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The adenovirus tripartite leader sequence can alter nuclear and cytoplasmic metabolism of a non-adenovirus mRNA within infected cells

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ABSTRACT

All mRNAs encoded by the adenovirus major late transcription unit share a common 5' noncoding region, 200 nucleotides in length, termed the tripartite leader sequence. To assess function of the tripartite leader, recombinant viruses were prepared which carried either a bona fide herpes simplex virus thymidine kinase gene or a modified thymidine kinase gene whose normal 5' noncoding domain was replaced with the adenovirus leader sequence. The tripartite leader simultaneously decreased the nuclear half-life and increased the cytoplasmic half-life of the thymidine kinase-specific mRNA. The tripartite leader stabilized the non-adenovirus mRNA only within the environment of an adenovirus-infected cell during the late phase of the infectious cycle.

INTRODUCTION

During the late phase of adenovirus infection, the preponderance of viral mRNAs are encoded by the major late transcription unit. Transcription of this unit initiates at 16.4 map units on the viral chromosome and gives rise to a primary transcript about 29,000 nucleotides in length. The primary transcript is processed by differential splicing and poly(A) addition to produce more than 30 mRNAs. These mRNAs all share a common 5' noncoding region, 200 nucleotides in length, termed the tripartite leader sequence (1,2), because it is coded in three spacially separated segments on the viral chromosome that are joined by splicing of the primary transcript.

The function of the tripartite leader sequence is not yet entirely clear. It has enhanced translation when appended to some mRNAs in virus-infected cells (3-5), but not others (6). Translational enhancement requires the tripartite leader be placed in close proximity to the AUG start codon of the mRNA (7), and this position requirement likely explains the instance in which the leader failed to enhance translation (6). Here we demonstrate two effects of the noncoding region on mRNA metabolism. When its normal 5' noncoding domain is replaced by the tripartite leader sequence, the nuclear half-life of a transcript encoding herpes simplex virus thymidine kinase (tk) is shortened and the cytoplasmic half-life of the resulting tk mRNA is substantially increased within adenovirus-infected cells.

MATERIALS AND METHODS

Plasmids, viruses and cells

The recombinant plasmid pMPCV2 (3) contains a left-end segment of the adenovirus type 5 (Ad5) genome inserted between the unique *Eco*RI and *Sal*I sites of pBR322. The Ad5 segment extends from sequence position 1 to 5788, but lacks the E1A and most of the E1B coding regions (the segment between an *Sst*II site at position 353 and a *Hin*dIII site at position 2805). The Ad2 major late transcriptional control region (sequence position 5791-6032), the first 172 bp of the tripartite leader coding sequence (from pJAW-43, ref. 8), and a unique *Xho*I cleavage site have been inserted at the site of the E1A/E1B deletion. p373 was produced by removing the major late promoter plus tripartite sequence from pMPCV2 by partial digestion with *Sst*II, followed by insertion of a *Pvu*II-generated fragment prepared from pAGO (9) that contains the entire herpes simplex virus type 1 tk gene with its normal 5' and 3' flanking regions. p374 contains a *Bgl*II to *Pvu*II fragment derived from pAGO inserted into pMPCV2 between its unique *Xho*I site and the *Sst*II site at sequence position 3824, removing the normal tk 5' flanking domain and appending the tk coding region to the tripartite leader sequence. p375 contains the tk *Bgl*II to *Pvu*II fragment inserted at the unique *Xho*I site in pMPCV3 (pMPCV3 is a gift from J. Logan, American Cyanamid, Inc.). In this case the *Xho*I site is located 5 bp upstream of the normal Ad2 major late transcriptional start site. p375 is, therefore, isogenic to p374 except that it lacks the segment coding the tripartite leader sequence.

*Sub*373, *sub*374 and *sub*375 viruses were derived from p373, p374 and p375, respectively. Plasmids were linearized with *Cla*I and used to co-transfect 293 cells with *Cla*I-cleaved *dl*327, generating recombinant viruses by overlap recombination (10). *Dl*327 is a phenotypically wild-type virus which lacks the majority of the E3 transcription unit. The E3 deletion is identical to that previously described for *dl*324 (11). Herpes simplex virus type 1 was a gift of A. Levine, Princeton University. HSV-D2, which lacks 850 bp of the tk gene (12) was a gift of S. Silverstein, Columbia University.

293 cells, human cells which contain and express the Ad5 E1A and E1B genes (13), were maintained in medium containing 10% calf serum. HeLa spinner cells were propagated in medium containing 7% calf serum. The tk-minus 143b cell line was obtained from the American Type Culture Collection, Rockville, MD, and grown in medium containing 10% calf serum.

RNA preparation and analysis

Protocols for isolation of cytoplasmic, polyadenylated RNA from infected cells, northern-type analysis and 5' or 3' S1 analysis have been described (3,14). Recombinant M13 DNAs (single-stranded or double-stranded replicative form, as appropriate) carrying region-specific Ad5 segments were used as

probes: E1A, 0 to 4.4 map units; E1B, 5.6 to 7.9 map units; E2A, 63.6 to 68 map units; E4, 92 to 97.1 map units; L1, 31.5 to 37.3 map units; L2, 41 to 50.1 map units; L3, 53 to 58.5 map units; L5, 89.1 to 92 map units. The tk probe DNA was an 850 bp segment of the herpes simplex virus type 1 tk coding region, and the actin probe DNA, pA1 (15), was a segment of chicken actin coding sequences.

Transcription rates were measured in isolated nuclei essentially as described by Hofer and Darnell (16) and Groudine *et al.* (17). Nuclei prepared from infected cells were incubated for 15 min at 30°C in the presence of [α^{32}P] UTP (750μCi/ml, 410Ci/mmole), and nuclear RNA was isolated, degraded by treatment with 0.2N NaOH for 10 min at 0°C, and hybridized to single-stranded DNA probes bound to nitrocellulose filters (100μg of genome equivalents per spot) by the method of McKnight and Palmiter (18). After one round of hybridization, a second DNA-containing filter was added to the hybridization mix to insure quantitative results.

Cytoplasmic accumulation of mRNAs was measured by previously described procedures (19,20). In brief, infected cells were labeled with [^{3}H] uridine (200μCi/ml, 50Ci/mmole) in the presence of added unlabeled uridine (14μM). Approximately 10^7 infected cells were harvested at each interval, cytoplasmic RNA was prepared by the guanidinium isothiocyanate procedure of Chirgwin *et al.* (22), and then hybridized to single-stranded DNA probes as described for nuclear RNAs.

Analysis of polypeptides

One hour before labeling, infected cells were placed in medium lacking methionine and supplemented with 2% calf serum. Cultures were labeled with [^{35}S] methionine (50μCi/ml, 1100Ci/mmole) for 60 min. Preparation of cellular extracts, immunoprecipitations, and SDS-polyacrylamide gel electrophoresis were performed as described by Sarnow *et al.* (23). The herpes simplex virus tk-specific goat polyclonal antibody was the gift of T. Silhavy, Princeton University.

RESULTS

Construction and propagation of recombinant viruses

To assess the impact of the tripartite leader sequence on a non-adenovirus mRNA, three viruses were constructed which carried the herpes simplex virus tk gene in place of the adenovirus type 5 (Ad5) E1A and E1B genes (Figure 1). The first, *sub*373, carried an intact tk gene under control of its own promoter sequences. The second, *sub*374, contained a hybrid tk gene in which the normal tk promoter region and a substantial portion of its 5' noncoding domain were replaced with the adenovirus major late transcriptional control region and tripartite leader sequence. The final construct, *sub*375,

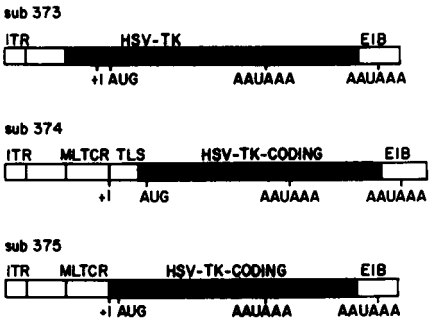

Figure 1. Diagrams of Ad5 recombinants that contain the herpes simplex virus tk gene. Solid blocks designate segments derived from the tk gene and open blocks represent segments from the Ad2 or Ad5 chromosome. Transcriptional start sites (+1), translational start sites (AUG) and polyA addition motifs (AAUAAA) are designated. ITR, Ad5 inverted terminal repeat; MLTCR, Ad2 major late transcriptional control region; TLS, segment encoding the first 172 base pairs of the Ad2 tripartite leader sequence.

was isogenic to *sub*374 except that no tripartite leader sequence was added after removal of the tk 5′ noncoding segment.

The recombinant viruses were propagated on 293 cells which supplied the products of the E1A and E1B genes that had been deleted in the recombinants. *Sub*373 and *sub*375 grew as well as wild-type virus on these cells. *Sub*374 grew somewhat more slowly producing smaller plaques, but ultimately generated infectious yields equivalent to wild-type virus. This reduced growth rate might result from toxicity due to the relatively high level of tk expression in *sub*374-infected cells (see below).

Structure of tk mRNAs produced by recombinant viruses

Cytoplasmic tk mRNAs in 293 cells infected with the recombinant viruses were analyzed by RNA blot analysis (Figure 2B). Two bands were detected by a tk-specific probe DNA for all three recombinants. The faster-migrating species corresponded to bona fide tk mRNA (see Figure 2C). The larger species also hybridized to an E1B-specific probe (data not shown) and resulted from polyadenylation at the E1B poly(A) site located downstream of the tk insert in the recombinant viruses (see Figure 1). Curiously, the proportion of mRNA molecules polyadenylated at the E1B-specific site was consistently greater in *sub*374-than in *sub*373- or *sub*375-infected cells, although this effect was much more pronounced at early than at late times after infection.

The structures of tk mRNAs coded by *sub*373 and *sub*374 were confirmed by 5′ and 3′ S1 endonuclease analysis. 5′-end-labeled, single-stranded probe DNAs were prepared specific for mRNAs coded by *sub*373 (natural tk 5′ noncoding region) or 374 (tripartite leader sequence appended to the tk coding region).

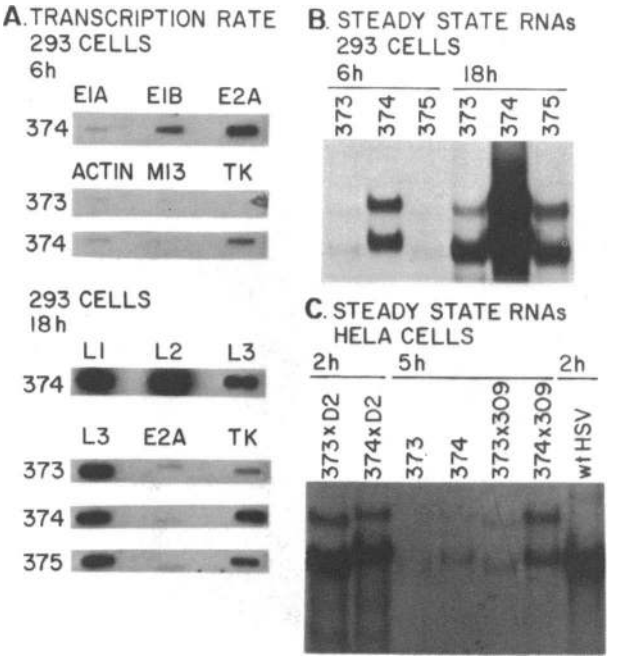

Figure 2. Transcription rates and steady state cytoplasmic RNA levels in cells infected with Ad5 recombinants. (A) Transcription rates were determined at 6 or 18 h after infection of 293 cells at a multiplicity of 10 pfu/cell. Isolated nuclei were incubated for 10 min at 30° in the presence of [α-^{32}P]UTP, nuclear RNA was prepared and hybridized to indicated probe DNAs immobilized on nitrocellulose sheets. Autoradiograms were generated by exposure of the nitrocellulose sheets to pre-flashed x-ray film. (B and C) Steady-state levels of tk mRNAs were assayed by RNA blot analysis of cytoplasmic, polyadenylated RNA isolated from infected cells. Panel B displays the analysis of mRNAs from the infected 293 cell cultures employed in panel A. Panel C quantitates tk mRNAs in HeLa cells present at either 5 h after infection with Ad5 recombinants at a multiplicity of 10 pfu/cell or 2 h after co-infection with Ad5 recombinants and HSV-D2 at a multiplicity of 5 pfu/cell. Excess tk-specific probe DNA and pre-flashed x-ray film were employed to insure quantitative results in the RNA blot analyses.

Figure 3A displays the protected bands. The *sub*373- and *sub*374-specific probes generated major products of 51 and 172 nucleotides, respectively. The fragments mapped 5′ ends to the normal start sites used by the tk and major late transcriptional control regions. It is not clear whether the minor products observed in the case of *sub*374 result from anomolous start sites or S1 cleavage within the 172 base-pair duplex. 3′ ends were monitored using a 3′-end-labeled, single-stranded probe DNA. Both *sub*373- and *sub*374-coded mRNA populations contained two 3′ ends producing 97 nucleotide (Figure 3B) and 875

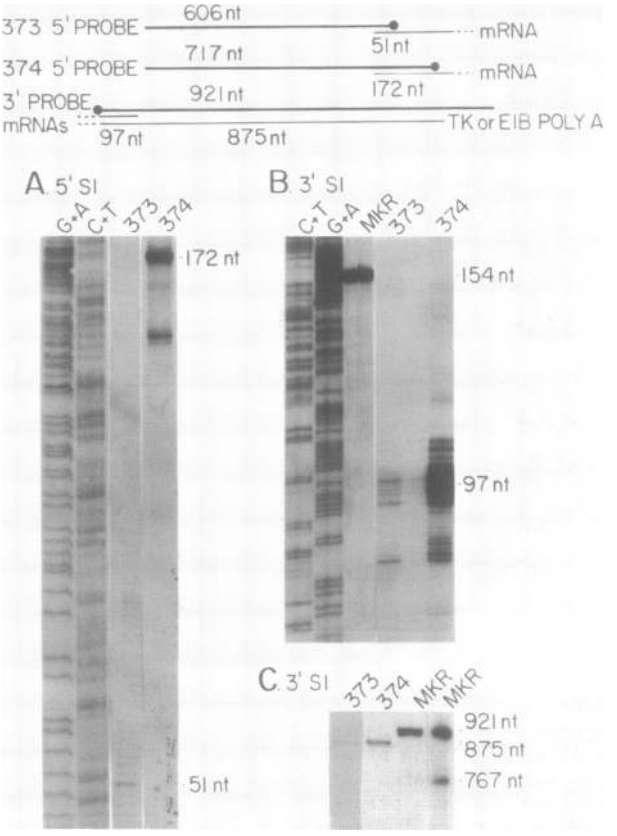

Figure 3. 5' and 3' analysis of tk-specific, polyadenylated RNAs which accumulate in the cytoplasm of 293 cells infected with Ad5 recombinants. Single-stranded probe DNAs are diagrammed at the top of the figure. mRNA segments are represented by thin lines and probe DNAs by thick lines. Solid circles designate [^{32}P] and the length of each probe that is protected by tk mRNA is indicated in nucleotides. (A) 5' S1 analysis utilized cytoplasmic, polyadenylated RNA prepared 18 h after infection of 293 cells with Ad5 recombinants at a multiplicity of 10 pfu/cell. Protected probe DNA fragments were analyzed by electrophoresis in an 8% poly-acrylamide gel containing 8M urea. DNA sequencing reactions were included as size markers. The lengths of DNA fragments are indicated. (B and C) 3' S1 analysis employed the same RNA preparations. Protected fragments were subjected to electrophoresis in either 8% (B) or 5% (C) polyacrylamide gels containing 8M urea, and their sizes determined relative to marker DNAs.

nucleotide (Figure 3C) fragments. These two ends correspond to polyadenylation at either the tk or downstream E1B sites.

Expression of tk mRNAs by recombinant viruses

Transcription rates of the tk units carried by the recombinant viruses

were measured by run on assay in isolated 293 cell nuclei. Autoradiograms of [^{32}P]UTP-labeled nuclear RNA hybridized to specific probe DNAs laid out on nitrocellulose with a slot blot apparatus are displayed in figure 2A. Early after infection (6 h), tk-specific hybridization was barely detectable in *sub*373-infected cells, where the tk gene was expressed on board the Ad5 chromosome under control of its own promoter elements. At this time, tk-specific RNA was produced at about 20-fold higher levels in *sub*374- and *sub*375-infected cells where its transcription was mediated by the adenovirus major late control region. Late after infection (18 h), tk-specific transcription was evident in cells infected with all mutants. However, tk sequences were expressed at about eight-fold higher levels under control of the adenovirus promoter (*sub*374 and *sub*375) as compared to the native tk control region (*sub*373). Additional early and late time points were assayed (data not shown) with similar results. At early times after infection (4,6,8 h), the tk promoter showed little activity on board the adenovirus chromosome. Later (16,17,18 h) it became more active, mediating transcription at about one-eighth the level of an adenovirus major late control region.

Steady state levels of tk mRNA produced in infected 293 cells were monitored by RNA blot analysis (Figure 2B). The *sub*373 mRNA levels were about 20-fold lower than *sub*374-coded tk mRNA levels early (6 h) after infection, mimicking their relative early transcription rates. Even though *sub*375 exhibited transcription rates equivalent to *sub*374, tk-specific mRNA accumulated to a much lower level in *sub*375- than in *sub*374-infected 293 cells. *Sub*375 encodes a variant RNA that lacks a normal 5′ noncoding region and a substantial portion of it is degraded within the nucleus (demonstrated below).

In contrast to 293 cells, HeLa cells contained similar levels of tk-specific mRNAs early (5h) after infection with *sub*373 or *sub*374 (Figure 2C). *Sub*374 but not *sub*373-coded tk mRNA levels were enhanced by co-infection with a phenotypically wild-type virus, *dl*309 (Figure 3C). Both 293 cells and *dl*309 provide E1A *trans*-activating functions which the recombinant viruses lack. It appears likely that enhanced tk-specific transcription rates and steady state mRNA levels early after infection with *sub*374 as compared to *sub*373 result from E1A-mediated transcriptional stimulation. Apparently, the major late transcriptional control region in *sub*374 responds to the E1A *trans*-activating function more efficiently than the normal tk control region in *sub*373. Both *sub*373 and *sub*374-infected HeLa cells accumulate similar levels of mRNAs very early (2 h) after co-infection with HSV-D2, a herpes simplex virus which carries a deletion within its tk gene allowing detection and quantitation of tk-specific mRNA encoded by the Ad5 recombinants (Figure 2C). The normal and hybrid adenovirus major late control region - tk genes

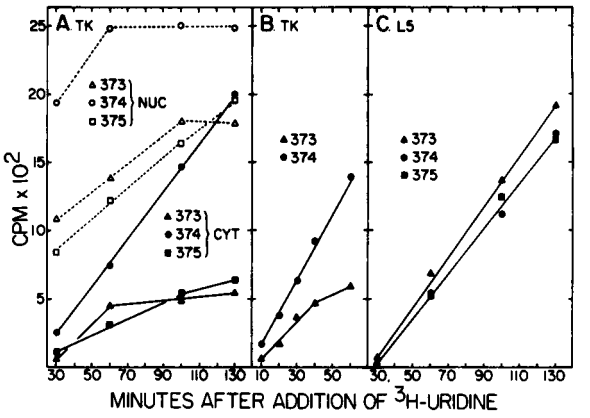

Figure 4. Kinetics of tk mRNA accumulation in the nucleus and cytoplasm of 293 cells infected with Ad5 recombinants. Cultures were infected at a multiplicity of 10 pfu/cell and labeled with [^{3}H]uridine beginning at 16 h later. Portions of the cultures were harvested at the indicated intervals, and RNAs were prepared and hybridized to single-stranded probe DNAs specific for either tk mRNA (A and B) or Ad5 L5 mRNA (C). Symbols: ▲ , cytoplasmic *sub*373-coded RNA; ● , cytoplasmic *sub*374-coded RNA; ■ , cytoplasmic *sub*375-coded RNA. Nuclear RNAs are designated by the corresponding open symbols.

both can respond efficiently to a herpes simplex virus immediate early *trans*-activating function.

Whereas early steady state tk mRNA levels faithfully tracked relative transcription rates in *sub*373 as compared to *sub*374-infected 293 cells, late mRNA levels did not (Figure 2B). At 18 h after infection, *sub*374-infected 293 cells contained approximately 25-fold more tk-specific mRNA than *sub*373-infected cells. The difference in transcription rates at this time was only about 8-fold (Figure 2A), suggesting that the tripartite leader sequence modulated steady state mRNA levels post-transcriptionally late after infection.

The tripartite leader sequence can shorten nuclear and increase cytoplasmic half-life of tk mRNA

To rigorously document a post-transcriptional effect of the tripartite leader sequence, 293 cell cultures were labeled continuously with [^{3}H]uridine from 16 to 18.2 h after infection, and the accumulation of labeled tk-specific RNA in the nucleus and cytoplasm was monitored (Figure 4). Curiously, the 30 min time points for nuclear accumulation (Figure 4A) do not fit with tk transcription rates measured by *in vitro* run on experiments in which *sub*374 and *sub*375 displayed eight-fold higher rates than *sub*373 (Figure 2A). After a 30 min *in vivo* labeling period, *sub*374 accumulated only two-fold

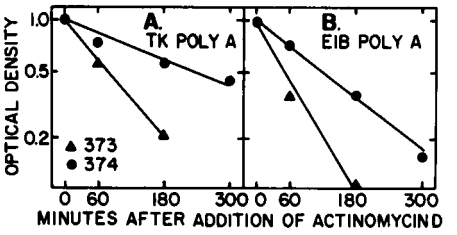

Figure 5. Rate of tk mRNA decay in 293 cells infected with *sub*373 and *sub*374. Cultures were infected at a multiplicity of 10 pfu/cell and actinomycin D (4 μg/ml) was added to cultures 18 h later. Samples were harvested immediately after addition of the drug and then at indicated time intervals thereafter. mRNAs were analyzed by RNA blot analysis using a tk-specific probe DNA. Bands representing mRNAs were quantitated with an LKB densitometer, and decay curves for mRNA species polyadenylated at the tk site (A) and E1B site (B) were plotted. Symbols: ▲, *dl*373; ●, *dl*374.

more and *sub*375 accumulated somewhat less [^{3}H] labeled tk mRNA than *sub*373-infected nuclei. Further, the nuclear accumulation curves do not extrapolate linearly to zero cpm at the time of label addition. Perhaps there are multiple kinetic classes of RNA molecules produced by an individual transcription unit. As yet, we haven't investigated the basis for these apparently anomalous results.

The point at which a plateau is reached in nuclear accumulation provides a measure of nuclear half-life. At this point, newly transcribed sequences are entering the nuclear pool at the same rate as sequences exit either by degradation or transport to the cytoplasm. Each tk-specific RNA displayed a different nuclear half life (Figure 4A). The transcript encoded by *sub*374 reached a steady-state level most quickly (60 min) while that coded by *sub*375 failed to reach a plateau during the 130 min experiment. Assuming that the rate at which nuclear steady state levels are reached is dictated at least in part by the speed of nuclear processing and transport to cytoplasm, our data suggests the tripartite leader-containing transcript (*sub*374) is more efficiently matured than either transcript lacking it (*sub*373 or *sub*375).

Cytoplasmic tk mRNA accumulation within *sub*374-infected cells failed to plateau until about 180 min (Figure 4A and 6B) while the *sub*373-coded tk mRNA reached steady state after only 60 min (Figure 4A, 4B and 6B). Thus, the cytoplasmic stability of the tk-specific mRNA was substantially increased by addition of the tripartite leader sequence. *Sub*375-coded tk mRNA accumulated at a slower rate than the other tk-specific species (Figure 4A). We are unable to comment with confidence on its cytoplasmic half-life from our present data. Accumulation of a virus-specific mRNA (L5) was monitored as an internal control and found to be very similar for the three viruses (Figure 4C).

Table 1. Transcription rates and steady state tk mRNA levels in 143b cells carrying normal (p373) and modified (p374) HSV-tk genes

Cell Line	Transcription Rate tk	Transcription Rate actin	Steady State tk
373	1.17	1.00	1.00
374	1.00	1.00	0.90

Transcription rates were determined by *in vitro* nuclear run on analysis and steady state mRNA levels by RNA blot analysis. Autoradiograms were prepared using pre-flashed x-ray film and then quantitated by densitometry. Rates are tabulated relative to the density of actin bands.

It was conceivable that the two species of tk mRNA produced by utilizing different poly(A) addition sites were differentially stable. The tk mRNA utilizing the downstream E1B poly(A) site could be more or less stable than that polyadenylated at the upstream tk-specific site. To directly test the effect of the two 3′ noncoding regions on stability, mRNA half-lives were monitored by RNA blot analysis following blockage of transcription at 18 h after infection with actinomycin D. This enabled the two mRNA species to be monitored separately. Bands representing the two mRNAs were quantitated by densitometry and decay curves plotted (Figure 5). In the case of *sub*373, mRNAs polyadenylated at the tk and E1B sites displayed half-lives of about 80 and 54 min, respectively, while the corresponding half-lives for *sub*374 mRNAs were 240 and 125 min. The different 3′ non-coding regions did, indeed, influence mRNA stability. However, both *sub*373 tk mRNAs were less stable than either *sub*374 species. Thus, we conclude that the tripartite leader sequence is responsible for the increased half-life exhibited by *sub*374 as compared to *sub*373-coded tk mRNA.

The mRNA half-lives determined by accumulation kinetics (*sub*373=60 min; *sub*374=180 min; Figures 4 and 6) are very similar to the average for individual mRNA half-lives determined by the actinomycin D chase method (*sub*373=[80+54 min]/2=67 min; *sub*374=[240+125min]/2=183 min; Figure 5). This strongly argues that the two poly(A) addition sites are chosen with equal frequency at 18 h after infection.

The tripartite leader sequence can stabilize mRNAs only within late adenovirus-infected cell

To assess the effect of the tripartite leader sequence on mRNA stability in an uninfected cell, human tk-minus 143b cells were transfected with a plasmid carrying either the normal tk gene (p373) or the modified gene comprised of the adenovirus major late transcriptional control region, tripartite leader and tk coding region (p374). The first pair of tk-plus

Table 2. Transcription rates and steady state tk mRNA levels in infected HeLa cells.

Infection	Transcription Rate				Steady State
	tk	E4	L3	actin	tk
*sub*373	0.10	ND	ND	1.00	1.0
*sub*374	0.13	ND	ND	1.00	1.2
*sub*373 x HSV-D2	1.05	0.92	<0.10	1.00	18.0
*sub*374 x HSV-D2	1.88	0.84	<0.10	1.00	22.0
*wt*HSV	1.58	ND	ND	1.00	24.0

Transcription rates were determined at 5 h after infection at a multiplicity of 10 pfu/cell with *sub*373 or *sub*374, and at 2 h after infections that included HSV-D2 at a multiplicity of 5 pfu/cell. Nuclear run on analysis employed specific DNA probes bound to nitrocellulose. Radioactive RNA hybridized to immobilized DNA was detected by autoradiography using pre-flashed x-ray film, and quantitated by densitometry. Rates are tabulated relative to the density of actin bands. Relative steady state levels of tk mRNAs were determined by RNA blot analysis of cytoplasmic, polyadenylated RNA prepared at 5 h after infection. Quantitation was by densitometric analysis of the resulting autoradiogram. Levels are reported relative to amount of tk mRNA present in *sub*373-infected cells.
ND: not determined.

transformants examined (termed 373 and 374) each contained a single insert and exhibited identical tk-specific RNA transcription rates and very similar tk mRNA steady state levels (Table 1). Similar results were obtained for several cell lines derived by introduction of p373 or p374 into 293 cells through co-transfection with pSV2-gpt (24) and selection for resistance to mycophenolic acid (data not shown). Thus, in the absence of infection, the tripartite leader sequence does not influence stability of RNAs.

The tripartite leader does not exert its effect until the late phase of the infectious cycle. The lack of an effect early after infection was initially suggested by examination of tk-specific transcription rates and steady state mRNA levels 6 h after infection with *sub*373 or *sub*374 (Figure 2A and B). Early steady state levels of tk mRNA were not enhanced by the presence of the leader sequence. The tripartite leader also failed to influence the level of tk mRNA at 2 h after co-infection of HeLa cells with both a recombinant adenovirus and HSV-D2, a herpes simplex virus that lacks the majority of its tk gene (Figure 2C and Table 2). In this case, co-infection with HSV-D2 has dramatically increased and nearly equalized transcription of the *sub*373- and *sub*374-coded tk mRNAs (Table 2), facilitating

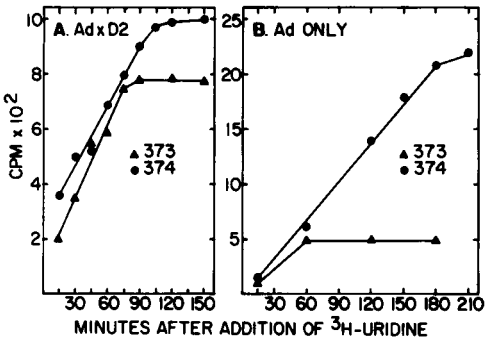

Figure 6. Kinetics of tk mRNA accumulation in the cytoplasm of infected 293 cells. Cultures were either co-infected with Ad5 recombinants plus HSV-D2 (A) or infected with Ad5 recombinants alone (B). HSV-D2 and Ad5 recombinants were used at multiplicities of 5 and 10 pfu/cell, respectively. Cultures were labeled with [^{3}H]uridine beginning at either 4 h (A) or 16 h (B) after infection. Portions of the cultures were harvested at the indicated intervals, and cytoplasmic RNA was prepared and hybridized to a single-stranded tk-specific probe DNA.

their accurate comparison. An early Ad5 unit (E4) was actively transcribed while a late gene (L3) was not, indicating that adenovirus gene expression was indeed in its early phase in the co-infected cells. As a final check, accumulation of cytoplasmic tk mRNAs was monitored between 4 to 6.5 h after co-infection (Figure 6A). The transcription rate of the tk gene did not change during this time interval (data not shown). The tripartite leader-bearing tk mRNA coded by *sub*374 exhibits a reduced half-life (about 90 min) in the coinfected cell early during the adenovirus infectious cycle (Figure 6A), as compared to the 180 min half-life measured late after adenovirus infection (Figure 6B). The half-life of bona fide tk mRNA encoded by *sub*373 was essentially unchanged in this comparison (Figure 6A and B) as was the adenovirus E2A mRNA which was monitored as a control (data not shown).

We conclude that the tripartite leader sequence can stabilize an mRNA only within the environment of a late adenovirus-infected cell. It does not alter mRNA stability within an uninfected cell or during the early phase of adenovirus infection.

The tripartite leader sequence does not enhance translation of the tk mRNA

Translation of the various tk mRNAs was followed by immunoprecipitation of tk polypeptide from extracts of 293 cells at 18 h after infection (Table 3). By this assay, *sub*374-infected cells contained about 18-fold more tk polypeptide than *sub*373-infected cells and 38-fold more than *sub*375-infected cells. Thus, the quantity of tk polypeptide closely tracked tk mRNA levels,

Table 3. Relative levels of tk polypeptide in 293 cells subsequent to infection with recombinant viruses.

Infection	Ad5 Polypeptide II	tk
*sub*373	100.	0.23
*sub*374	100.	4.20
*sub*375	100.	0.11

Proteins were labeled for 60 min with [^{35}S]-methionine 18 h after infection at a multiplicity of 10 pfu/cell. Whole cell extracts were prepared and subjected to immunoprecipitation using a polyclonal goat antibody to the tk polypeptide. Immunoprecipitates as well as unprecipitated aliquots were analyzed by electrophoresis in SDS-containing polyacrylamide gels. Radioactivepolypeptides were visualized by fluorography and then quantitated by densitometry. Levels of tk polypeptide are reported relative to the amount of Ad5 polypeptide II present in each extract.

and no translational enhancement by the tripartite leader sequence was evident.

DISCUSSION

We have documented three different, but likely related, effects of the tripartite leader sequence. This report demonstrates that the tripartite leader can reduce the nuclear half-life of transcripts and increase the cytoplasmic stability of mRNAs. Previously, we (3) and others (4,5,7) have shown that the tripartite leader sequence can enhance translation of mRNAs. The nuclear half-life of a tk transcript is reduced from about 100 min to 60 min by substitution of its normal 5' noncoding region with the tripartite leader (Figure 4A). It is difficult, as yet, to definitively interpret this result. mRNA containing the tripartite leader sequence appeared in the cytoplasm somewhat more rapidly than mRNA lacking it (e.g. 10 min time point, Figure 4B), tempting us to suggest that the reduced nuclear half-life might well reflect an enhanced rate of processing or transport to the cytoplasm, and not an increased rate of degradation within the nucleus. The slightly enhanced rate of cytoplasmic mRNA accumulation observed for the tk mRNA containing the tripartite leader (Figure 4B) is also consistent with this interpretation. It is possible, however that the cytoplasmic accumulation rates are influenced by the different tk-specific transcription rates in *sub*374- as compared to *sub*373-infected cells.

Cytoplasmic half-life increased from about 60 min for bona fide tk mRNA to 180 min for tk mRNA containing the tripartite leader (Figures 4 and 6). The stabilization only occured within the milieu of a late adenovirus-infected

cell. We observed no stabilization in uninfected cells (Table 1) or early after adenovirus infection (Figure 2 and Table 2). Some increase in half-life was observed for tk mRNA bearing the tripartite leader at 4.5 to 6 h after co-infection of cells with *sub*374 and herpes simplex virus (Figure 6), but the extent of stabilization was considerably less than observed late after adenovirus infection (90 versus 180 min).

The three effects which have been observed for the tripartite leader do not always occur in concert when the sequence is appended to novel coding regions. Although nuclear and cytoplasmic metabolism of tk mRNAs were altered, the efficiency of tk mRNA translation was indifferent to the tripartite leader (Table 3). The leader sequence has previously been shown to enhance translation of the adenovirus E1A (3), dihydrofolate reductase (4) and hepatitis B virus surface antigen (5) coding regions, but not the SV40 T antigen coding domain (6). Berkner et al. (7) have recently shown that translational enhancement requires the tripartite leader be placed in close proximity to the AUG start codon of the mRNA as is the case for normal late adenovirus mRNAs. Such a requirement could explain the lack of a translational effect for both the tk and SV40 hybrid constructs where the spacing was 51 and 48 nucleotides, respectively.

Whereas the tk constructs reported here failed to exhibit a translational effect, our earlier constructs in which E1A coding sequences were appended to the tripartite leader exhibited no change in hybrid mRNA half-lives (3). Conceivably, some structural feature specific to the fusion of leader to E1A coding region prevented stabilization. For example, the secondary structure of E1A coding sequences might fortuitously interfere with leader function. It seems more likely that the E1A mRNA contains a signal which mediates its rapid turnover, and it is dominant to the tripartite leader in the hybrid construct. Destabilization signals have been described in several mRNAs which, like E1A, display short half-lives (c-fos, ref. 25; GM-CSF, ref. 26).

The experiments described here and in our earlier report on the translational effect of the tripartite leader sequence (3) employed constructs that encode nucleotides 1-172 of the 200 nucleotide long 5' noncoding sequence. Inclusion of the additional 28 nucleotides normally present at the 3' end of the third leader element might modify activity of the tripartite leader. As yet, this possibility has not been explored.

The adenovirus E1B-55K gene product has been demonstrated to facilitate cytoplasmic accumulation of viral mRNAs late after infection (27-29). Given the apparent similarity in function of the *trans*-acting E1B-55K polypeptide and *cis*-acting tripartite leader, it seemed possible that the E1B product might act either directly or indirectly at the tripartite leader sequence to facilitate cytoplasmic transport of late viral mRNAs. To test this notion, HeLa cells were coinfected with *sub*374 and either wild-type Ad5 or *dl*338,

which does not express the E1B-55K polypeptide. The resulting tk mRNA levels were indifferent to the presence of E1B product (data not shown). Although we can't yet rule out an interrelationship, this experiment suggests the tripartite leader and E1B product can function independently to enhance cytoplasmic accumulation of mRNAs. Thus, the mechanism underlying the multiple effects displayed by the tripartite leader in hybrid mRNA constructs remains unclear.

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