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The Adenovirus Tripartite Leader May Eliminate the Requirement for Cap-Binding Protein Complex during Translation Initiation

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The adenovirus tripartite leader is a 200-nucleotide 5′ noncoding region that is found on all late viral mRNAs. This segment is required for preferential translation of viral mRNAs at late times during infection. Most tripartite leader-containing mRNAs appear to exhibit little if any requirement for intact cap-binding protein complex, a property previously established only for uncapped poliovirus mRNAs and capped mRNAs with minimal secondary structure. The tripartite leader also permits the translation of mRNAs in poliovirus-infected cells in the apparent absence of active cap-binding protein complex and does not require any adenovirus gene products for this activity. The preferential translation of viral late mRNAs may involve this unusual property.

At late times after adenovirus infection, the majority of viral mRNAs are transcribed from the major late promoter. Five families of 3′ coterminal mRNAs are derived from a primary transcript, all of which contain an identical 5′ noncoding tripartite leader segment 200 nucleotides in length joined to the various coding regions by the splicing of three small exons (8). It has been demonstrated that the tripartite leader enhances translation of mRNAs at late but not early times after adenovirus infection (9, 37). Deletion analysis has indicated that all three leader segments are required for this effect. The leaders were also found to enhance translation of mRNAs in transfected cells (29).

Adenovirus infection results in a complex series of host metabolic alterations including the significant inhibition of cellular protein synthesis at late times after infection. Viral late mRNAs constitute the vast majority of those found in polyribosomes, although they probably account for less than half of the total cytoplasmic mRNAs (21). Most tripartite leader-containing mRNAs are therefore preferentially translated at late times during adenovirus infection (9, 37).

Preferential translation may occur, at one level, by the rate at which an mRNA complexes with ribosomes, especially under competitive conditions (57). In this regard, there is growing evidence that mRNA 5′ noncoding regions can influence levels of protein synthesis. Stable secondary structures in 5′ noncoding regions may act as energy barriers to translation initiation. Experimentally, the introduction of stable hairpin loops decreases ribosome binding, whereas reduced secondary structure promotes interaction (20, 31, 32, 42, 43). There is some evidence that translation may even be controlled predominantly by the degree of secondary structure involving a small stretch of about 15 nucleotides adjacent to the cap (33).

Considerable attention has been directed toward identifying factors which may be responsible for enhanced or preferential translation of mRNAs. There is experimental evidence to suggest that regulation may involve a presumptive ATP-dependent mRNA-unwinding activity of initiation factor eIF-4F (33, 47, 48), which is significantly inhibited by RNA secondary structure. This factor appears to be identical to the cap-binding protein complex, which binds cap structures in the presence of ATP and stimulates protein synthesis (35; reviewed in reference 53). CBP complex consists of at least three polypeptides, of 24, 46 to 50, and 220 kilodaltons (kDa). The 24-kDa polypeptide was originally identified as CBPI, and the 46- to 50-kDa species was identified as initiation factor eIF-4A.

CBP complex has been shown to be required for the translation of most capped mRNAs. In poliovirus-infected cells, the selective inhibition of host protein synthesis has been roughly correlated with cleavage of a 220,000-Da protein (p220) (18), a component of CBP complex (16, 24). Although proteolysis of p220 appears to be required, it is not totally sufficient for the complete inhibition of cellular protein synthesis during poliovirus infection, suggesting that at least one other event is also required (11). The specific cleavage of p220 is thought to inactivate CBP complex and inhibit translation of capped mRNAs (for reviews, see references 51 and 53). Interestingly, capped mRNAs with reduced secondary structure display a reduced requirement for CBP complex and continue to initiate translation in poliovirus-infected cell extracts (54).

To understand the function of the adenovirus tripartite leader during the viral life cycle, we have investigated the mechanism by which the leader enhances translation. It has been known for some time that the translation of at least several adenovirus late mRNAs is resistant to inhibition by poliovirus infection (6, 13). The mechanism by which this occurs, however, has remained obscure. We demonstrate that the tripartite leader may reduce or eliminate the requirement for intact CBP complex. Most tripartite leader-containing mRNAs continue to translate despite proteolysis of CBP p220, a property previously established only for uncapped poliovirus mRNAs and capped mRNAs with little secondary structure. We also demonstrate that the tripartite leader permits translation in the absence of intact CBP complex in poliovirus-infected cells and does not require the participation of any adenovirus gene products for this activity. This unusual property of the tripartite leader may be involved in the preferential translation of late mRNAs in adenovirus-infected cells.

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MATERIALS AND METHODS

Viruses and cells. Adenovirus mutant H5d1309 was grown in 293 cells (23) and is a phenotypically wild-type (wt) virus that contains a series of altered restriction enzyme cleavage sites (28). Cos1 cells are a CV1 monkey cell line transformed with a replication origin-defective simian virus 40 (22). HeLa and Cos cells were grown in monolayers in Dulbecco modified Eagle medium containing 10% calf serum. DNA transfections of Cos cells were performed by the DEAE-dextran technique (38). Infections were performed with 5 to 10 PFU of viruses for either 30 min (poliovirus) or 60 min (adenovirus).

RNA preparation and analysis. A cytoplasmic RNA fraction was prepared by suspending cells in cold, sterile Nonidet P-40 lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40), incubating them for 5 min on ice, and subjecting them to Dounce homogenization. Nuclei and mitochondria were pelleted by Microfuge centrifugation. Supernatants were phenol-chloroform extracted and ethanol precipitated. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography.

Northern (RNA) type analysis of poly(A)$^+$ RNA was performed with formaldehyde-agarose gels, and the RNA was transferred to nitrocellulose paper (55). DNA probes for Northern analysis were prepared by using α-^{32}P-deoxynucleoside triphosphates by the method of Feinberg and Vogelstein (19). DNA probes consisted of the following adenovirus type 5 (Ad5) restriction fragments, in map units: E1B (*Kpn*I [5.8] to *Hin*dIII [7.9]), E2A (*Bgl*II [63.6] to *Sma*I [68.0]), L3 (*Sma*I [52.6 to 54.8]), and L5 (*Hpa*I [89.0] to *Sma*I [91.9]).

Analysis of polypeptides. Cells were labeled with [^{35}S] methionine as indicated in the figure legends, by using 50 μCi of *trans*-[^{35}S]methionine (ICN) per ml in Dulbecco modified Eagle medium without methionine. Equal numbers of cells were used for preparation of cell extracts, immunoprecipitations, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, as described (49). Immunoprecipitations were carried out with monoclonal antibodies directed against adenovirus DBP (72 kDa) and protein IX (a gift of A. J. Levine, Princeton University), hexon (Chemicon), and hepatitis B surface antigen (HBsAg) (a gift of Imclone Systems, Inc.). Fluorography was performed with En^{3}Hance (New England Nuclear Corp.). Western immunoblot analysis of CBP p220 was performed by the method of Bernstein et al. (10) with SDS–10% polyacrylamide gels. Approximately 100 μg of cytoplasmic protein extract was loaded per sample. Rabbit polyclonal serum raised against p220 was a gift of R. Lloyd and E. Ehrenfeld (University of Utah). Autoradiograms were quantitated by densitometric scan with an LKB Ultrospec 2.

Plasmid and poliovirus constructions. Plasmid pSV-HBV was constructed from plasmid p415 (37), which contains the Ad5 E1a enhancer (base pairs 1 to 357) fused to the Ad2 major late promoter (nucleotides [nt] 5805 to 6038) and a full-length tripartite leader cDNA. A *Sal*I linker is located immediately downstream of the third leader. The human hepatitis B virus (HBV) (subtype ayw) surface antigen coding region was derived from a *Hha*I (nt 146)-to-*Dra*I (nt 831) fragment, which was fused to the *Sal*I site of the third leader, and an Ad5 E1a fragment (*Hpa*I [nt 1572] to *Kpn*I [nt 2048]), which restores the HBsAg stop codon and provides a polyadenylation signal. About 18 nucleotides separate the end of the third leader and the HBsAg translational start codon.

Poliovirus mutant R2-2A^{Pro}-2 contains a 2-amino-acid insertion in polypeptide 2A^{Pro}. This mutant was constructed by partial digestion of P2 Lansing cDNA (45) with *Sna*BI, followed by insertion of a 6-base *Hpa*I linker. P2 Lansing cDNA, containing a linker inserted at nt 3767, was transfected into HeLa cells, and viruses were isolated. Mutant virus R2-2A^{Pro}-2 forms small plaques on HeLa cell monolayers. The expected 6-base insertion at nt 3767 was verified by nucleotide sequence analysis of R2-2A^{Pro}-2 viral RNA.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was carried out with a microdilution well system to determine levels of HBsAg in cell lysates. Microdilution dishes were prepared and ELISA was performed essentially as described by Patzer et al. (41). Purified HBsAg standards (Abbott Laboratories) were used to quantitate the analysis.

RESULTS

Construction of a poliovirus mutant defective in proteolysis of CBP p220. The construction of mutant R2-2A^{Pro}-2, which contains a 2-amino-acid insertion in polypeptide 2A^{Pro}, is described in detail in Materials and Methods. R2-2A^{Pro}-2 appears to be defective in inhibition of host cell translation (V. Racaniello and R. J. Schneider, manuscript in preparation). It has a phenotype similar to that of a previously reported poliovirus mutant, HF121 (10), which contains a single-amino-acid insertion in polypeptide 2A^{Pro} between amino acids 102 and 103. The 2-amino-acid insertion in R2-2A^{Pro}-2 is between amino acids 129 and 130. At late times after infection, both R2-2A^{Pro}-2 and HF121 induce a global inhibition of cellular and viral protein synthesis (10; Racaniello and Schneider, in preparation).

Translation of adenovirus mRNAs during poliovirus superinfection. We analyzed viral polypeptide synthesis in HeLa cells infected with adenovirus for 16 to 18 h and then superinfected with poliovirus. Cells were labeled with [^{35}S]methionine, and polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 1). As expected, cellular protein synthesis was severely inhibited by infection with wt poliovirus (lane 3). Inhibition of cellular polypeptide synthesis is evidenced by the complete absence of the actin band (Ac in Fig. 1) and the background cellular polypeptides normally present in cells infected only with adenovirus (lane 8). Surprisingly, the majority of late adenovirus mRNAs continue to translate in cells superinfected with wt poliovirus.

At late times in cells coinfected with wt adenovirus and poliovirus, late adenovirus polypeptides II (hexon), III (penton base), V (core protein 1) and pVII (precore protein 2) were all present at levels reduced about one-half from those found in cells infected only with adenovirus (compare lanes 6 and 8). Levels of late polypeptides 100k, IIIa, and IV (fiber) were reduced to about one-third to one-fourth those of cells infected with only wt adenovirus, but were still significant. Only two of nine late polypeptides (pVI-VI and pVIII) had significantly reduced levels (about 10- to 15-fold). Conspicuously absent in late coinfected cells are the 72k DNA-binding protein, protein IVa2, and protein IX, which are all translated from mRNAs that lack the tripartite leader, but which are quite abundant in late adenovirus-infected cells (21).

Since the majority of late adenovirus mRNAs continue to be translated in cells superinfected with wt poliovirus, the inhibition in accumulation of pVI and pVIII polypeptides is surprising. The late adenovirus mRNAs encoding pVI and pVIII are present at lower levels than those for most other

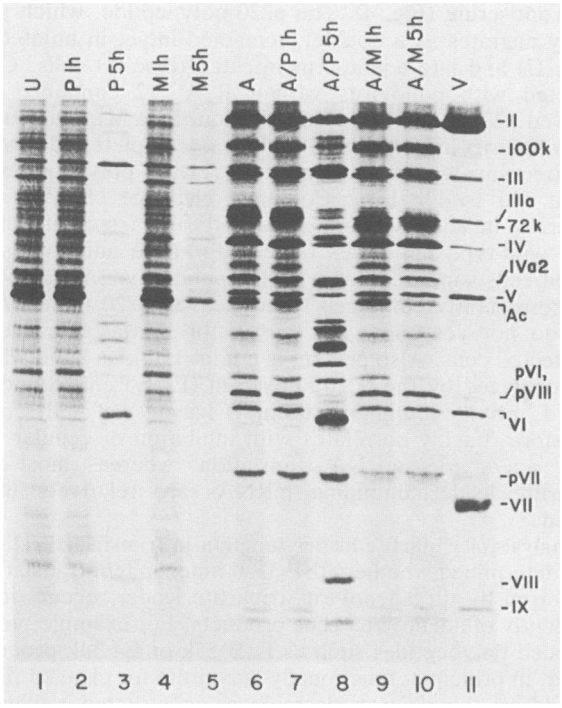

FIG. 1. Electrophoretic analysis of polypeptides synthesized in HeLa cells after infection with adenovirus and poliovirus. Cells were infected and labeled with [^{35}S]methionine (50 μCi/ml) for 1 h at 24 h after infection with adenovirus *dl*309, or 1 or 5 h after poliovirus infection. Equal numbers of cells were loaded in each sample. Extracts were prepared, and electrophoresis was carried out for 18 h at 15 mA in an SDS–15% polyacrylamide gel as described (49). Lanes: U, uninfected cells; P, wt poliovirus labeled 1 or 5 h after infection; M, poliovirus mutant 2A-2; A, adenovirus *dl*309; A/P, late adenovirus *dl*309 plus wt poliovirus labeled 1 or 5 h after poliovirus superinfection; A/M, late adenovirus *dl*309 plus poliovirus mutant 2A-2 labeled 1 or 5 h after poliovirus superinfection; V, labeled virion proteins included as size markers. Labeled polypeptides were quantitated by densitometric scans of original autoradiograms.

late polypeptides described above (2, 12). Although it remains to be tested, one possible explanation of these results is that poliovirus mRNAs simply dilute those for pVI and pVIII, so that their products are barely represented.

The accumulation of early and late adenovirus polypeptides was also analyzed after superinfection of late adenovirus-infected cells with poliovirus mutant R2A^{Pro}-2. This virus is phenotypically similar to another 2A mutant, HF121, previously described by Bernstein et al. (10). Cells infected with the poliovirus 2A mutant fail to accumulate cleaved CBP p220 (see Fig. 5). When late adenovirus-infected cells are superinfected by the poliovirus 2A variant, there is at best only a slight block to the translation of cellular and early adenovirus mRNAs (Fig. 1, compare lanes 8 and 10). The translation of tripartite leader-containing mRNAs remains relatively unchanged, except for an unexplained decrease in pVII protein levels.

In cells infected with poliovirus variant R2A^{Pro}-2 alone, both viral and cellular protein synthesis is significantly inhibited (lanes 5 and 10), in a manner which is not yet understood. Interestingly, prior infection with adenovirus partially reverses this block. One possible explanation of this effect concerns the controversial role of the interferon-induced P1/eIF-2 kinase during poliovirus infection (3, 40). However, since adenovirus VA1 RNA blocks activation of this kinase (50, 52) it is tempting to speculate that elevated kinase levels may be present in cells infected with poliovirus mutant 2A^{Pro}-2. Lastly, it should be noted that in general, larger amounts of poliovirus proteins seem to accumulate in cells coinfected with adenovirus, possibly as a result of lengthening the poliovirus replication cycle.

Immunoprecipitation analysis demonstrates that only late adenovirus mRNAs escape the translation block created by wt poliovirus infection. Cells were labeled with [^{35}S]methionine at late times after infection with adenovirus, with adenovirus plus wt poliovirus, or with adenovirus plus poliovirus mutant 2A^{Pro}-2. Specific adenovirus polypeptides were immunoprecipitated from infected-cell extracts and resolved by gel electrophoresis (Fig. 2). As expected, roughly comparable levels of late hexon protein II were produced. Translation of mRNAs encoding adenovirus DBP and protein IX, which do not contain the tripartite leader, was inhibited by infection with wt poliovirus (lane A/P), but not by the poliovirus variant (lane A/M). Immunoprecipitation analysis also demonstrated that translation of adenovirus early E1B mRNA (55k protein) was inhibited in cells superinfected by wt poliovirus (data not shown).

Synthesis of adenovirus mRNA at late times after infection. Infection of animal cells with poliovirus has been shown to result in inhibition of cellular RNA synthesis (39), presumably by inhibiting transcription factor activity in some manner (14). It was therefore important to determine whether adenovirus early or late mRNAs were differentially affected by poliovirus superinfection. Northern-type analysis was performed on cytoplasmic poly(A)$^+$ RNAs purified from cells late after infection with adenovirus, with adenovirus plus poliovirus, or with poliovirus alone (Fig. 3). RNA

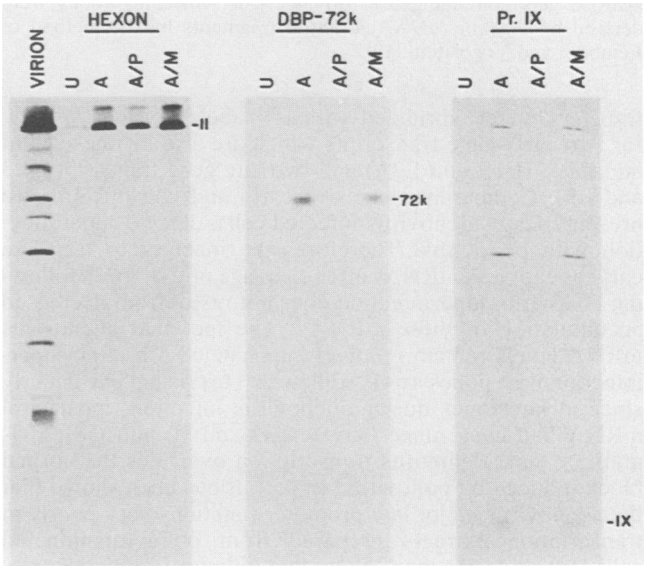

FIG. 2. Electrophoretic analysis of specific adenovirus polypeptides synthesized in HeLa cells at late times after infection. Cells were infected with adenovirus *dl*309 for 18 h (lanes A), superinfected with wt poliovirus (lanes A/P) or poliovirus mutant 2A^{Pro}-2 (lanes A/M) for 5 h, and then labeled with [^{35}S]methionine (50 μCi/ml) for 1 h. Extracts were prepared and immunoprecipitations were performed by using monoclonal antibodies specific for late hexon protein II, early polypeptide 72k (DBP), or early polypeptide IX. Immunoprecipitations and electrophoresis in an SDS–15% polyacrylamide gel were performed as described (49).

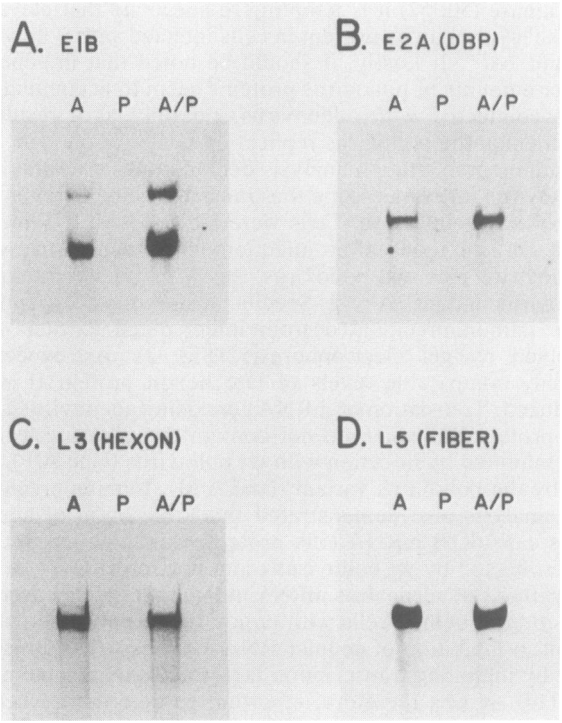

FIG. 3. Analysis of adenovirus mRNAs present at late times after infection with wt poliovirus. Northern-type analyses of adenovirus early mRNAs E1B (A) and E2A (B) and late mRNAs L3 (C) and L5 (D) are shown. Cytoplasmic poly(A)$^+$ RNA was prepared from HeLa cells after infection for 23 h with adenovirus *dl*309 (lanes A), for 5 h with wt poliovirus (lanes P), or for 18 h with adenovirus followed by superinfection for 5 h with wt poliovirus (lanes A/P). RNAs were subjected to electrophoresis at 100 V for 4 h in 1% agarose gels containing 6% formaldehyde. Specific probes were derived by labeling DNA restriction fragments by the method of Feinberg and Vogelstein (19).

transfers were hybridized with ^{32}P-labeled probes specific for two early gene transcripts which are also synthesized at late times (E1B and E2A) and two late gene transcripts (L3 and L5). Comparable levels of early and late mRNAs are present in late adenovirus-infected cells, despite superinfection with poliovirus. Therefore, the inability to translate early adenovirus mRNAs after cleavage of CBP p220 following poliovirus superinfection does not result from decreased accumulation of these mRNAs. The fact that adenovirus mRNA levels remain relatively unchanged 5 h after superinfection with poliovirus is somewhat surprising, particularly since at late times during adenovirus infection, most viral mRNA half-lives range between 30 and 90 min (58). It is unlikely that adenovirus transcription overrides the normal block induced by poliovirus. In fact, it has been shown that the adenovirus major late promoter functions very poorly in transcription extracts prepared from poliovirus-infected cells (14). It seems more likely that poliovirus infection may increase mRNA stability (36) in a manner similar to that observed with protein synthesis inhibitors (15).

Cleavage of CBP p220. The inhibition in translation of cellular and early adenovirus mRNAs following superinfection with wt poliovirus suggests that CBP complex is inactivated in these cells, presumably by poliovirus-induced cleavage of CBP p220. Characterization of p220 was performed on cell lysates subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analyses with a p220 antiserum (Fig. 4). The p220 polypeptide, which generally migrates as a doublet, remained intact in uninfected (lane U) and late adenovirus-infected (lane A) cells. Cells infected with poliovirus variant R2A^{Pro}-2 contained uncleaved p220 at late times in infection (lane M), whereas in wt poliovirus-infected cells, p220 was completely degraded to three smaller, but antigenically related polypeptides of about 115 to 130 kDa. Complete cleavage of p220 also occurred in adenovirus-infected cells upon superinfection with wild-type poliovirus (lane A/P). Late adenovirus-infected cells superinfected with the poliovirus 2A gene variant consistently contained some cleaved p220 (lane A/M). We do not yet know the reason for partial cleavage in coinfected cells. Also present is a faint 150-kDa band which is recognized by the p220 antiserum (E. Ehrenfeld and R. Lloyd, private communication). Cleavage of CBP p220 therefore roughly correlates with inhibition of cellular and early adenovirus mRNA translation, whereas most late tripartite leader-containing mRNAs are relatively unaffected.

Analysis of tripartite leader function in transfected cells. It was determined whether CBP p220-independent translation, conferred by the adenovirus tripartite leader, occurs independently of adenovirus gene products. For example, virus-encoded polypeptides such as E1B 55k or E4 34k products could, in principle, functionally substitute for cleaved p220. To address this point, a plasmid was constructed in which a full-length cDNA of the tripartite leader was fused to the HBsAg-coding region (Fig. 5). The HBsAg ATG is 18 nucleotides downstream of the third leader. Transcripts are generated from the Ad5 major late promoter and use the viral E1A polyadenylation signal. Construction of this vector is described more fully in Materials and Methods. The pSV-HBV clone expresses easily detectable levels of S antigen after transfection into Cos cells.

Cells were transfected with pSV-HBV and 48 h later were infected with wt poliovirus (Fig. 6A). High levels of the nonglycosylated (p24) and glycosylated (gp27) forms of S antigen were detected by immunoprecipitation of [^{35}S]methionine-labeled polypeptides from uninfected cells (lane S-Ag). To determine the effect of cleavage of CBP p220 on the production of S-antigen polypeptides, we labeled trans-

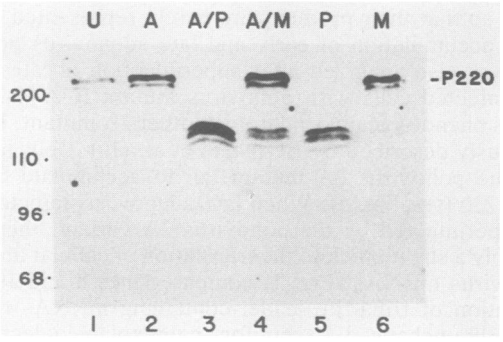

FIG. 4. Immunoblot analysis of p220 cleavage in late-infected HeLa cells. HeLa cells were infected and cytoplasmic extracts were prepared as described in Materials and Methods. A 100-μg portion of each extract was analyzed by electrophoresis in an SDS–10% polyacrylamide gel at 40 V for 20 h. Proteins were transferred to nitrocellulose by the method of Towbin et al. (56). Filters were probed with a polyclonal serum against p220. Numbers on the left side refer to nonradioactive high-molecular-weight standards that were transferred and stained with amido black. Sample nomenclature is as described in the legend to Fig. 1.

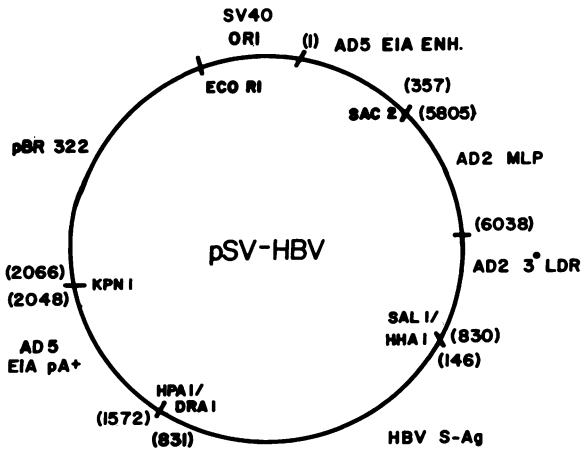

FIG. 5. Diagram of pSV-HBV which expresses HBsAg under the control of the adenovirus major late promoter. Nucleotide sequence numbers correspond to sources of DNA segments as indicated. The vector expresses HBsAg mRNA which contains a full-length cDNA of the tripartite leader. The construction of the vector is described in Materials and Methods. Abbreviations: SV40, simian virus 40; MLP, major late promoter; LDR, leader.

fected cells with [^{35}S]methionine from 4 to 7 h after infecting them with wt poliovirus. Cleavage of CBP p220 did not alter translation of S-antigen mRNAs, whereas cellular protein synthesis was inhibited (data not shown). Analysis of immunoprecipitations performed at lower stringency (Fig. 6B) further demonstrated that comparable amounts of HBsAg are produced, despite inhibition of host cell translation. Cells transfected with a control plasmid (pUC18) contained no immunologically cross-reactive polypeptides in the 24- to 27-kDa range.

Cos cells were also transfected with a plasmid that expresses the adenovirus DNA-binding protein E2A mRNA (Fig. 6C). Immunoprecipitation of labeled 72k polypeptide could be demonstrated only in cells that were not infected by poliovirus (compare lanes + and −). It is clear that in poliovirus-infected cells the tripartite leader promotes translation of mRNAs, despite inactivation of CBP complex. We conclude that adenovirus products do not appear to be necessary for the CBP p220-independent translation conferred by the tripartite leader.

TABLE 1. Quantitation of HBsAg polypeptides by ELISA[a]

Plasmid	Amt of HBsAg (ng) per 5 × 10^5 cells[b]	
	−Poliovirus	+Poliovirus
pSV-HBV	90.0	100.0
pSV-S	4.8	0.3
None	0.01	0.01

[a] pSV-HBV S-antigen mRNAs contain adenovirus tripartite leader 5′ NCR; pSV-S mRNAs contain native 5′ noncoding regions.
[b] All values are ±10%.

Lastly, we determined whether the S-antigen coding region itself contributes, in some manner, to p220-independent translation. Plasmid pSV-S contains the complete S-antigen mRNA (S, preS1, and preS2) expressed from the simian virus 40 late promoter. Cos cells were transfected with pSV-S and 48 h later were infected with wt poliovirus for 8 h. Cell lysates were prepared, and the levels of intracellular S antigen were determined by quantitative ELISA (Table 1).

The half time for secretion of S antigen in cultured cells has been determined to be about 5 h (41). If translation of native S-antigen mRNAs is inhibited by inactivation of CBP complex, at best 25% of the steady-state protein level should remain 8 h after poliovirus infection. Quantitative ELISA indicates that the intracellular level of S antigen in the poliovirus-infected sample declines to about 7% that of the uninfected control (0.3 ng compared with 4.8 ng). We conclude that native mRNAs encoding S antigen require CBP complex for translation. S-antigen mRNAs that contain the adenovirus tripartite leader, however, can be efficiently translated despite inactivation of CBP complex and inhibition of cellular protein synthesis.

DISCUSSION

In this study we have begun an investigation into the molecular mechanism by which the adenovirus tripartite leader enhances translation at late times during virus infection. We have demonstrated that most adenovirus late mRNAs, which contain the tripartite leader sequence, are translated in cells in which CBP p220 has been cleaved by poliovirus superinfection (Fig. 1). Cleavage of CBP p220 clearly blocks translation of cellular and adenoviral mRNAs that lack the tripartite leader sequence, demonstrated in Fig.

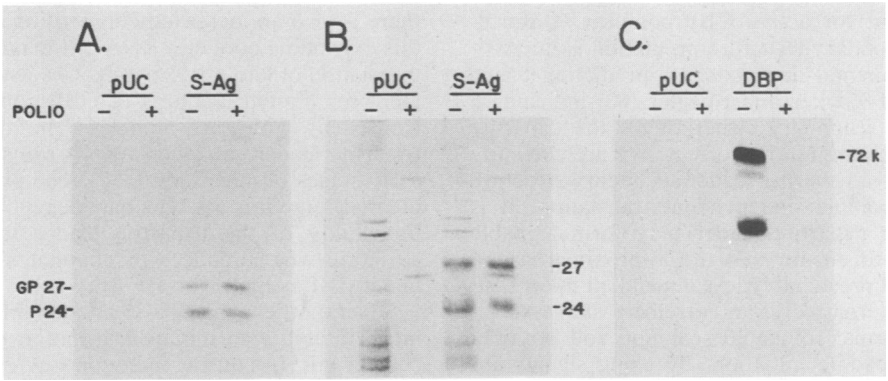

FIG. 6. Analysis of tripartite leader requirement for adenovirus gene products. Cos1 cells were transfected with pSV-HBV and then infected 48 h later with wild-type poliovirus for 5 h. Cells were labeled with 100 μCi of [^{35}S]methionine per ml for 3 to 5 h after poliovirus infection. Extracts were prepared and immunoprecipitations were performed by using a monoclonal antibody specific for HBsAg. Polypeptides were resolved by electrophoresis in an SDS–15% polyacrylamide gel. Immunoprecipitation washes were carried out in 0.5 M NaCl (A) or 0.3 M NaCl (B). Cos cells were transfected with pDBP and then infected with poliovirus and processed as described above (C).

1 and 2 by the immunoprecipitation of labeled adenoviral polypeptides. CBP p220 appears to be entirely cleaved after infection with wt poliovirus (Fig. 4). It seems unlikely that small amounts of residual p220 could be responsible for translation of the large number of adenovirus late mRNAs present in cells coinfected with poliovirus. In fact, overexposure of the immunoblot shown in Fig. 5 failed to reveal any uncleaved p220 in wt poliovirus-infected samples (data not shown).

Our results indicate that the tripartite leader promotes translation independent of active CBP complex and in the absence of any adenovirus gene products. This was demonstrated in Fig. 6, in which cleavage of CBP p220 failed to diminish the translation of a tripartite leader-containing mRNA in transfected cells. mRNAs that lack the tripartite leader were inhibited from translating under the same conditions (Fig. 6; Table 1). We doubt that after p220 cleavage tripartite leader-containing mRNAs are translated simply because large amounts of these mRNAs are present. High levels of test mRNAs were provided in transfected cells, but only those containing the tripartite leader were translated after CBP complex inactivation. In addition, several studies have shown that extracts prepared from wt poliovirus-infected cells do not actively translate most capped mRNAs, even if excessive amounts are added (11, 24).

We cannot be absolutely certain that the tripartite leader permits translation in a manner completely independent of CBP complex activity. The results of Etchison et al. (17) indicate that cleavage of p220 most probably inactivates the entire CBP complex, since complexes purified from poliovirus-infected cells fail to permit translation of capped mRNAs in an in vitro translation system and cannot be cross-linked to capped mRNAs (17). However, it has not been firmly established whether the p220 component is absolutely required in vivo or merely assists CBP complex, for example, by enhancing the putative mRNA-unwinding activity (16, 24, 48). In this regard, it is interesting that capped mRNAs with minimal 5′ secondary structure can still bind ribosomes in extracts in which CBP p220 has been cleaved (54). These results indicate that intact CBP complex may not be absolutely required for translation of mRNAs with minimal 5′ secondary structure. In a separate study, it was found that reduced secondary structure was required only within the first 15 nucleotides from the cap to enhance translation of an mRNA (33).

It is tempting to speculate that the tripartite leader may form a peculiar secondary structure that reduces or eliminates the requirement for active CBP complex. Unfortunately, a reliable secondary-structure prediction is impossible, since no experimental data exist. A prediction based upon the program of Zucker and Stiegler (60) indicates a surprising degree of secondary structure for the tripartite leader (Fig. 7), which may not be correct. We are currently investigating the role of tripartite leader secondary structure in conferring CBP complex-independent translation. It is conceivable that the tripartite leader may form a stable secondary structure to ensure that the 5′-proximal region remains unpaired and presumably less dependent upon CBP complex activity. Alternatively, an extremely stable structure may impede normal ribosome scanning and possibly promote internal ribosome initiation, by using signals encoded within the tripartite leader which have not yet been identified.

The preferential expression of late adenovirus polypeptides most probably results from a combination of events. First, there is enhanced transcription from the viral major late promoter during the onset of the late phase of infection. Second, newly synthesized cellular transcripts fail to accumulate in the cytoplasm (7, 59). The block to cellular accumulation has been related to the activity of two adenovirus gene products, the E1B 55k (5, 44) and the E4 34k (25) polypeptides, which may inhibit the nucleus-to-cytoplasm transport of cellular RNAs. Third, there is preferential translation of viral late mRNAs, which occurs even before the block to cellular RNA transport (reviewed in reference 21). The enhanced translation of late mRNAs may result in part from their reduced requirement for intact CBP complex. During the late stages of infection, when large amounts of viral structural proteins must be produced, it is likely that there is also an increased competition for the translation of this expanding pool of mRNAs. It makes sense that efficient translation of late mRNAs might be assured if their requirement for limited amounts of CBP complex were reduced. Consistent with this model is the observation that the tripartite leader enhances mRNA translation at late but not early stages of infection (9, 37). The preferential translation of late adenovirus mRNAs may depend, to a large extent, on the ability of the tripartite leader to form a secondary structure which reduces or eliminates the requirement for intact CBP complex or eIF-4F.

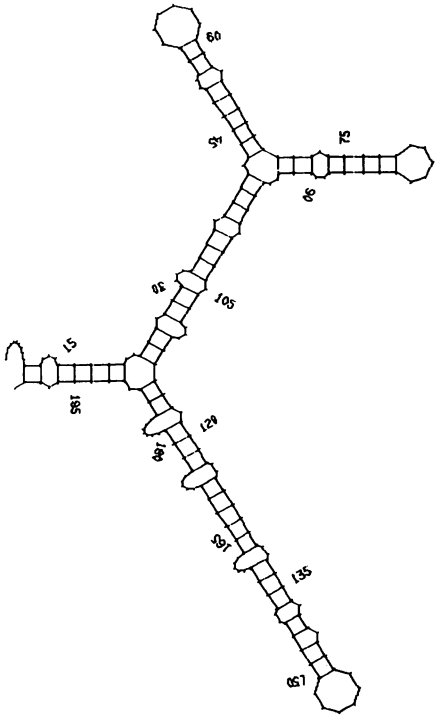

FIG. 7. Secondary structure model for Ad2 tripartite leader. The secondary structure of the Ad2 tripartite leader predicted by the program of Zucker and Stiegler (60) performed on a VAX 750 computer is shown. Nucleotide positions are relative to the start of transcription. The predicted free energy is −55.3 kcal (−231.4 kJ).

Several other viruses also encode mRNAs which have an intrinsic ability to initiate translation more efficiently than cellular mRNAs during infection (reviewed in reference 51). In particular, this has been demonstrated for mRNAs encoded by two picornaviruses, encephalomyocarditis virus (27) and mengovirus (1). The *Drosophila melanogaster* heat shock mRNAs, which are preferentially translated (reviewed in reference 4), contain long A-rich leader sequences which

may play a role in the selective translation of these mRNAs in heat-shocked cells (26, 30). In all of these examples, however, it is not yet understood how the mRNA itself contributes to the intrinsically high levels of translation initiation.

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LITERATURE CITED

1. **Abreu, S. L., and J. Lucas-Lenard.** 1976. Cellular protein synthesis shut-off by mengovirus: translation of nonviral and viral mRNAs in extracts from uninfected and infected Ehrlich ascites tumor cells. J. Virol. **18**:182–194.
2. **Akusjarvi, G., and H. Persson.** 1981. Gene and mRNA for precursor polypeptide VI from adenovirus type 2. J. Virol. **38**: 469–482.
3. **Andrews, N. C., and D. Baltimore.** 1986. Purification of a terminal uridylytransferase that acts as host factor in the *in vitro* poliovirus replication reaction. Proc. Natl. Acad. Sci. USA **83**: 221–225.
4. **Atkinson, B. G., and D. B. Walden.** 1985. Changes in eukaryotic gene expression in response to environmental stress. Academic Press, Inc., Orlando, Fla.
5. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50**:202–212.
6. **Bablanian, R., and W. C. Russell.** 1974. Adenopolypeptide synthesis in the presence of non-replicating poliovirus. J. Gen. Virol. **24**:261–279.
7. **Beltz, G. A., and S. J. Flint.** 1979. Inhibition of HeLa cell protein synthesis during adenovirus infection. J. Mol. Biol. **131**: 353–373.
8. **Berget, S. M., C. Moore, and P. Sharp.** 1977. Spliced segments at the 5′ terminus of Ad2 late mRNA. Proc. Natl. Acad. Sci. USA **74**:3171–3175.
9. **Berkner, K. E., and P. A. Sharp.** 1985. Effect of tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. Nucleic Acids Res. **13**:841–857.
10. **Bernstein, H., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.
11. **Bonneau, A. M., and N. Sonenberg.** 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J. Virol. **61**:986–991.
12. **Broker, T. R., L. T. Chow, A. R. Dunn, R. E. Gelinas, J. A. Hassell, D. G. Klessing, J. B. Lewis, R. J. Roberts, and B. S. Zain.** 1977. Adenovirus-2 messengers—an example of baroque molecular architecture. Cold Spring Harbor Symp. Quant. Biol. **42**:531–553.
13. **Castrillo, J. L., and L. Carrasco.** 1987. Adenovirus late protein synthesis is resistant to the inhibition of translation induced by poliovirus. J. Biol. Chem. **262**:7328–7334.
14. **Crawford, N., A. Fire, M. Samuels, P. Sharp, and D. Baltimore.** 1981. Inhibition of transcription factor activity by poliovirus. Cell **27**:555–561.
15. **Dani, C., J. M. Blanchard, M. Piechaczyk, S. E. Sabouty, L. Marty, and P. Jeanteur.** 1984. Extreme instability of myc mRNA in normal and transformed human cells. Proc. Natl. Acad. Sci. USA **81**:7046–7050.
16. **Edery, I., M. Humbelin, A. Darveau, K. Lee, S. Milburn, J. Hershey, H. Trachsel, and N. Sonenberg.** 1983. Involvement of eukaryotic initiation factor 4A in the cap recognition process. J. Biol. Chem. **258**:11398–11403.
17. **Etchison, D., J. Hansen, E. Ehrenfeld, I. Edery, N. Sonenberg, S. C. Milburn, and J. W. B. Hershey.** 1984. Demonstration in vitro that eucaryotic initiation factor 3 is active but that cap-binding protein complex is inactive in poliovirus-infected HeLa cells. J. Virol. **51**:832–837.
18. **Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey.** 1982. Inhibition of Hela cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000 dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. **257**:14806–14810.
19. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
20. **Gehrke, L., P. E. Avron, A. J. Quigly, A. Rich, and N. Sonenberg.** 1983. 5′ conformation of capped alfalfa mosaic virus ribonucleic acid 4 may reflect its independence of the cap structure or of cap binding protein for efficient translation. Biochemistry **22**:5157–5164.
21. **Ginsberg, H. S.** 1984. The adenoviruses. Plenum Publishing Corp., New York.
22. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
23. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
24. **Grifo, J. A., S. M. Tahara, M. A. Morgan, A. J. Shatkin, and W. C. Merrick.** 1983. New initiator activity required for globin mRNA translation. J. Biol. Chem. **258**:5804–5810.
25. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56**: 250–257.
26. **Hultmark, D., R. Klemenz, and W. J. Gehring.** 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp 22. Cell **44**:429–438.
27. **Jen, G., C. H. Birge, and R. E. Thach.** 1978. Comparison of initiation rates of encephalmyocarditis virus and host protein synthesis in infected cells. J. Virol. **27**:640–647.
28. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
29. **Kaufman, R. J.** 1985. Identification of the components necessary for adenovirus translation control and their utilization in cDNA expression vectors. Proc. Natl. Acad. Sci. USA **82**:689–693.
30. **Klemenz, R., D. Hultmark, and W. J. Gehring.** 1985. Selective translation of heat shock mRNA in Drosophila melanogaster depends on sequence information in the leader. EMBO J. **4**: 2053–2060.
31. **Kozak, M.** 1980. Influence of mRNA secondary structure on binding and migration of 40S ribosomal subunits. Cell **19**:79–80.
32. **Kozak, M.** 1986. Influence of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. USA **83**:2850–2854.
33. **Lawson, T. G., B. K. Ray, J. T. Dodds, J. A. Grifo, R. D. Abramson, W. C. Merrick, D. F. Betsch, H. L. Weith, and R. E. Thach.** 1986. Influence of 5′ proximal secondary structure on the translational efficiency of eukaryotic mRNAs and on their interaction with initiation factors. J. Biol. Chem. **261**:13979–13989.
34. **Lee, K. A. W., I. Edery, and N. Sonenberg.** 1985. Isolation and structural characterization of cap-binding proteins from poliovirus-infected HeLa cells. J. Virol. **54**:515–524.
35. **Lee, K. A. W., and N. Sonenberg.** 1982. Inactivation of cap binding proteins accompanies the shut-off of host protein synthesis by poliovirus. Proc. Natl. Acad. Sci. USA **79**:3447–3451.
36. **Leibowitz, R., and S. Penman.** 1971. Regulation of protein synthesis in HeLa cells. III. Inhibition during poliovirus infection. J. Virol. **8**:661–668.
37. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection.

Proc. Natl. Acad. Sci. USA **81**:3655–3659.
38. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level expression of a chloramphenicol acetyltransferase gene by DEAE-dextran mediated DNA transfection coupled with dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
39. **Lucas-Lenard, J. M.** 1979. Inhibition of cellular protein synthesis after virus infection, p. 73–99. *In* R. Perez-Berioff (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
40. **Morrow, C. D., G. F. Gibbons, and A. Dasgupta.** 1985. The host protein required for in vitro replication of poliovirus is a protein kinase that phosphorylates eukaryotic initiation factor-2. Cell **40**:913–921.
41. **Patzer, E. J., G. R. Nakamura, and A. Yaffe.** 1984. Intracellular transport and secretion of hepatitis B surface antigen in mammalian cells. J. Virol. **51**:346–353.
42. **Pelletier, J., and N. Sonenberg.** 1985. Photochemical cross-linking of CAP-binding proteins to eucaryotic mRNAs: effect of mRNA 5′ secondary structure. Mol. Cell. Biol. **5**:3222–3230.
43. **Pelletier, J., and N. Sonenberg.** 1985. Insertion mutagenesis to increase secondary structure within the 5′ non-coding region of a eukaryotic mRNA reduces translational efficiency. Cell **40**: 515–526.
44. **Pilder, S., J. Logan, and T. Shenk.** 1986. The adenovirus E1B-55k transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. **6**:470–476.
45. **Racaniello, V. R.** 1984. Poliovirus type II Lansing produced from cloned cDNA is infectious in mice. Virus Res. **1**:669–675.
46. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **78**:4887–4891.
47. **Ray, B. K., T. G. Brendke, S. Adya, S. D. McQueen, J. K. Miller, J. W. B. Hershey, J. A. Grifo, W. C. Merrick, and R. E. Thach.** 1983. Role of mRNA competition in regulating translation: further characterization of mRNA discriminatory initiation factors. Proc. Natl. Acad. Sci. USA **80**:663–667.
48. **Ray, B. K., T. G. Lawson, J. C. Kramer, M. H. Cladaras, J. A. Grifo, R. D. Abramson, W. C. Merrick, and R. E. Thach.** 1985. ATP dependent unwinding of messenger RNA structure by eukaryotic initiation factors. J. Biol. Chem. **260**:7651–7658.
49. **Sarnow, P., Y. S. Ho, J. Williams, and A. Levine.** 1982. Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28**:387–394.
50. **Schneider, R. J., B. Safer, S. Munemitsu, C. E. Samuel, and T. Shenk.** 1985. Adenovirus VA1 RNA prevents phosphorylation of the eukaryotic initiation factor 2 alpha subunit subsequent to infection. Proc. Natl. Acad. Sci. USA **82**:4321–4325.
51. **Schneider, R. J., and T. Shenk.** 1987. Impact of virus infection on host cell protein synthesis. Annu. Rev. Biochem. **56**:317–332.
52. **Siekierka, J., T. M. Mariano, P. A. Reichel, and M. B. Mathews.** 1985. Translational control by adenovirus: lack of virus-associated RNA_1 during adenovirus infection results in phosphorylation of initiation factor eIF-2 and inhibition of protein synthesis. Proc. Natl. Acad. Sci. USA **82**:1959–1963.
53. **Sonenberg, N.** 1987. Regulation of translation by poliovirus. Adv. Virus Res. **33**:175–204.
54. **Sonenberg, N., D. Guertin, and K. Lee.** 1982. Capped mRNAs with reduced secondary structure can function in extracts from poliovirus-infected cells. Mol. Cell. Biol. **2**:1633–1638.
55. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
56. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
57. **Walden, W. E., T. Godefroy-Colburn, and R. E. Thach.** 1981. The role of mRNA competition in regulating translation. J. Biol. Chem. **256**:11739–11746.
58. **Wilson, M. C., and J. E. Darnell.** 1981. Control of messenger RNA concentration by differential cytoplasmic half-life. J. Mol. Biol. **148**:231–251.
59. **Yoder, S. S., B. L. Robberson, E. J. Leys, A. G. Hook, M. Al-Ubaidi, C. Y. Yeung, R. E. Kellems, and S. M. Berget.** 1983. Control of cellular gene expression during adenovirus infection: induction and shut-off of dihydrofolate reductase gene expression by adenovirus type 2. Mol. Cell. Biol. **3**:819–828.
60. **Zucker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamic and auxillary information. Nucleic Acids Res. **9**:133–148.