Goldenberg et al., 1981; Crossland & Raskas, 1983). To determine whether the dl338 phenotype extended to products of these late viral promoters, cytoplasmic mRNA levels in wild-type (dl309)-and Elb mutant dl338-infected cells were determined by RNase protection assays using probes transcribed from appropriate cloned fragments of Ad5 genomic DNA (Fig. 2a). Results of these assays are shown in Fig. 2(b).

Control early RNAs from the early E2 (E2-E) and Elb promoters were present at similar levels in both wild-type and dl338 infections at 8 and 12 h p.i. In contrast, IX, IVa2 and E2-L mRNAs were first detected at 12 h p.i. and, at this time, cytoplasmic levels were depressed by between twofold for E2-L and sixfold for IVa2 in the dl338 infection. Later, E2-E mRNA levels increased in dl338 infection relative to wild-type infection. This effect is due, at least in part, to the increased E2 transcription rate of dl338 compared to wild-type in the late phase, as observed previously (Pilder et al., 1986). The cytoplasmic level of total Elb mRNA shows an increase in wild-type over mutant in the late phase. Levels of Elb mRNA may be directly affected, however, by the dl338 lesion in this transcription unit. It is not possible to determine from these data whether accumulation of Elb and E2 mRNA, newly synthesized from the Elb and E2-E promoters after the onset of replication, is sensitive to Elb 55K protein function.

Previous experiments have shown that transcription rates across the major late transcription unit, determined by nuclear run-on assays, do not vary significantly between wild-type and dl338 at this early stage of the late phase of infection (Pilder et al., 1986). The E2-L and IX promoters are embedded within the active E2-E and Elb transcription units respectively (Fig. 1), complicating analysis of the transcription rate from these promoters. Although the IVa2 promoter is similarly embedded within the E2b segment of the E2 transcription unit, the rate measured across E2b was negligible, making possible an estimate of the transcription rate across IVa2 due to its own promoter. Results of this analysis are shown in Fig. 3. At 12 h p.i. the transcription rate ratio, wild-type to dl338, was 3. After taking account of this transcription
rate difference, there remains a twofold decrease in \textit{dl338} IVa2 cytoplasmic mRNA expression, attributable to a post-transcriptional defect. This result suggests that E1b dependence is not restricted to MLP-derived transcripts, but may be a more general feature of mRNAs expressed from the viral genome at late times.

\textbf{Major late transcripts at early times are E1b-independent}

The MLP is weakly active at early times, giving rise exclusively to an mRNA encoding the L1 52K/55K proteins (Lewis & Mathews, 1980; Akusjarvi & Persson, 1981). Production of this mRNA continues into the late phase, when its accumulation is dependent on E1b 55K protein function (Pilder et al., 1986). When assayed by RNase protection (Fig. 4), the cytoplasmic levels of this RNA at both 8 and 12 h.p.i. were essentially the same for \textit{dl338} as for wild-type virus, although they diverged thereafter, in agreement with previous data. In contrast, L1 IIIa mRNA, produced only after replication, shows E1b-dependent accumulation from its earliest detection. These results show that expression of L1 52K/55K mRNA is not intrinsically E1b-dependent, but rather that its expression in the late phase of infection renders its accumulation E1b 55K-dependent.

\textbf{mRNAs within major late families differ in E1b dependence}

If an adverse environment for late gene expression was the only factor determining the phenotype of viruses such as \textit{dl338}, then one might expect all late mRNAs to show a similar defect at any given time p.i. Previous data have suggested this is not the case, with significantly different levels of defect between the five major late gene segments being reported (Pilder et al., 1986). To examine this point further, the levels of individual mRNA products of major late regions L1, L2 and L3 in \textit{dl309} and \textit{dl338} infections were determined. Details of the RNase protection probes used and the results of these analyses are shown in Fig. 4 (L1) and Fig. 5 (L2, L3).

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\textbf{Fig. 2.} (a) Schematic representation of the probes used for the RNase protection assays of mRNA levels as shown in (b). Ad5 genomic fragments cloned into pGEM vectors are shown as thick lines, with nucleotide (nt) positions according to the numbering of Chroboczek et al. (1992). Uniformly labelled antisense probe RNA transcribed from these clones \textit{in vitro} is shown as \ldots and mRNA as thin lines. Protected fragment lengths expected after hybridization of probe and mRNA are indicated above each mRNA. (b) RNA was prepared from wild-type (\textit{dl309}, 309 on this and subsequent figures) and mutant (\textit{dl338}) virus-infected cells at the times (h) p.i. indicated, and probed for specific mRNAs as on the left of the panel.
Transcription rates across the L1, L2 and L3 major late segments were determined by run-on assays in nuclei isolated at the time of cytoplasmic RNA preparation from a set of parallel infections (Fig. 6). mRNAs detected in Fig. 4 and 5 were quantified by densitometric scanning of suitable film exposures. These data are shown in Fig. 7, expressed as a wild-type/d1338 ratio, together with the ratio of transcription rates derived from Fig. 6.

For each segment of the major late unit assayed, the transcription rate ratio rose with time p.i., as has been seen previously (Pilder et al., 1986), although the ratio at 12 h p.i. is higher than seen before. This finding does not reflect errors in the relative multiplicity of infection since the cytoplasmic mRNA levels for early transcripts at 8 h p.i. are approximately equal (ratio 0.8 to 1.0) for E1b and E2-E (Fig. 2) and also for other early mRNAs (data not shown). Differences in transcription rate ratio between experiments may reflect differing extents of nascent RNA degradation during the assay since a deficiency in either component of the E1b 55K/E40RF6 complex affects nuclear RNA stability (Sandler & Ketner, 1989; Leppard & Shenk, 1989).

In the absence of any other effects, the ratios of cytoplasmic levels for all MLP mRNAs would be expected to follow the relevant rate curves, subject to a time lag due to the cytoplasmic levels being steady-state measurements. Instead, Fig. 7 shows that individual MLP mRNAs are either favoured or discriminated against in
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Fig. 6. Transcription rates across the L1, L2 and L3 (a to c) segments of the major late region. Details as for Fig. 3. Counts specifically bound to each probe were corrected for differences in the numbers of uridine residues in each region probed. □, dl309; △, dl338.

Fig. 7. dl309/dl338 ratios for individual late cytoplasmic mRNA species from major late segments L1, L2 and L3 (a to c). Amounts of individual mRNA species were quantified by scanning densitometry of suitable autoradiographic exposures of primary data from RNase protection assays (Fig. 4 and 5) and data used to produce ratios, shown here as a function of time p.i. Also shown are transcription rate ratios derived from data shown in Figure 6.

dl338 as compared to dl309 infection. The longest mRNA in each of the L1, L2 and L3 families (52K/55K, III and pVI) becomes progressively less abundant relative to wild-type levels as the infection proceeds. In contrast, the shortest mRNAs in each family (IIIa, mu and 23K protease) are less severely affected (IIIa) or become more abundant relative to wild-type levels. RNAs of intermediate length, pVII in L2 and II in L3, display behaviour similar to the shortest species from these families; protein V mRNA levels exhibit no clear trend.

These data could be explained if altered splice site usage occurred during MLP RNA processing in a dl338
Fig. 8. (a) RNase protection assays of L3 nuclear RNA species from dl309- and dl338-infected cells. Probes as shown in Fig. 5(a). (b) dl309/dl338 ratios for individual L3 nuclear RNA species. Details as for Fig. 7.

infection. A previous study of dl338 (Pilder et al., 1986) did not reveal any effect on MLP RNA splicing efficiency, although only the L5 region, where differential splicing does not occur, was examined. The same study also examined expression of artificial gene constructs within the Ad5 genome that either did or did not require splicing to form mRNA, and found no difference in the extent of dependence of expression from these constructs on E1B 55K function. Again, no differential splicing was expected from either construct.

To examine further the possibility that the pattern of splice site usage might be altered during a dl338 infection, total nuclear RNA was prepared from both wild-type and dl338-infected cells and levels of individual spliced L3 RNAs were determined. As shown in Fig. 8, the wild-type/dl338 ratios for each L3 product were essentially constant, discounting the possibility that altered relative usage of the L3 splice acceptor sites was the basis of the results in Fig. 7. It appears therefore that the expression of a subset of MLP mRNA species is differentially sensitive to the absence of the E1b 55K protein, resulting in an increasingly severe reduction in the cytoplasmic levels of these mRNAs as the late phase of a dl338 infection proceeds.

Discussion

In previous studies (Moore et al., 1987; Leppard & Shenk, 1989) it was proposed that some feature of the local environment in the cell nucleus, in which late viral gene expression occurred, necessitated the provision of a function from E1b to achieve optimum cytoplasmic mRNA accumulation. The idea was founded on a proposal by Blobel (1985) that expression of specific genes occurred in a restricted local environment in the nucleus which was ‘gated’ to the cytoplasm so that random diffusion of specific RNAs within the nucleus did not occur. This last point was subsequently demonstrated, for expression from an integrated Epstein–Barr virus genome, by in situ hybridization (Lawrence et al., 1989). According to this model, an early viral transcription complex would occupy a vacant expression microenvironment in the nucleus, its products therefore being expressed independently of E1b 55K function. In the absence of sufficient vacant sites, late complexes would, in contrast, have either to compete with pre-existing complexes for a site or be expressed in a relatively unfavourable position elsewhere in the nucleus. In either case, expression would be facilitated in some way by the E1b 55K/E4 ORF6 complex.

Data presented here demonstrate that the E1b dependence of late viral mRNA accumulation extends from MLP products to other viral mRNAs expressed exclusively during the late phase of infection, and that the single MLP mRNA that is expressed at early times is not dependent on E1b function at this time, although it becomes so at late times. These results indicate that it is the expression of an mRNA from a late viral transcription complex that renders its accumulation dependent on E1b 55K function. They counter the idea that specific sequence features of mRNA mediate E1b dependence, and support the hypothesis that some aspect of the environment in which late viral transcription complexes function creates a requirement for E1b 55K protein to achieve optimum gene expression.

If expression in a somehow unfavourable location were the sole determinant of E1b dependence, then all mRNAs expressed from late viral transcription complexes would be expected to show a similar measure of dependence. Data presented here, however, show that accumulation of the longest mRNA in each of MLP families L1, L2 and L3 shows greater dependence on E1b function than does accumulation of the shortest mRNA in each family, which become disproportionately
abundant. These findings were not explained by altered patterns of splice site usage in the absence of Elb 55K function. Furthermore, they cannot be due to a slower progression of dl338 infections through the late phase because any such delay would favour the accumulation of the longest mRNAs (Flint, 1986).

Expression from various early promoters continues into the late phase of infection. If this expression were from replicated 'late' template molecules then, according to the model described above, accumulation of these mRNAs would also be expected to become Elb 55K-dependent in the late phase. It is also possible, however, that expression from early and late promoters in the late phase occurs from different template pools, with the early promoters continuing to be transcribed on functionally or spatially distinct early templates. In this case, dependence of these mRNAs on Elb function, even in the late phase, would not be expected. The data reported here cannot be used to distinguish these possibilities.

In the uninfected cell, systems exist which prevent the exit from the nucleus of unspliced or incompletely spliced transcripts, these being retained in tight association with the nuclear matrix (Ciejk et al., 1982). The significance of this control system is illustrated by the fact that retroviruses of the lentivirus family such as human immunodeficiency virus type 1 (HIV-1) encode a function (Rev), essential for viral infectivity, which is required to overcome the action of this mechanism on viral unspliced and partially spliced mRNAs (for reviews, see Cullen & Greene, 1989; Chang & Sharp, 1990). In this system, it has been shown that the presence of a single functional splice site in an mRNA can cause its retention in the nucleus (Chang & Sharp, 1989). It may be significant, therefore, that a common feature of the most severely Elb-dependent MLP mRNAs is the presence within them of functional 3' splice acceptor sites, used in other transcripts of the same gene to produce shorter mRNAs (Fig. 4 and 5). Although no normally functional 5' donor site is present, these residual intron/acceptor site sequences may be sufficient to specify mRNA retention.

Ad5 MLP transcripts have been shown to associate with the nuclear matrix (Mariman et al., 1982). They spend longer in the nucleus than do transcripts of an early gene, E2a, and, measured as a group, their impaired release from the nuclear matrix is a feature of the dl338 phenotype (Leppard & Shenk, 1989). The pattern of differential sensitivity to lack of Elb 55K function reported here, considered in the light of these previous findings, is strongly suggestive of a role for the Elb 55K/E4 ORF6 complex in countering the action of host nuclear retention systems on a subset of late viral RNAs.

The dual requirement for functional Elb 55K protein, generally for efficient expression of any gene transcribed from a 'late' viral transcription complex and, more specifically, for the efficient release from the matrix of certain mRNAs that carry nuclear retention signals, may be facets of the same function. If the most significant unfavourable feature of the environment in which late gene expression occurs is absent or poor linkage to a pathway for export of mature mRNA from the nucleus, then accumulation of all mRNAs produced from late viral transcription complexes would benefit from the action of a viral function that facilitated mRNA release and/or movement away from the matrix. However, accumulation of any mRNA specifically retained on the matrix due to elements of its sequence would experience the greatest effect.

Given the variety of mRNAs that are dependent on Elb 55K/E4 ORF6 complex function, it is unlikely that the complex acts through a single RNA target sequence analogous to that used by HIV-1 Rev protein. Instead, it is more likely that the complex acts indirectly, altering the interaction of cellular components with mRNA in or close to the nuclear matrix compartment. The emphasis of future work must therefore be to reveal the identity of these putative cellular targets.

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References


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