
Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant

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Received 29 October 1984; Revised and Accepted 31 December 1984

ABSTRACT

The E1a region of an Adenovirus 5 recombinant has been substituted by a modular gene encoding dihydrofolate reductase (DHFR). In this recombinant, the mouse DHFR cDNA was positioned behind sequences of the major late promoter and the complete tripartite leader. The leader sequences end in the normal 5' splice site (SS) of the third leader, so that RNA splicing joins the tripartite leader to a 3' splice site immediately upstream of the DHFR cDNA. At late stages of infection, high levels of DHFR mRNAs were synthesized. At early times in the late stage, this mRNA was efficiently translated; however, at later times translation of DHFR decreased probably due to poor competition with other late mRNAs. Synthesis of DHFR protein from an analogous Adenovirus 5 recombinant containing only the first late leader was studied in parallel. Equivalent levels of DHFR mRNA were expressed after infection with this recombinant virus; however, the efficiency of DHFR translation was at least 20 fold lower than that of the DHFR mRNA containing the tripartite leader. This suggests that the tripartite leader sequence is important for translation in the late stage of infection. As reported previously, the Ad5 recombinant containing only the first leader vastly overexpresses polypeptide IX from a novel mRNA, formed by the splicing of the first leader in the modular DHFR gene to the 3' splice site in the E1b region. Cells infected with this recombinant synthesize very little normal mRNA from the E1b region. Here, we demonstrated that coinfection of 293 cells with this recombinant and wild type Adenovirus 5 also results in decreased E1b mRNA synthesis. We propose that the overproduction of polypeptide IX suppresses mRNA expression from the E1b and IX promoter sites, probably by an autoregulation loop active during lytic growth.

INTRODUCTION

One of the hallmarks of adenovirus infection of human cells is the efficient expression of viral late proteins. This high level of synthesis is a consequence of both the amount of RNA transcribed from the adenovirus major late promoter (MLP) and the selective translation of late viral mRNAs. In the late stage of infection, most of the cell's polyribosomes contain viral mRNAs. However, the mechanism by which viral messages are preferentially translated is not well understood. This control must involve the RNA polymerase III product V.A. RNA-I, as virus containing a mutation in

this gene no longer efficiently synthesizes late adenovirus proteins (1). Since late mRNAs as a group are preferentially translated, they must possess common recognition elements. A candidate element is the tripartite leader. This 203 nucleotide sequence, which is generated by the splicing of sequences from three distinct regions of the genome (2, 3), is spliced onto the late viral messages.

We recently described an Ad5 recombinant, Ad5 (DHFR-I), in which the EIa region was replaced by a modular gene containing sequences from the major late promoter, a cDNA copy of the mouse dihydrofolate reductase gene (DHFR) and the polyadenylation site from the early region of SV40 (4). The DHFR containing transcript synthesized off of this recombinant only possessed sequences from the first leader [16.4 map units (mu)]. Although this DHFR mRNA was produced at levels comparable to other late adenovirus mRNAs, it was poorly translated in vivo. However, the same message was translated efficiently in an in vitro system. Similar conclusions have been drawn by others, studying genes encoding EIa and SV40 Tag (5, 6). This observation suggested that translation of late mRNAs in vivo might require sequences from the second and third leaders. In order to assess whether this was the case, we refined the DHFR modular gene by insertion of all of the tripartite leader sequences. We show here that the presence of the tripartite leader increases the efficiency of in vivo translation of the DHFR mRNA.

MATERIALS AND METHODS

Construction of the Ad5 (DHFR-III) Recombinant Virus

The 5.9 kb plasmid pDHFR-I, whose construction has been described (4), was treated with HindIII, S1 nuclease, and then with T4 DNA ligase and XhoI linkers in order to convert the HindIII site to an XhoI site (Figure 1). This DNA was partially cleaved with PvuII, then completely digested with XhoI, and the desired product (see Figure 1) was purified using agarose gel electrophoresis. The fragment was ligated with a 140 bp PvuII-XhoI fragment isolated from the plasmid pJAW (7). Positive colonies were identified by colony hybridization (8), using as a probe the 140 bp PvuII-XhoI fragment. The product plasmid, pDHFR-II, was cleaved with XhoI, treated with calf intestinal phosphatase, and then ligated with a gel purified fragment from 9690 bp (XhoI) to 9830 bp (SalI) of the Ad2 genome. A plasmid with the correct orientation, designated pDHFR-III, was prepared, and 5 ug of XbaI-cleaved plasmid DNA was ligated to 4 ug of sucrose gradient purified

4-100 mu Ad5 (309) DNA (9), and transfected onto 293 cells (10). Virus was harvested after 8-10 days and recombinants were detected by restriction enzyme screening of Hirt isolates (11). The virus was plaque purified twice, on 293 cells, and stocks were prepared. Ad5 (DHFR-III) DNA is 103 mu in length.

RNA and Protein Analyses

Protocols for the analysis of protein either *in vivo* or by *in vitro* translation have previously been described (4). Likewise, the procedures for RNA detection by Northern or SI analysis have been detailed (4). Three different types of RNA isolation were used. Total polyA(+) mRNA was prepared as described (4). In addition, cytoplasmic RNA was isolated from cells washed in PBS and lysed by suspension in 0.3% NP40, 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl₂. The lysate was spun at 2000 g for 3 min at 4°C and the supernatant was mixed with an equal volume of 40% urea, 1% SDS, 350 mM NaCl, 100 mM Tris pH 7.4, 10 mM EDTA, then phenol extracted twice and ethanol precipitated. PolyA(+) mRNA was also isolated from this preparation. The nuclear pellet was resuspended in 1% SDS, 10 mM Tris pH 8, 1 mM EDTA and Hirt extracted (11).

Materials

Restriction enzymes and T4 DNA ligase were from New England Biolabs and Bethesda Research Laboratories. T4 DNA kinase and XhoI linkers were from Collaborative Research. SI nuclease and calf intestinal phosphatase were from Miles. All radioisotopes were supplied by New England Nuclear.

RESULTS

Insertion of the Ad2 Tripartite Leader into a Modular Gene Encoding DHFR

The progenitor plasmid (pDHFR-I) that was used in construction of a gene with the entire tripartite leader has been described in detail (4). This modular gene is composed of segments containing the leftmost 350 bp of Ad5 (0-1 mu), the Ad2 major late promoter (MLP) and adjacent first leader sequences, a 3' splice site from an immunoglobulin gene, a mouse cDNA segment encoding DHFR, and the SV40 early polyadenylation signal (Figure 1). The complete tripartite leader was inserted into pDHFR-I by subcloning into it the PvuII-XhoI fragment from pJAW DNA and the Ad2 XhoI-SalI segment from 26 mu. This reconstructed the tripartite leader with its normal 5' splice site. In order to assure that inadvertent base changes had not occurred in the sequences during manipulation, the pDHFR-III plasmid was end-labeled at the XhoI site and sequenced in both directions for approximately 150 bases.

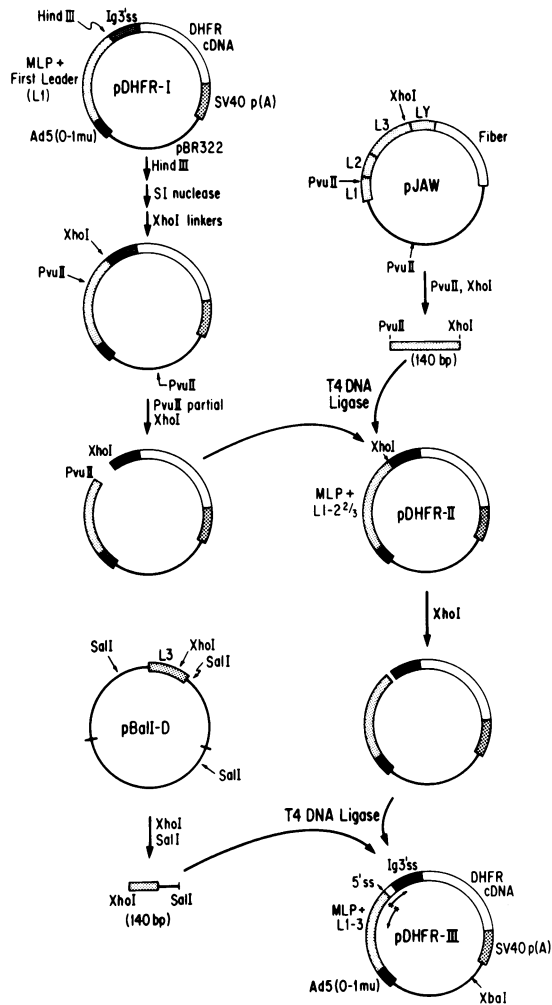


Figure 1. Construction of a modular gene encoding DHFR that contains the entire Ad2 tripartite leader

The details for the construction of pDHFR-III are given in the Experimental Procedures. Plasmids pBalID and pJAW (7) are Ad2 subclones. pDHFR-III was sequenced in the region around the XhoI site, as indicated by asterisks and arrows.

No unanticipated changes were observed. Plasmids pDHFR-I and pDHFR-III differ, therefore, only in the sequence content of the leader and in the 5' splice site sequences: pDHFR-I contains the first leader 5' splice site, while pDHFR-III contains the 5' splice site of the third leader.

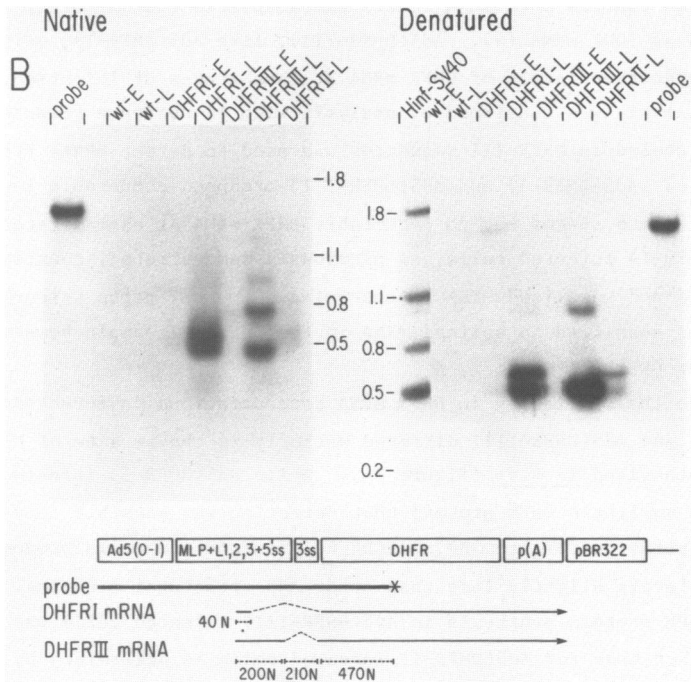
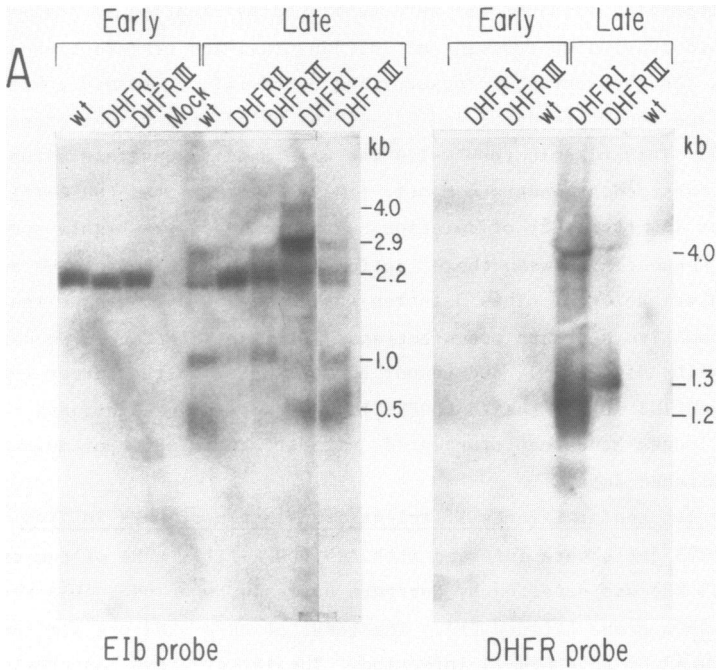
Ad5(DHFR-III) virus stocks were generated by ligation of XbaI-cleaved pDHFR-III to a 4-100 mu fragment of Ad5(309) DNA, and transfection onto 293 cells (9). In the course of constructing pDHFR-III, a plasmid containing the first and second leaders, and 2/3 of the third leader, was prepared (Figure 1). This plasmid (pDHFR-II) was also used to generate virus.

When passaged at moderate m.o.i. (10-20 PFU/cell), Ad5(DHFR-III) exhibited a low frequency of deletion. This tendency was highly specific: 1.3 kb of sequences between the 3' splice site in the modular gene and the E1b region was deleted. This deletion was detected in 15 plaque isolates derived from five separate transfections. Similar deletions were not observed with Ad5(DHFR-I), and cannot be attributed to the larger than genome size (103 mu) of the Ad5(DHFR-III). Other Ad5 recombinants several map units longer have been propagated, without accumulation of deletions (our unpublished data).

The Tripartite Leader Effects Efficient Translation of DHFR In Vivo

When 293 cells were infected with Ad5 (DHFR-III), mRNA of the expected length (1.3 kb) was detected by Northern analysis, when DHFR cDNA sequences were used as a probe (Figure 2A). The level of DHFR mRNA was similar to that generated by Ad5(DHFR-I) infection. The latter virus has previously been shown to express DHFR mRNA levels comparable to that of other late mRNAs, such as 100K mRNA (4). Ad5(DHFR-III), like Ad5(DHFR-I), only produced detectable levels of DHFR mRNA at late stages of infection. The Northern analysis was confirmed by analysis with S1 nuclease (Figure 2B): a probe end labeled in DHFR III sequences was used to detect mRNAs from the modular gene. Ad5(DHFR-I) and Ad5(DHFR-III) produced comparable levels of DHFR mRNA at late stages but no detectable DHFR mRNA at early stages. RNA from Ad5(DHFR-I) infected cells, as previously demonstrated, consisted of both spliced and unspliced mRNA in approximately equal parts (Figure 2B). The ratio of unspliced to spliced mRNA in the DHFR-III sample however was much smaller (Figure 2B).

Despite the similarity in DHFR mRNA concentrations in infected cells, Ad5(DHFR-I) and Ad5(DHFR-III) differed markedly in the amounts of DHFR protein synthesized in vivo (Figure 3A). While Ad5(DHFR-I) infected cells synthesized so little DHFR protein that detection was possible only after immunoprecipitation (Figure 3B), Ad5(DHFR-III) infected cells produced DHFR protein at levels slightly less than other Ad5 structural proteins. The level of DHFR protein synthesis in Ad5(DHFR-III) infected cells was at least 20-fold higher than for Ad5(DHFR-I) infected cells as determined by



densitometry tracing of the autoradiograph. Both viruses encoded a functional DHFR, since both were capable of transforming a DHFR negative CHO cell line to a DHFR positive phenotype (data not shown; see ref. 4 for assay).

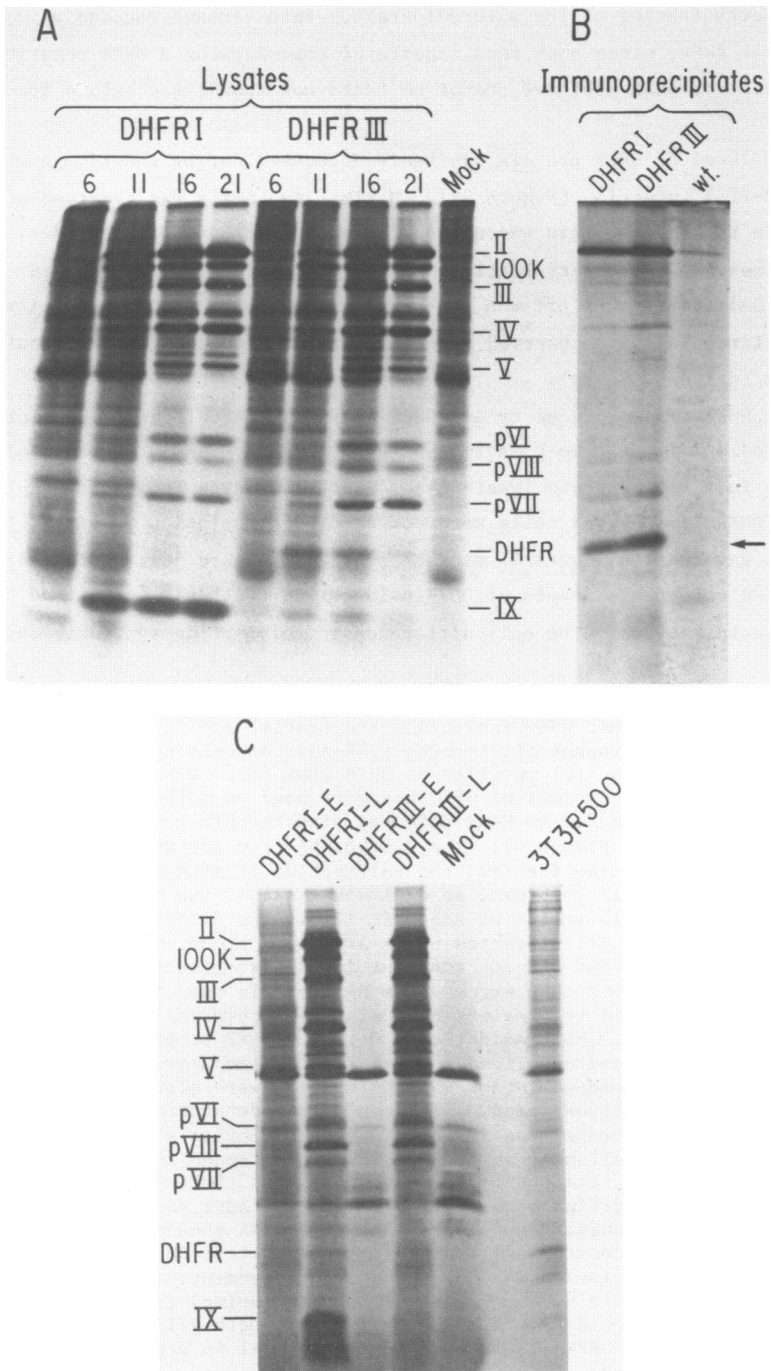
The level of DHFR protein synthesis decreased during the time course of Ad5(DHFR-III) infection (Figure 3A). Maximal synthesis was observed at 11 hr, where the DHFR protein was one of the more prominent polypeptides. At later times (21 hrs), very little DHFR protein synthesis was observed (Figure 3A). This shut off was probably not due to protein instability, as identical results were observed with short periods (5') of ^{35}S -methionine labeling (data not shown).

The DHFR mRNA produced by Ad5(DHFR-I) infection, although inefficiently translated in vivo, is as competent as other Ad5 late mRNAs when translated in vitro in a reticulocyte lysate (4). When late mRNAs from Ad5(DHFR-III) and Ad5(DHFR-I) infected cells were compared by translation in vitro, the two RNAs gave nearly identical product profiles (Figure 3C). Both RNAs stimulated comparable levels of DHFR polypeptide synthesis (confirmed by immunoprecipitation). The only difference in polypeptide synthesis observed

Figure 2. RNAs produced in Ad5(DHFR-I) and Ad5(DHFR-III) infected 293 cells

(A). Cytoplasmic RNAs isolated 6 hrs (early) and 21 hrs (late) were analyzed after electrophoresis through 1.4% agarose gels using as probes Ad5 sequences from 4.5 to 11.1 mu (E1b) or DHFR cDNA (4). HindIII digestions of Ad5 DNA and HindI digestions of SV40 DNA were used as molecular weight markers. The 1.0 and 2.2 kb RNAs detected with the E1b probe are the usual E1b transcripts (see Figure 4). The 0.5 kb RNA from Ad5(DHFR-I)-infected cells is composed of two species, the polypeptide IX mRNA and a spliced L1-IX mRNA. As discussed in the text, an equivalent set of two mRNAs of 0.5 kb is expressed from the E1b region of Ad5(DHFR-III). Two different preparations of RNA from Ad5(DHFR-III) infected cells are shown since, for unknown reasons, the 0.5 kb band was not apparent in one experiment. The 2.9 and 4.0 kb RNAs are transcripts that extend from DHFR or E1a into E1b sequences. Similar E1a-E1b hybrid transcripts have also been observed by Montell *et al.* (12). The 1.2 and 1.3 kb RNAs detected with the DHFR probe are the anticipated spliced and unspliced species from the modular gene.

(B). SI nuclease resistant RNA/DNA hybrids were electrophoresed in a 1.4% agarose gel or glyoxylated and run on a 1.4% denaturing agarose gel, as described (4). The probe used in the SI nuclease analysis was prepared from pDHFR-III by end-labeling at an Acc-I site. It extended to the Eco RI site, corresponding to the terminus of the viral DNA. Ad5(DHFR-I) and Ad5(DHFR-III) mRNAs differ both in the length of leader sequences, and in the 5' splice site sequences. Thus, spliced DHFR-I mRNA should yield a 510 N (native) and 470 N (denaturing) fragment, and unspliced DHFR-I mRNA should generate a 610 N (native) and 570 N (denaturing) segment. Spliced DHFR-III mRNA should yield a 670 N (native) or 470 N (denaturing) fragment, while unspliced DHFR-III would give a 880 N fragment in both gel systems. The 0.5 kb band observed for DHFR-III mRNA in the native gel is probably due to cleavage of SI nuclease at the splice site.



with RNAs from the two viruses was the relative levels of polypeptide IX synthesis (see below).

Ad5(DHFR-I) and Ad5(DHFR-III) Differ in Transcriptional Activity of the E1b Region

Substitution of E1a sequences in Ad5(DHFR-I) had previously been shown to result in several aberrant transcriptional events in the adjacent E1b region at late stages (ref. 4; see Figure 4C). Read through of mRNAs from the DHFR region into the E1b region were observed. The normal E1b 1.0 kb mRNA was synthesized at a 10-fold lower level than that from w.t. virus, and the normal polypeptide IX mRNA was not detected. A novel short mRNA generated by splicing of the first leader in the DHFR-I modular gene to the E1b 3' splice site was observed (Figure 4C). This mRNA, L1-IX, accumulated to levels equivalent to that of normal polypeptide IX mRNA. This L1-IX mRNA was more efficiently translated both *in vivo* (40 fold) and *in vitro* (10 fold) than normal polypeptide IX mRNA (4). In this study, we have confirmed these previous results and additionally established that the E1b 2.2 kb mRNA levels in Ad5(DHFR-I) late infected cells were less than that of w.t. infected cells (Figures 2A and 4A).

In contrast to the Ad5(DHFR-I) results, Ad5(DHFR-III) infected cells synthesized wt levels of both 1 kb and 2.2 kb E1b mRNAs (Figure 4A) and very little DHFR-E1b fusion mRNAs (Figures 2 and 4A). The DHFR-E1b read through mRNAs yield ³²P-labeled bands migrating slower than 2.2 kb in Figure 4A. The levels of polypeptide IX mRNAs were examined by resolving the S1 nuclease digested hybrids on a sequencing gel (Figure 4B). The initiation site for polypeptide IX mRNA is 13 N upstream of the 3' splice site in E1b and thus the probe protected by this mRNA can be detected by electrophoresis

Figure 3. Synthesis of DHFR protein in vivo and in vitro

(A) and (B). 293 cells were infected with recombinant virus at m.o.i.s of 20 and pulsed labeled with ³⁵S-methionine for 1 hour, at the times indicated above. Extracts (16 hr) were then prepared and either analyzed by gel electrophoresis on a 12.5% acrylamide (38:1 acrylamide:bis) gel, or 5 times the amount of the ³⁵S-methionine pulsed extract was immunoprecipitated with antisera against DHFR (the antisera did not give quantitative immunoprecipitation). Lysates from Ad5(DHFR-II) were also treated as in B, and immunoprecipitable protein was observed (data not shown). The levels of DHFR protein were quantitated by scanning the 11 and 16 hr gel lanes.

(C). Cytoplasmic mRNA isolated 6 (E) or 21 (L) hours postinfection was translated *in vitro* in a rabbit reticulocyte lysate as previously described (4). RNA from 3T3 R500 cells, which have been methotrexate-selected for high DHFR gene copy numbers (13), was translated in parallel. The ³⁵S-labeled protein migrating at the position expected for DHFR, indicated above, was immunoprecipitable with α -DHFR (data not shown).

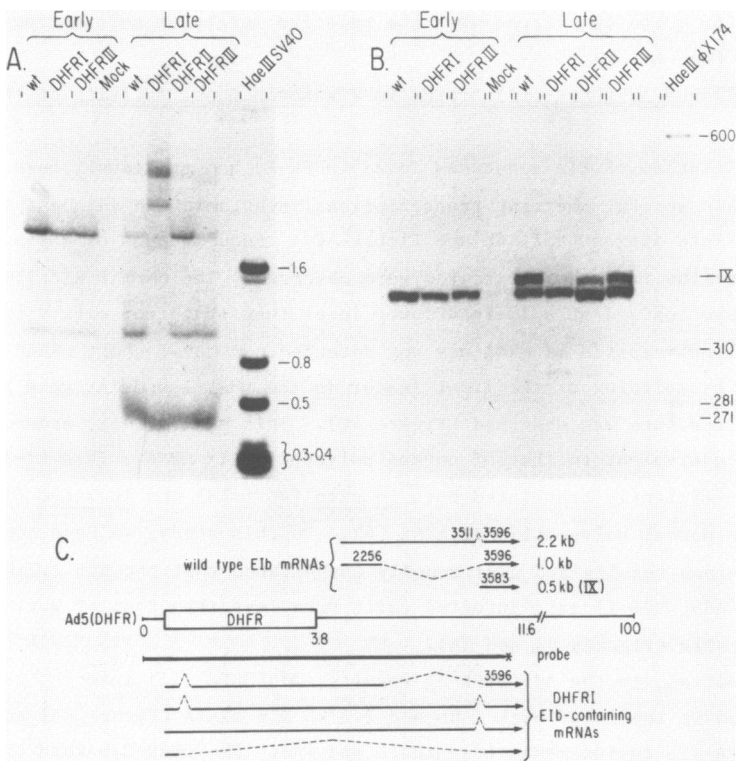


Figure 4. SI nuclease analysis of RNAs from the EIB region of wt Ad5, Ad5(DHFR-I) and Ad5(DHFR-III) infected 293 cells

The above analysis was performed on cytoplasmic mRNA prepared 21 hrs post infection; identical results were obtained with polyA(+) cytoplasmic mRNA or total polyA(+). The probe, prepared from pDHFR-I DNA, was end-labeled at the 11.1 mu SmaI site and extended to the left end of the viral DNA. The DHFR modular gene was substituted between 1.0 and 3.8 mu and generated genome lengths of 102.6 mu [Ad5(DHFR-I)] and 103 mu [Ad5(DHFR-III)]. Hybridization of this probe to mRNAs from the EIB region will generate DNAs 0.4 kb, 0.9 kb and 2.1 kb in length. SI nuclease resistant hybrids were electrophoresed in a neutral 1.4% agarose gel (A) or in a 6% polyacrylamide-8 M urea sequencing gel (B). The anticipated mobility of the polypeptide IX mRNA product in (B) is indicated. (C) Diagram of the anticipated RNAs from the EIB region. The 3' splice site of EIB is at nucleotide 3596 while the site of initiation of polypeptide IX mRNA is at nucleotide 3583. Thus RNAs initiated at the latter site protect 13 additional nucleotides of the probe as compared to RNAs spliced at the EIB site.

in a high resolution denaturing gel. Ad5(DHFR-III) infected cells synthesized 30-60% of w.t. levels of this mRNA. In accord with previous results, Ad5(DHFR-I) infected cells did not synthesize polypeptide IX mRNA. The levels of polypeptide IX synthesis in Ad5(DHFR-I) and Ad5(DHFR-III)

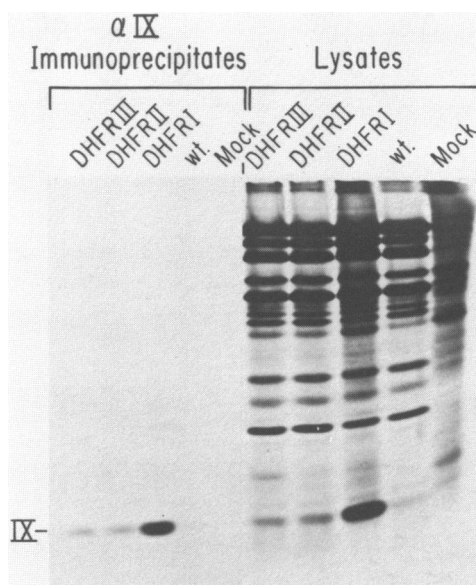
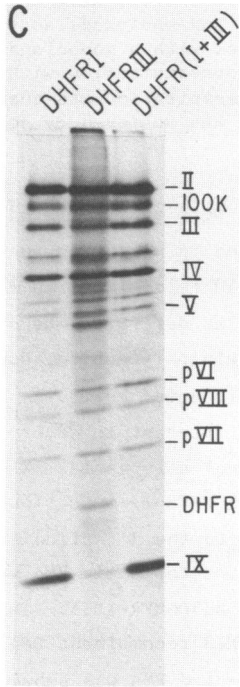
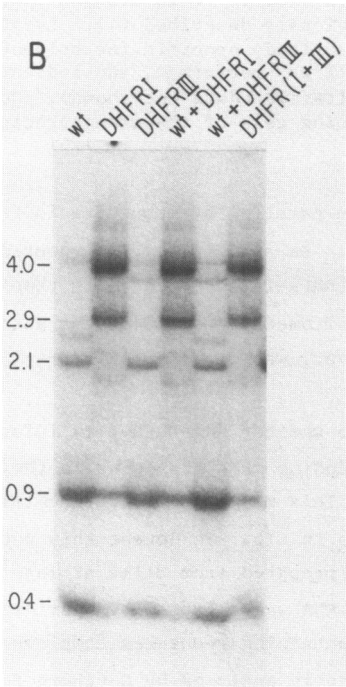
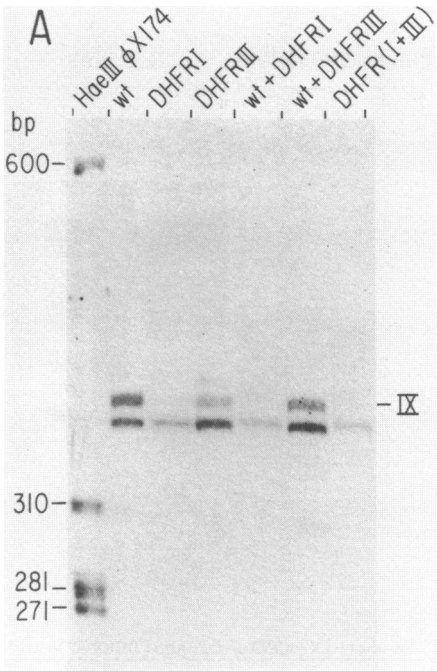


Figure 5. Analysis of leader-IX mRNAs in Ad5(DHFR-I) and Ad5(DHFR-III) infected 293 cells

Infected 293 cells were labeled from 14 to 15 hrs postinfection with ^{35}S methionine and analyzed, as previously described (4). Lysates were immunoprecipitated with a monoclonal antibody specific for polypeptide IX (14). A second immunoprecipitation with αIX was performed, and indicated that the first immunoprecipitation was quantitative (data not shown). Quantitation of polypeptide IX was achieved by scanning gels of the immunoprecipitated samples.

infected cells were also compared in parallel by immunoprecipitation of extracts labeled *in vivo* (Figure 5). As anticipated, polypeptide IX synthesis was 40 fold higher in Ad5(DHFR-I) infected cells than in w.t. infected cells as determined by densitometry tracing of the autoradiogram. Synthesis of this polypeptide, however, was elevated only 3-4 fold in Ad5(DHFR-III) infected cells.

It was of interest to determine whether Ad5(DHFR-III) infected cells contained a novel polypeptide IX encoding mRNA analogous to the L1-IX mRNA observed in Ad5(DHFR-I) infection. This mRNA would have the tripartite leader spliced to the 3' splice site in E1b. To detect this putative mRNA, i.e. L(1-3)-IX, cytoplasmic RNA was prepared from cells at late times after infection with Ad5(DHFR-III). This RNA was selected by hybridization to single strand M13 recombinant DNA containing sequences complementary to L1. The hybrid selected RNA was subsequently analyzed by Northern for enrichment



in 0.5 kb RNA complementarity to sequences of a E1b probe (data not shown). This hybrid selected RNA was also analyzed by S1 nuclease protection for sequences spliced to the 3' splice site of the E1b region (data not shown). Both the Northern and the S1 nuclease protection results suggested that a mRNA of the structure L(1-3)-IX was synthesized in Ad5(DHFR-III) infected cells and that the level of this mRNA was 10 fold lower than the L1-IX mRNA in Ad5(DHFR-I) infected cells. Since the level of polypeptide IX synthesis in Ad5(DHFR-III) infected cells is 3-4 fold higher than in w.t. Ad5 infected cells (Figure 5), it is likely that the L(1-3)-IX mRNA is translated with the same high efficiency as the L1-IX mRNA.

E1b Transcription May Be Regulated by Polypeptide IX

As described in the previous section, the levels of E1b 1 kb and 2.2 kb mRNAs were quite low in Ad5(DHFR-I) infected cells, and normal polypeptide IX mRNA could not be detected. All three E1b mRNAs, however, are made at or near wt levels in Ad5(DHFR-III) infected cells. Concomitantly, polypeptide IX protein synthesis in Ad5(DHFR-I) infected cells was 10-fold higher than in an Ad5(DHFR-III) infection. It was possible that the overproduced IX polypeptide was suppressing transcription from the E1b region. To test whether inhibition of E1b transcriptional activity could be effected in trans, cells were infected with combinations of wt Ad5 and Ad5(DHFR) viruses, and analyzed for RNA and protein. When either wt Ad5 or Ad5(DHFR-III) was coinfecting with Ad5(DHFR-I), expression of normal polypeptide IX mRNA was abolished (Figure 6A), and the levels of E1b 1 kb and 2.2 kb mRNAs were reduced (Figure 6B). [These two mRNAs generate 0.9 and 2.1 kb bands respectively after hybridization to the probe from the E1b

Figure 6. E1b mRNA and polypeptide IX expression in 293 cells coinfecting with Ad5(DHFR-I) and either Ad5 or Ad5(DHFR-III)

Cytoplasmic mRNA isolated from 293 cells 21 hrs after (co)infection with Ad5(DHFR-I) and wt Ad5 or Ad5(DHFR-III), with each at a m.o.i. of 20, was hybridized to the E1b probe illustrated in Figure 4. After S1 nuclease digestion, the hybrids were resolved on a 6% sequencing gel under denaturing conditions (A), or a 1.4% agarose gel under nondenaturing conditions (B). The band generated by hybridization to the polypeptide IX mRNA is indicated in A. Note that the lower band, produced by hybridization to 1.0 and 2.2 kb E1b mRNAs, is reduced after coinfections with Ad5(DHFR-I). The molecular weights indicated in B were determined using HindIII digests of Ad5 DNA and HaeIII digests of ϕ X174 DNA as markers. The various RNAs yielding particular S1 nuclease resistant bands can be deduced from Figure 4. (C). 293 cells were pulsed for 1 hr with 35 S-methionine, 20 hr after (co)infection, then lysed and analyzed on a 12.5% acrylamide (38:1 acrylamide:bis) gel. The mobility of the DHFR protein is indicated. The decrease in synthesis of DHFR protein after coinfection is due to inefficient translation of this mRNA during the latter stages of infection (see text).

region (see Figure 4C).] In mixed infections with Ad5(DHFR-I), polypeptide IX protein synthesis was not suppressed by the second virus, w.t. Ad5 or Ad5(DHFR-III) (Figure 6C). To demonstrate that both viral genomes were replicating in coinfecting cells, late infected cell nuclei were Hirt extracted and analyzed with restriction endonucleases diagnostic for each viral DNA (data not shown). Equal amounts of each viral genome were observed, in each coinfection combination.

DISCUSSION

Two Adenovirus 5 recombinants have been compared for efficiency of expression of a cDNA segment encoding dihydrofolate reductase. The E1a region of both recombinants has been substituted by modular genes, which contain sequences for: the major late promoter (MLP), either the first leader or the complete tripartite leader, with their normal 5' splice sites, a short segment specifying a 3' splice site, a cDNA encoding mouse DHFR and the polyadenylation signal from early SV40. The recombinant Ad5(DHFR-I), which only contains sequences for the first leader, has been previously described (4). The second recombinant, Ad5(DHFR-III), contains the complete tripartite sequence with the third leader 5' splice site intact.

At late stages of infection, Ad5(DHFR-I) and Ad5(DHFR-III) specify equivalent and high levels of DHFR mRNAs. The Ad5(DHFR-I) mRNA, with only the first leader, was inefficiently translated in infected cells, while the Ad5(DHFR-III) mRNA, containing the tripartite leader, was efficiently translated at early times in the late stage. In particular, the level of DHFR polypeptide synthesis was comparable to that of late viral polypeptides at 11-16 hrs post infection with Ad5(DHFR-III). By 21 hrs postinfection, however, DHFR polypeptide synthesis decreased significantly. These results suggest that the DHFR mRNA containing the tripartite leader was more efficiently recognized for translation in late infected cells than mRNA with only the first leader. This effect was greater than 20 fold. The decrease in synthesis of DHFR polypeptide, as the late stage progresses, is probably explained by competition for an initiation factor by a large pool of other late viral mRNAs which have a higher affinity for this factor. One potential explanation for the poor competition of the tripartite form of the DHFR mRNA with other late viral mRNAs is the distance between the initiation codon and tripartite leader in this mRNA, 138 N. This distance in viral mRNAs for the prominent late polypeptides hexon (II) and fiber (IV) is 36 or

0 nucleotides, respectively. In another study using a different gene, we have constructed a recombinant with a shorter distance between the initiation AUG and tripartite leader and have observed more efficient translation. Whether this result can be generalized awaits further experiments.

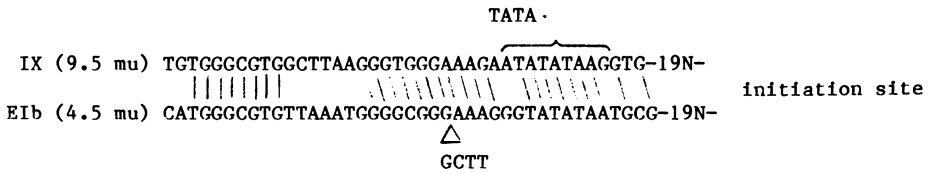
During the late stage of adenovirus infection, polyribosomes preferentially translate viral mRNAs even though cellular mRNAs are present at comparable levels. This preferential recognition requires the small viral RNAs, V.A. RNA I and II. Ad5 mutants defective for V.A. I RNA synthesis are deficient for translation of viral mRNAs, but still efficiently suppress translation of cellular mRNAs (1). Some feature of late viral mRNAs must specify their common recognition for translation. The tripartite leader which is spliced to almost all late mRNAs is an obvious candidate and our results support this supposition. Both Logan and Shenk (15) and Thummel *et al.* (16) have also concluded that the tripartite leader is important for translation in late infected cells. In the former study, mRNAs containing the tripartite leader were only 5-fold more efficiently translated than mRNA with only the first leader sequence. However, this ratio was not examined at early times in the late stage and could be an underestimate. In the latter study, each adenovirus recombinant specified multiple types of spliced mRNAs making it difficult to correlate levels of expression with sequence content. The manner by which the tripartite leader specifies translation is not known. Although V.A. RNA I synthesis is essential for translation of late viral mRNA, its stimulation of translation is not restricted to mRNAs containing the tripartite leader (17). In any case, it is likely that some factor or combination of factors in late infected cells preferentially recognize the tripartite leader sequence for translation.

Substitution of the E1a region by the modular genes in Ad5(DHFR-I) and Ad5(DHFR-III) produced markedly different effects on the transcription activity of the adjacent E1b region. The 10-fold decrease in levels of E1b 1 kb and 2.2 kb mRNAs in the late stage of Ad5(DHFR-I) infected cells was not observed with Ad5(DHFR-III). In addition, near wt levels of polypeptide IX mRNA were produced during Ad5(DHFR-III) infection, while this mRNA was not detected during Ad5(DHFR-I) infection. A novel mRNA, L1-IX, generated by splicing of the first leader in the modular gene to the 3' splice site of E1b, was synthesized during Ad5(DHFR-I) infection. This mRNA was translated

40 and 10-fold more efficiently in vivo and in vitro, respectively, than the normal polypeptide IX mRNA (4). An analogous mRNA, L(1-3)-IX, was synthesized in Ad5(DHFR-III) infected cells and also appeared to be efficiently translated in vivo. However, this mRNA only accumulated to 1/10th the level in Ad5(DHFR-III) infected cells as its counterpart in Ad5(DHFR-I) infection. Consistent with these findings, Ad5(DHFR-I) infected cells synthesized 10 fold more polypeptide IX than did Ad5(DHFR-III) infected cells.

We had previously suggested that the reduction in mRNA synthesis from the E1b region in Ad5(DHFR-I) infected cells might be due to the presence of the strong upstream MLP. However, this explanation now seems unlikely, since the transcriptional activity of the MLP in Ad5(DHFR-I) and Ad5(DHFR-III) was the same, and cells infected with the latter virus synthesized wt levels of mRNA from the E1b region. A more likely explanation for the reduction in mRNA synthesis from the E1b region of Ad5(DHFR-I) is a suppression effect due to the overproduction of the IX polypeptide. Consistent with this hypothesis is the finding that the suppression can be observed in trans: when wt Ad5 or Ad5(DHFR-III) virus was coinfecting with Ad5(DHFR-I), synthesis of all three normal mRNAs from the E1b region of both viruses was suppressed. In all coinfections, polypeptide IX levels were comparable to that observed after single infection with Ad5(DHFR-I).

Synthesis of mRNAs from the E1b region is due to initiation at two sites: the E1b promoter at 4.5 mu and the polypeptide IX promoter at 9.5 mu. As mentioned above, mRNAs from both initiation sites are suppressed during Ad5(DHFR-I) infection, probably by the high levels of IX polypeptide. This suggests the presence of an autoregulation loop between IX protein levels and synthesis of IX and E1b mRNAs. The role of this autoregulation during the lytic cycle is not clear, as functions of proteins encoded by the E1b region are not well understood. Polypeptide IX is associated with hexon in virions, and thus its intracellular concentration may reflect the level of virion components and the rate of virus assembly. Although we have no direct evidence, it is likely that mRNA synthesis from the E1b region is suppressed at the level of initiation of transcription. If so, a common sequence element of the E1b and IX initiation sites must be recognized in the suppression. As shown below, the sequence in the vicinity of the TATA box (18) of these two sites is remarkably similar.



Perhaps polypeptide IX autoregulation and suppression of transcription of the Eib region are mediated through a subset of these sequences.

ACKNOWLEDGMENTS

K.L.B would like to acknowledge support from a Charles A. King Trust fellowship from The Medical Foundation, Inc. This work was supported by grants for National Science Foundation (PCM-8200309), from National Institutes of Health (PO1-CA26717) to P.A.S. and partially from National Institutes of Health Core grant (PO1-CA14051).

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