Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells

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Translation initiation on eukaryotic mRNAs usually occurs by 5′-processive scanning of 40S ribosome subunits from the m^7GTP-cap to the initiating AUG. In contrast, picornavirus and some specialized mRNAs initiate translation by internally binding ribosomes. A poorly described third mechanism of initiation, referred to as ribosome shunting or jumping, involves discontinuous scanning by 40S ribosome subunits, in which large segments of the 5′ noncoding region are bypassed. Ribosome shunting has only been observed to date on a cauliflower mosaic virus mRNA. In this report we show that the family of adenovirus late mRNAs, which are preferentially translated during infection, use a ribosome jumping mechanism to initiate protein synthesis. Late adenovirus mRNAs contain a common 5′-noncoding region known as the tripartite leader, which confers preferential translation by reducing the requirement for the rate-limiting initiation factor eIF-4F (cap-binding protein complex). Adenovirus inhibits cell protein synthesis largely by inactivating eIF-4F. We show that the tripartite leader directs both 5′ linear ribosome scanning and ribosome jumping when eIF-4F is abundant but exclusively uses a ribosome jumping mechanism during late adenovirus infection or heat shock (stress) of mammalian cells, when eIF-4F is altered or inactivated. Shunting is directed by a complex group of secondary structures in the tripartite leader and is facilitated by one or more unidentified viral late gene products. We propose that shunting may represent a widespread mechanism to facilitate selective translation of specialized classes of capped mRNAs, including some stress and developmentally regulated mRNAs, which possess little requirement for eIF-4F but do not initiate by internal ribosome binding.

[*Key Words*: Translation initiation; adenovirus late mRNA; ribosome jumping; protein synthesis; cap-binding protein; heat shock]

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Translation in eukaryotes is thought to initiate in either of two ways. For the majority of mRNAs, 40S ribosome subunits bind in a cap (m^7GTP)-dependent manner and scan linearly in a nucleotide-by-nucleotide search for the initiating AUG codon, in a process that requires the activity of a series of initiation factors (for review, see Merrick and Hershey 1996; Sonenberg 1996). In contrast, a small but growing collection of mRNAs initiate through an alternate mechanism in which ribosomes (presumably 40S subunits) bind the mRNA from an internal ribosome entry site (IRES), rather than enter by 5′ scanning from the cap (for review, see Jackson 1996). Internal ribosome entry has been described for mRNAs with long 5′ noncoding regions (NCRs) and multiple unused upstream AUGs. mRNAs that initiate translation using IRES elements include picornavirus (Jang et al. 1988; Pelletier and Sonenberg 1988), hepatitis C virus (Tsukiyama-Kohara et al. 1992), cellular Bip (Macejak and Sarnow 1991), and *Drosophila antennapedia* mRNA (Oh et al. 1992). IRES-directed initiation is distinguished from 5′ scanning by the internal binding of 40S ribosomes to mRNA independently of the 5′ cap and without an essential requirement for cap-binding protein complex, also known as initiation factor eIF-4F (Merrick and Hershey 1996; Jackson 1996).

Translation factor eIF-4F is a cap- and ATP-dependent RNA helicase that promotes initiation by unwinding RNA duplexes near the cap, as well as internally in the RNA (Rozen et al. 1990), thereby facilitating the entry and scanning of 40S ribosomes (for review, see Sonenberg 1996). eIF-4F consists of three polypeptide subunits, a 24-kD cap-binding protein (eIF-4E), a 50-kD ATP-dependent RNA helicase (eIF-4A), and a 220-kD protein (p220) of unknown function. The activity of eIF-4F is controlled by the phosphorylation state of eIF-4E. Increased phosphorylation of eIF-4E correlates with increased translation mediated by serum, mitogens, and transformation, whereas decreased phosphorylation correlates with inhibition of initiation during cell stress, such as heat shock

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and nutrient deprivation, and adenovirus (Ad) infection (for review, see Schneider 1996).

A poorly understood additional form of initiation known as ribosome jumping or shunting occurs on the cauliflower mosaic virus (CaMV) 35S mRNA (Futterer et al. 1993). Shunting appears to combine features of both conventional 5′ scanning and internal initiation. Although ribosomes (presumably 40S subunits) initiate through 5′ cap-dependent scanning, a *cis*-acting shunting element promotes their direct translocation downstream, bypassing intervening segments of the 5′ NCR, including AUG codons and strong secondary structures that normally block translation (Futterer et al. 1993). The roles of different initiation factors and possibly of cell factors in ribosome shunting are not known.

In principle, ribosome shunting could reduce eIF-4F dependence during initiation by minimizing the need to unwind structured blocks of RNA, a step that is essential for initiation by 5′ scanning. Ribosome shunting might therefore promote selective translation of special classes of mRNAs that translate well when the activity of eIF-4F has been impaired, such as cell stress mRNAs (Joshi-Barve et al. 1992) and all late Ad mRNAs (Dolph et al. 1988; Jang et al. 1988; Dolph et al. 1990). Neither heat shock nor late Ad mRNAs bind ribosomes internally, at least as described for picornaviral IRES elements (Jang et al. 1988; Dolph et al. 1990; Joshi-Barve et al. 1992), which to date is the only established mechanism for initiating translation without eIF-4F. Independence from eIF-4F is conferred on late Ad mRNAs by the viral 5′ NCR known as the tripartite leader, which is found on all late viral mRNAs (Dolph et al. 1988, 1990). Secondary structure analysis and mutagenic mapping of the tripartite leader demonstrated that it possesses an unstructured 5′ conformation, followed by a complex arrangement of stable hairpin structures (Zhang et al. 1989; Dolph et al. 1990). The structurally relaxed 5′ end of the tripartite leader is essential for the reduced dependence on eIF-4F during translation (Dolph et al. 1990). Studies have shown that mRNAs with relaxed 5′ secondary structure no longer compete for the limited amounts of eIF-4F in cells, which restricts translation of most mRNAs (e.g., Lawson et al. 1986; Fletcher et al. 1990). With respect to Ad, studies have shown that host cell protein synthesis is inhibited during the late phase of infection by viral inhibition of eIF-4E phosphorylation, which in turn impairs eIF-4F activity (Huang and Schneider 1991; Zhang et al. 1994). Late Ad mRNAs therefore preferentially recruit ribosomes because the tripartite leader continues to direct translation despite inactivation of eIF-4F (Dolph et al. 1990; Huang and Schneider 1990, 1991; for review, see Schneider 1996). It has been shown that the tripartite leader does not function as an IRES element (Dolph et al. 1990).

This report describes studies carried out to understand further the mechanism by which the Ad tripartite leader 5′ NCR directs eIF-4F independent translation. We show that the Ad late tripartite leader promotes two independent modes of ribosome initiation according to the availability of active eIF-4F. When eIF-4F is abundant, the tripartite leader promotes both 5′ linear scanning and ribosome shunting. However, when eIF-4F is depleted by heat shock, or cells are infected by Ad, the tripartite leader directs ribosome initiation exclusively by shunting. Studies are presented that characterize the *cis*-acting RNA elements involved and show that one or more *trans*-acting, late Ad factors facilitate shunting by ribosomes on the tripartite leader.

Results

In uninfected cells, the tripartite leader initiates translation by both 5′ scanning and ribosome jumping

Studies explored whether ribosomes scan all, part, or none of the tripartite leader 5′ NCR during initiation. To do so, ribosome initiation was blocked using a strong stem–loop structure, created by linking five copies of a 12-nucleotide *Bam*HI linker ($\Delta G = -80$ kcal/mole). The stability of this hairpin is in considerable excess of that required to block scanning completely by 40S subunits and elongation by 80S ribosomes ($\Delta G > -40$ kcal/mole; Pelletier and Sonenberg 1985; Kozak 1986). The hairpin was inserted in the 5′-unstructured region of the tripartite leader (position 34, mutant B34), or at the 3′ end of the leader (position 202, mutant B202). All mRNAs contain the wild-type or a mutant cDNA copy of the tripartite leader 5′ NCR fused to the hepatitis B virus (HBV) surface antigen (S-Ag)-coding region. Studies first determined that insertion of the stable hairpin in the single-stranded 5′ portion of the tripartite leader did not alter the downstream secondary structure of the leader. To do so, the conformation of wild-type and mutant B34 leaders was examined in vitro by comparing the sensitivity of 5′[γ^{32}P]ATP-labeled RNAs to single-strand-specific endonuclease S1 (Zhang et al. 1989). The wild-type and B34 leader RNAs possessed identical single-strand cleavage patterns (shown for the first half of the leader), which in mutant B34 was shifted to larger RNA fragments beyond position 40 by the hairpin (Fig. 1A). Structural analysis of the 3′ half of the two leaders showed them to be identical as well (data not shown). These data demonstrate that insertion of a strong hairpin in the unstructured 5′ end does not detectably alter the downstream structural conformation of the tripartite leader. By extension, insertion of the hairpin outside the leader at the 3′ end should also have no effect on 5′NCR structure. Strong hairpins were not inserted interally within the structured regions of the tripartite leader because they might disrupt its conformation and alter its activity.

Translation of wild-type and mutant tripartite leader S-Ag mRNAs was examined in transfected 293 cells labeled with [^{35}S]methionine. S-Ag was immunoprecipitated and quantitated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In some cases, cells that were transfected for 24 hr were then infected with wild-type Ad to block eIF-4E phosphorylation and eIF-4F activity, which in turn inhibits cell protein synthesis. The transfection/infection protocol was shown to measure accurately the requirement of an

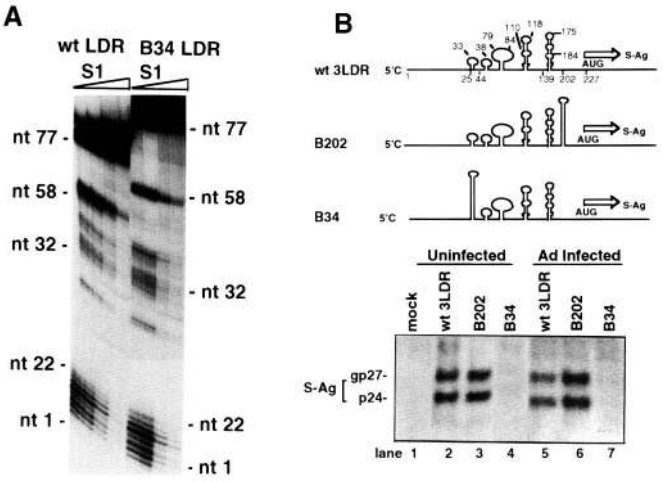

Figure 1. Strong secondary structure in the 5′ but not the 3′ end of the tripartite leader directly blocks translation initiation. (*A*) Secondary structure analysis of wild-type and B34 tripartite leaders. 5′-γ-^{32}P-end-labeled tripartite leaders were treated with increasing concentrations of single-strand-specific endonuclease S1 and the products resolved by urea gel electrophoresis as described previously (Zhang et al. 1989). (*B*) 293 cells were transfected with plasmids expressing mRNAs containing either wild-type or mutant forms of the tripartite leader (3LDR) 5′ NCR linked to the HBV S-Ag coding region. Transfection efficiencies were monitored, and translation of mRNAs determined by immunoprecipitation of S-Ag from equal protein amounts (equal counts per minute) of [^{35}S]methionine-labeled cell extracts followed by SDS–PAGE. For Ad-infected samples, cells were infected 24 hr after transfection with 25 PFU/cell of Add*l*309 for 24 hr before labeling with [^{35}S]methionine. Data were quantitated by autoradiography and densitometry. S-Ag synthesizes a 24-kD and a related glycosylated 27-kD polypeptide, as indicated. Diagrams represent the experimentally determined secondary structure of the tripartite leader (nucleotides 1–202; Zhang et al. 1989) in linear form linked to the S-Ag coding region (AUG at nucleotide 227). Strong secondary structure was created by ligating five copies of a 12-nucleotide *Bam*HI linker (ΔG = −80 kcal/mole), inserted at position 34 or 202. The slow migrating band in some samples is a nonspecific cellular polypeptide that coimmunoprecipitates with s-Ag.

mRNA for eIF-4F during late Ad infection (Dolph et al. 1988, 1990; Zhang et al. 1994). Insertion of the stable hairpin in the unstructured 5′ end (position 34) blocked mRNA translation in both uninfected and late Ad-infected cells (Fig. 1B). In contrast, the strong hairpin at the 3′ end of the tripartite leader (mutant B202) did not inhibit translation of the mRNA. Because identical results were obtained using two other reporters (luciferase and β-galactosidase; data not shown), it is unlikely that the particular coding region influences translation directed by the tripartite leader. Steady-state levels of the wild-type tripartite leader and mutant S-Ag mRNAs were also similar (within twofold) in uninfected cells (Fig. 2) and in late Ad-infected cells (Fig. 4, below), excluding an effect of secondary structure on mRNA accumulation and protein levels. In addition, primer extension analysis showed that mRNAs containing the hairpin were structurally intact and full length, excluding the possibility that fragmented mRNAs were translated (data not shown).

The tripartite leader contains an unstructured 5′ end and a complex internal arrangement of secondary structures. To determine whether ribosome shunting is promoted by *cis*-acting elements in the unstructured or structured portion of the 5′ NCR, the structured region was replaced with an equal-sized piece of another RNA (mutant Δ160) that is devoid of AUGs and extensive secondary structure (described in Materials and methods). Replacing the structured region of the tripartite leader (nucleotides 34–202) with another RNA (mutant Δ160) reduced translation of the S-Ag reporter by approximately threefold compared with wild-type in uninfected cells (Fig. 3, lanes 2,3), indicating that it could still direct initiation efficiently. However, translation of this mRNA was blocked fully if a stable hairpin was inserted at the 3′ end of the chimeric 5′ NCR (mutant Δ160-B202; Fig. 3, lane 4). The unstructured 5′ end of the tripartite leader is therefore not sufficient to direct ribosome shunting but must include part or all of the structured internal region of the leader. To further characterize ribosome jumping in the tripartite leader, the 160-nucleotide foreign RNA segment was inserted between the 3′ end of the leader and the downstream S-Ag AUG (mutant WT-160; Fig. 3, lane 5) to increase the distance to the AUG. This mutant was translationally impaired by approximately six- to sevenfold, consistent with less efficient initiation by long 5′ NCRs (Kozak 1988). However, increasing the distance to the AUG almost abolished initiation by ribosome shunting of secondary structure (mutant B202-160; Fig. 3, lane 6). It is noteworthy that all Ad serotypes constrain the initiating AUG of late mRNAs to a distance no greater than 35 nucleotides from the 3′ end of the tripartite leader, indicating con-

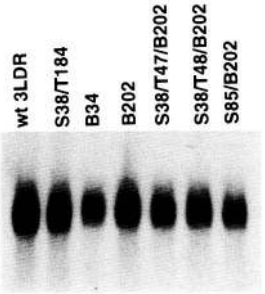

Figure 2. Steady-state accumulation of mRNAs containing a wild-type or mutant form of the Ad tripartite leader 5′ NCR. Poly(A)$^+$ mRNA was prepared from 293 cells 48 hr following transfection with expression plasmids, and equal amounts were analyzed by Northern analysis in formaldehyde–agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes prepared from the S-Ag coding region. Representative mutant mRNAs are shown. Autoradigraphs were quanitiated by densitometry. S-Ag mRNAs were compared with cellular GAPDH mRNA levels in each lane after stripping and reprobing blots to ensure that equal levels of mRNA were loaded (data not shown).

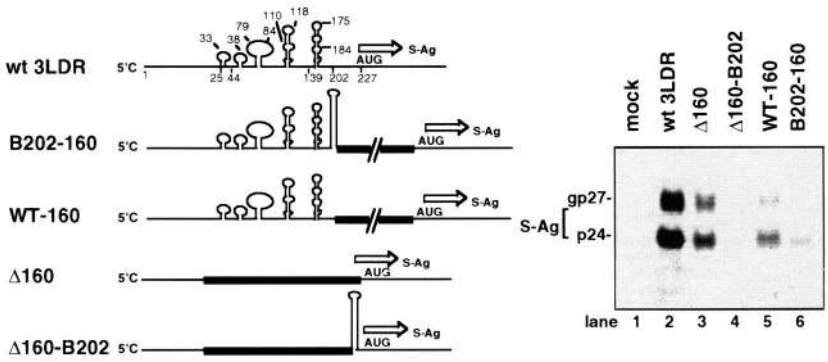

Figure 3. The complex structured region of the tripartite leader is essential for ribosome shunting. Tripartite leader mutants were generated that contain a foreign 160-nucleotide RNA segment that is devoid of AUG codons in place of the equal-sized structured region (described in Materials and methods). Constructs were made in the absence (WT-160) or the presence (B202-160) of the 3′ *Bam*HI hairpin at position 202. In mutants B202-160 and WT-160, a foreign 160-nucleotide RNA segment was used to expand the intersegmental distance between the 3′ end of the tripartite leader and the initiating AUG. S-Ag transfection of 293 cells and analysis of S-Ag translation were carried out as described in the legend to Fig. 1 (B).

siderable conservation in the site of ribosome initiation (Ginsberg 1984). If ribosomes had scanned through the hairpin and 160-nucleotide spacer RNA, then only a slight effect on initiation should have been observed, as for mutant Δ160. In late Ad-infected 293 cells, when eIF-4F activity is impaired, only mRNAs containing the wild-type tripartite leader were translated (data not shown), consistent with previous studies (Dolph et al. 1988, 1990). In uninfected cells, the tripartite leader therefore directs ribosomes to initiate by both 5′ scanning and jumping. The mechanism for ribosome jumping therefore involves translocation of a ribosome to a confined range from the 5′ NCR, reminiscent of some IRES elements (for review, see Jackson 1996).

Late Ad infection inhibits ribosome scanning through the structured region of the tripartite leader but does not affect ribosome jumping

In uninfected cells, the tripartite leader directs initiation by both ribosome scanning and jumping mechanisms. During late Ad infection or heat shock of cells active eIF-4F is depleted, which might limit linear scanning by ribosomes to the AUG. We therefore determined whether late Ad infection reduces initiation by 5′ ribosome scanning on the tripartite leader. An AUG in perfect context was inserted at position 44 (mutant S44) in the tripartite leader, in-frame with the downstream S-Ag AUG by disrupting a minor 5-bp stem, creating a presumptive long (44-nucleotide) relaxed 5′ end (Fig. 4). If all ribosomes enter the tripartite leader by 5′ nucleotide-by-nucleotide scanning, then initiation will be directed from position 44. An AUG was also inserted into an engineered weak stem at position 134 ($\Delta G = -20$ kcal/mole), in-frame with the downstream S-Ag AUG (mutant stem–ATG^{134}). If ribosomes scan the tripartite leader to this site, they will melt the weak stem and synthesize a larger fusion protein. Therefore, the ratio of S-Ag to fusion protein measures the proportion of ribosomes that initiate translation by 5′ scanning or jumping.

Cells were transfected with plasmids encoding the test mRNAs, infected with wild-type Ad to inhibit eIF-4E phosphorylation and host protein synthesis, labeled with [^{35}S]methionine, and the level of native and fusion forms of S-Ag protein compared by immunoprecipitation and SDS-PAGE (Fig. 4A). An AUG in the unstructured 5′ region (position 44, mutant S44) redirected ribosomes exclusively to this site in uninfected and late Ad-infected cells (Fig. 4A). Identical results were obtained for AUG insertions at other sites in the unstructured domain of the leader (data not shown). 40S ribosomes therefore enter the tripartite leader by a nucleotide-by-nucleotide scanning of the unstructured region, regardless of the level of eIF-4F.

Studies determined whether ribosomes initiate from within the structured region of the tripartite leader at an AUG in a weak stem at position 134 (mutant stem–AUG^{134}). An AUG within a weak stem midway in the leader (nucleotide 134) directed about half of ribosomes in uninfected cells to initiate from this upstream site (Fig. 4B), evidenced by a larger glycosylated and nonglycosylated fusion form of S-Ag protein. Although a native and fusion form of S-Ag comigrated, they could be resolved by high-resolution gel electrophoresis as shown. Therefore, in uninfected cells linear and nonlinear ribosome initation is directed with equal frequency by the tripartite leader. However, during late Ad infection, the tripartite leader directed ribosomes to initiate exclusively from the downstream AUG (Fig. 4B), which was corroborated by expressing the wild-type and stem–AUG^{134} tripartite leader mutant mRNAs from recombinant Ad vectors during late phase (Fig. 4C). These data demonstrate that during the late phase of infection, when eIF-4F was inactivated the tripartite leader directed initiation solely by ribosome shunting, whereas in uninfected cells both scanning and jumping occurred. Results presented later suggest that ribosomes cannot simultaneously scan through and shunt the structured region of the tripartite leader.

Heat shock impairs initiation by 5′ ribosome scanning but not jumping on the tripartite leader

Studies were carried out to characterize the molecular events that control whether translation will initiate by

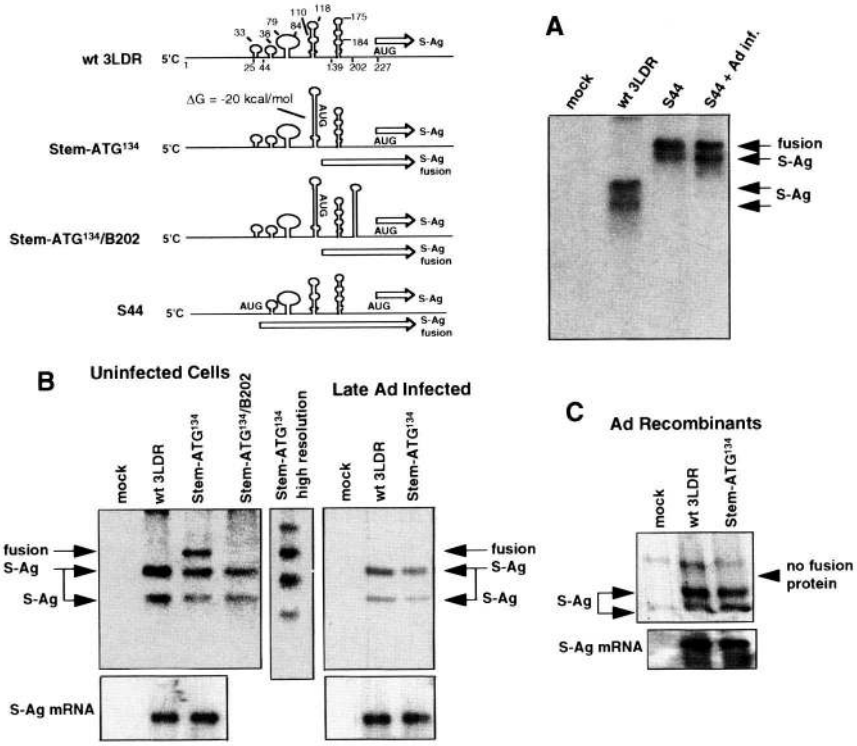

Figure 4. Late Ad infection prevents initiation by ribosome scanning but not shunting in the tripartite leader. (*A*) Mutant S44 contains an AUG in perfect context in the unstructured 5′ end of the tripartite leader at nucleotide 44, in-frame with the downstream S-Ag AUG. (*B*) Mutant stem–ATG^{134} contains an AUG in perfect context in-frame with S-Ag within a weak stem ($\Delta = -20$ kcal/mole) engineered by site-directed mutagenesis at position 134. (*C*) Recombinant Ad vectors were developed that express either the wild-type tripartite leader/S-Ag mRNA, or stem–ATG^{134}/S-Ag mRNA in place of region E1A/E1B. Transfection and subsequent infection of 293 cells, analysis of S-Ag polypeptides, and mRNA levels were performed as described in the legends to Figs. 1 (B) and 2. Open arrows indicate an in-frame open reading frame with S-Ag.

ribosome scanning or shunting in the tripartite leader during late Ad infection. In particular, we asked whether initiation by scanning or shunting is controlled by viral inactivation of eIF-4F, viral late gene expression, or both. Heat shock of mammalian cells blocks cap-dependent protein synthesis by inhibiting eIF-4E phosphorylation, which in turn depletes the cell of active eIF-4F (for review, see Duncan 1996). Cells were transfected with the stem-AUG^{134} plasmid, then subjected to heat shock (Fig. 5). To examine eIF-4E phosphorylation, cells were labeled in vivo with [^{32}P]orthophosphate, eIF-4E was purified by cap-affinity chromatography, resolved by SDS-PAGE, and visualized by autoradiography. Phosphorylation of eIF-4E was reduced ~20-fold by heat shock, to a level similar to that of late Ad infection (Fig. 5A). Control experiments found that equal amounts of eIF-4E were present in each lane by immunoblot analysis (data not shown). The level of S-Ag translation directed by the wild-type tripartite leader during heat shock and late Ad infection was similar (Fig. 5B, cf. lanes 2 and 5 with Fig. 4B). In contrast, during heat shock, translation of the tripartite leader stem–AUG^{134} mutant occurred exclusively from the downstream AUG (position 227), not from within the structured portion of the tripartite leader (Fig. 5B). Northern analysis showed that reporter mRNA levels were not altered by heat shock (Fig. 5C).

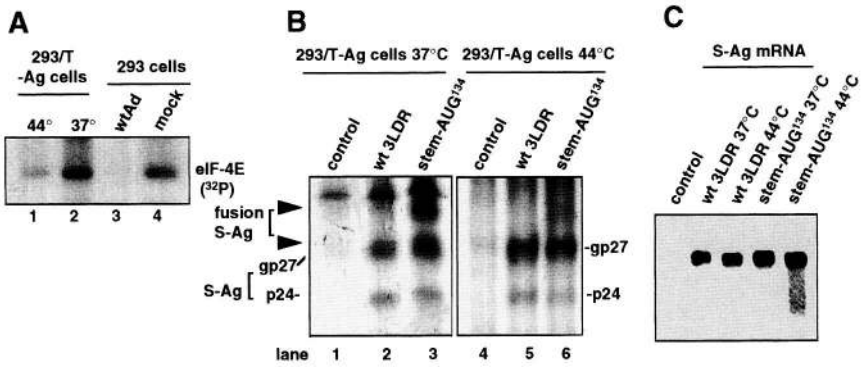

Figure 5. Heat shock of uninfected cells induces the tripartite leader to initiate ribosomes exclusively by shunting. 293/T-Ag cells are 293 cells that express the SV40 T-Ag gene. (*A*) 293/T-Ag cells were subjected to heat shock for 2 hr at 44°C or maintained at 37°C. 293 cells were infected with 25 PFU/cell of Ad*dl*309 for 24 hr. Cell lines were labeled with [^{32}P]orthophosphate and eIF-4E-purified by m^{7}GTP-agarose affinity chromatography and equal protein amounts analyzed by SDS-15%-PAGE and autoradiography. (*B*) 293/T-Ag cells were transfected with plasmids; 48 hr later duplicate plates were subjected to heat shock, labeled with [^{35}S]methionine, and immunoprecipitated S-Ag polypeptides were examined by SDS-PAGE and autoradiography. Heat shock results predominantly in the synthesis of the 27-kD glycosylated S-Ag form of the reporter protein. The position of the larger fusion S-Ag protein is indicated. (*C*) Northern analysis of transfected poly(A)$^+$ S-Ag mRNA from control and heat-shocked 293/T-Ag cells.

Mutant S44 still initiated translation during heat shock from the upstream AUG within the unstructured domain of the tripartite leader (data not shown), consistent with the results from Ad infection. Because late Ad infection and heat shock both inhibit eIF-4E phosphorylation and impair eIF-4F activity, these data imply (but do not prove) that inactivation of eIF-4F prevents translation mediated by ribosome scanning but not shunting. Other studies directed to this issue will be presented later.

5′ scanning through the structured region of the tripartite leader and ribosome shunting are mutually incompatible

Studies were conducted to better characterize the mechanism that determines whether ribosomes will scan or shunt in the tripartite leader. Because the results of the S44 mutant (Fig. 4) indicated that translation of the tripartite leader by 80S ribosomes blocked downstream initiation by 40S subunits, we asked whether the leader can simultaneously direct both ribosome scanning and shunting. To explore this issue, an AUG was inserted in the 5′-unstructured region of the tripartite leader at position 38 (mutant 38), out of frame with the downstream S-Ag AUG at position 227. AUG-38 redirected all 40S ribosomes to translate from this site and impeded downstream initiation, ablating S-Ag translation (Fig. 6, lane 3). Insertion of stable secondary structure into the 3′ end of the S38 tripartite leader did not restore S-Ag translation (data not shown). However, insertion of a stop codon downstream at position 47 or 184 restored initiation from the authentic S-Ag AUG to near wild-type levels (Fig. 6, lanes 4,5). Presumably, 40S ribosomal subunits were freed from the minicistron and efficiently reinitiated a short distance downstream at the next (S-Ag) AUG, as described previously for other mRNAs (Kozak 1987). Surprisingly, a strong hairpin at the 3′ end of the tripartite leader in mutant S38/T47, but not S38/T184, blocked translation of S-Ag by ribosome shunting (Fig. 6, lanes 6,7). These data indicate that 80S ribosome transit through the tripartite leader between position 47–184 blocks 40S ribosome jumping. Simultaneous initiation by ribosome scanning and jumping is therefore temporally incompatible on the same tripartite leader mRNA. These data also suggest that shunting is carried out by the 40S subunit but not the 80S elongating ribosome.

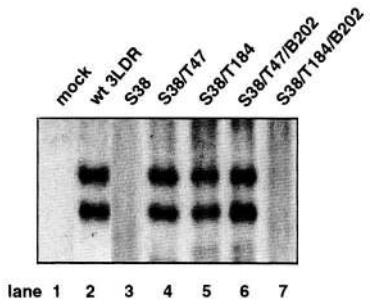

Figure 6. Shunting is carried out by 40S but not 80S translating ribosomes. 293 cells were transfected with plasmids expressing the wild-type tripartite leader or mutants containing an AUG start S codon in perfect context in the unstructured 5′ end at nucleotide 38 (S38), out of frame with the downstream S-Ag AUG, and termination codons ("T") at position 47 or 184 as shown. Insertion of the stable hairpin structure at the 3′ end of the tripartite leader is indicated by the designation B202. Analysis of labeled S-Ag synthesis was described in the legend to Fig. 1 (B). The positions of S-Ag proteins are indicated.

One or more late Ad gene products facilitate ribosome shunting in the tripartite leader

The inhibition of 40S subunit shunting by elongating (translating) 80S ribosomes provided a useful method for mapping the region of the tripartite leader that promotes 40S subunit jumping. Minicistrons were constructed in the tripartite leader out of frame with the S-Ag reporter, initiating translation at nucleotide 38 and terminating at nucleotides 85, 118, 139, and 184 within the leader. As expected from the results of Figure 6, ribosomes efficiently reinitiated at the downstream S-Ag AUG at position 227 (Fig. 7A). Insertion of a strong hairpin at the 3′ end of the tripartite leader in the minicistron mutants was used to map the boundaries of the leader that must be accessed by 40S subunits to promote shunting. Termination of translation at position 85 impaired ribosome shunting by 5- to 10-fold (Fig. 7B, lane 2), whereas termination beyond position 118 severely blocked shunting (Fig. 7B, lanes 3–5). Because termination of translation at position 47 did not block shunting (Fig. 6), taken collectively, these data indicate that most of the structured region of the tripartite leader is essential to direct ribosome shunting. Because all minicistron mutants, in the absence of a 3′ hairpin, reinitiated ribosomes efficiently at the S-Ag AUG, we can exclude that 40S subunits failed to be freed at the stop codon or could not reinitiate for other reasons. It is likely that 40S subunits must recognize particular structures or sequences in the tripartite leader, which are probably melted or masked by 80S elongating ribosomes, to shunt. Translation of the tripartite leader minicistron mutants was also examined during late Ad infection by transfection/infection. Late Ad infection largely rescued ribosome shunting on all mRNAs that failed to shunt in uninfected cells (Fig. 7C). mRNA levels for wild-type and mutant tripartite leader transcripts were unchanged in uninfected compared with late Ad-infected cells (Fig. 4B; data not shown). Experiments therefore characterized the mechanism by which late Ad infection rescued ribosome shunting in the tripartite leader mutants.

Ad rescue of ribosome shunting could result from viral inactivation of eIF-4F, from a late Ad gene product that facilitates ribosome jumping on the tripartite leader, or from both. To discriminate among these possibilities, three sets of studies were conducted. First, translation of the S38/T184/B202 shunting mutant mRNA was examined in control and heat-shocked 293 cells. Cells were transfected with expression plasmids and subjected to

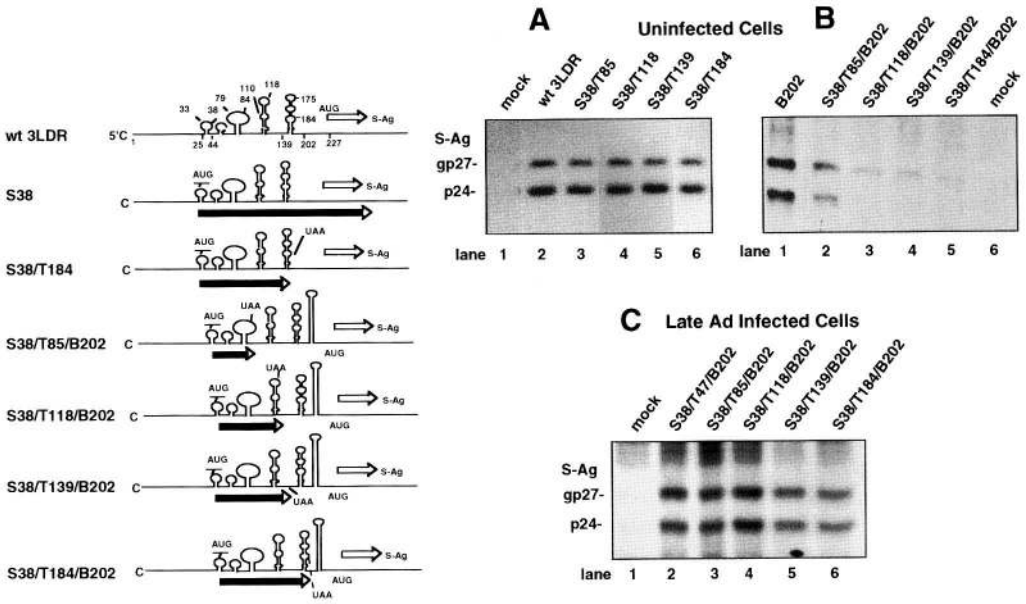

Figure 7. The complex structured region of the tripartite leader downstream of position 85 is essential for ribosome shunting. 293 cells were transfected with plasmids expressing wild-type or mutant forms of the tripartite leader linked to the S-Ag coding region and levels of [^{35}S]methionine-labeled S-Ag, in uninfected or late Ad-infected cells, determined as described in the legend to Fig. 1. (*A*) Mutant tripartite leader mRNAs containing start S AUG codons and termination T codons are indicated. (*B*) Tripartite leader mutants containing minicistrons and stable 3′ hairpin structure (B202) are indicated. Shaded arrows indicate the presence of an out-of-frame upstream open reading frame.

heat shock to block eIF-4E phosphorylation, as described above, and the level of [^{35}S]methionine-labeled S-Ag compared between wild-type and mutant tripartite leader mRNAs (Fig. 8A). Heat shock inhibited cell protein synthesis by 95% (not shown) and induced an approximately threefold increase in translation of both wild-type and shunting mutant tripartite leader mRNAs, but it did not selectively rescue the shunting mutant to wild-type levels (Fig. 8A). Studies therefore examined whether early or late Ad gene expression rescues ribosome shunting in the tripartite leader. 293 cells transfected with the wild-type tripartite leader, or a shunting mutant, were infected by an Ad virus containing a temperature sensitive mutation in the viral DNA-binding protein (Ad5*ts*125). At restrictive temperature (39.5°C), the Ad*ts*125 mutant remained in viral early phase and did not express late viral genes (Fig. 8B). At 32°C, Ad*ts*125 virus was indistinguishable from wild-type Ad in entering late phase and blocking eIF-4E phosphorylation and host cell protein synthesis (Fig. 8B; Zhang et al. 1994). Only at the nonrestrictive temperature (32°C), where Ad*ts*125 entered late phase and expressed late Ad genes (Fig. 8B), did it rescue shunting in the tripartite leader to a level similar to that of wild-type Ad (Fig. 8C). These results indicate that early Ad gene expression is not sufficient to rescue ribosome shunting in mutants in the tripartite leader.

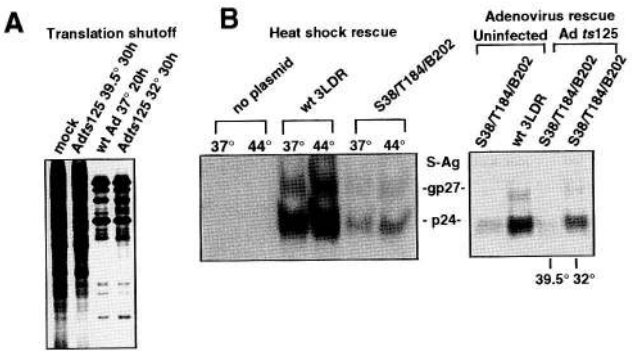

Figure 8. Late Ad infection, but not heat shock, efficiently rescues tripartite leader shunting mutants. (*A*) 293/T-Ag cells were transfected with plasmids expressing mRNAs with the wild-type tripartite leader or a shunting mutant (S38/T84/B202), duplicate plates of cells heat-shocked and the level of S-Ag synthesis were examined as described in the legend to Fig. 5. (*B*) 293 cells were infected by wild-type Ad for 24 hr, or Ad*ts*125 at the restrictive temperature (39.5°C) for 30 hr, labeled with [^{35}S]methionine, and shutoff of cell protein synthesis was examined by SDS-PAGE and autoradiography. (*C*) The level of S-Ag synthesized by complementing Ad*ts*125 virus infection was determined from labeled extracts by immunoprecipitation and SDS-PAGE.

A third group of studies determined whether both late Ad gene expression and inactivation of eIF-4F are required to rescue translation of the shunting mutants or whether late Ad gene expression alone is sufficient. During infection of 293/T-antigen cells, Ad enters late phase and produces abundant amounts of late polypeptides but only inhibits eIF-4E phosphorylation and host translation very late in the infectious cycle (~40 hr; Fig. 9A),

compared with 24 hrs in 293 cells. In 293/T-antigen cells Ad synthesizes abundant amounts of its late gene products without inhibiting eIF-4F for most of the late phase of infection. 293/T-antigen cells were transfected with plasmids expressing wild-type or a tripartite leader-shunting mutant mRNA, infected with wild-type Ad for 26 hr, and the effect of late viral gene expression on rescue of the shunting mutant examined before inhibition of eIF-4F. Translation of the shunting mutant mRNA was partially rescued at 24 hr after infection (Fig. 9B), without dephosphorylation of eIF-4E and inactivation of eIF-4F. These data indicate that late Ad gene expression facilitates ribosome shunting directed by the tripartite leader but that inactivation of eIF-4F also partially facilitates ribosome jumping in the tripartite leader (approximately threefold). Ongoing studies have yet to identify the Ad gene products that facilitate ribosome shunting. A likely viral candidate in shunting, the 100-kD nonstructural protein that binds cellular and viral mRNAs (Adam and Dreyfuss 1987), had no effect on shunting when expressed independently of other Ad genes by transfection (data not shown).

Discussion

It has been shown previously that ribosomes can search for the initiating AUG on eukaryotic mRNAs by 5′ cap-dependent scanning and by internal binding at an IRES element. In addition, ribosomes locate the initiating AUG on the CaMV 35S mRNA through a cap-dependent but discontinuous scanning process (Futterer et al. 1993). In this study it was demonstrated that ribosome shunting is not restricted to the CaMV 35S mRNA. Rather, all Ad late mRNAs use ribosome shunting, which is promoted by a common 5′ NCR and is promoted in response to changes in the physiological state of the cell, such as heat shock or virus infection, that inactivate cap-binding protein (eIF-4E) and reduce the availability of initiation factor eIF-4F.

40S ribosome subunits cannot normally bypass stable hairpin structures in the 5′ NCR of most mRNAs (e.g., Fig. 3; Pelletier and Sonenberg 1985; Kozak 1986). The thermodynamic barrier created by insertion of strong secondary structure is consistent with a nucleotide-by-nucleotide scanning search by 40S ribosomes for the initiator AUG (Kozak 1989). Although 40S ribosome shunting absolutely requires *cis*-acting tripartite leader elements (Fig. 3), it is clear from this study and that of Futterer et al. (1993) that shunting is a normal property of the translational apparatus in uninfected plant and mammalian cells. The role of cellular *trans*-acting factors in ribosome jumping is unknown, apart from the stimulation mediated by eIF-4F inactivation. It is clear that ribosome shunting takes place on the tripartite leader without an absolute requirement for Ad viral gene expression (Figs. 1B and 4), although late Ad gene expression promotes initiation through shunting by severalfold. This is most evident when translation of the B202 mRNA is directly compared in uninfected and late Ad-infected cells (e.g. Fig. 1B). Moreover, ribosome shunting on the tripartite leader was surprisingly efficient in both uninfected and late Ad-infected cells, displaying translation levels that were at least equal to that of 5′ scanning. This is in contrast to the low frequency of shunting on the CaMV mRNA in plant protoplasts, which averaged ~7.5% (Futterer et al. 1993). The difference in efficiency in these two systems could be attributable to inherently unrelated shunting mechanisms or to a higher overall efficiency of translation in intact cells in our studies compared with plant protoplasts. Ribosome shunting was also dependent on unique *cis*-acting elements in the tripartite leader 5′ NCR. Other 5′ NCRs carried out only conventional linear ribosome scanning, with no evidence for shunting of inhibitory secondary structure (Fig. 3; data not shown). Our studies also suggested that only the 40S ribosome subunit can jump (Fig. 6). Elongating 80S ribosomes formed by insertion of AUG codons in the tripartite leader actually inhibited ribosome shunting. A similar strategy was used to characterize the picornavirus IRES elements, from which it was also concluded that internal initiation is blocked by the movement of 80S ribosomes through the IRES (Hellen et al. 1994).

An important observation disclosed by this report is that physiological alterations to the cell that impair the cap-dependent eIF-4F helicase activity also impair translation initiation carried out by ribosome scanning but not by ribosome shunting. Both heat shock and late Ad infection mediated a block to eIF-4E phosphorylation of similar magnitude (Fig. 5), which in turn inhibited or

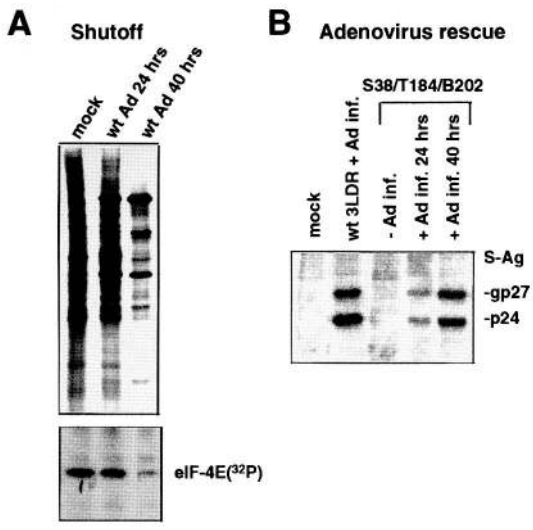

Figure 9. One or more late Ad gene products promote ribosome jumping in the tripartite leader. 293/T-Ag cells were transfected with plasmids expressing the wild-type tripartite leader or a shunting mutant, and duplicate plates were infected with wild-type for 24 hr or 40 hr. (*A*) Cells were labeled with [^{32}P]orthophosphate and eIF-4E phosphorylation examined as described in the legend to Fig. 5. (*B*) Cells were labeled with [^{35}S]methionine, and the synthesis of cell and viral late proteins examined by SDS-PAGE. S-Ag synthesis was examined by immunoprecipitation.

altered the activity of eIF-4F (for review, see Schneider 1996). In both cases, dephosphorylation of eIF-4E is associated with inhibition of initiation by ribosome scanning but not by ribosome jumping. These results are consistent with other studies demonstrating a requirement for eIF-4F to unwind the mRNA during scanning-dependent initiation (for review, see Merrick and Hershey 1996; Jackson 1996). Several lines of evidence indicate that inactivation of eIF-4F is the event that blocks scanning but not shunting initiation in the tripartite leader. First, ribosome scanning, but not shunting on the tripartite leader, was inhibited by dephosphorylation of eIF-4E and inactivation of eIF-4F during heat shock and late Ad infection. Second, in 293/T-antigen cells, which do not dephosphorylate eIF-4E until very late in the infectious cycle, initiation took place by both ribosome scanning and shunting, whereas in 293 cells dephosphorylation of eIF-4E is linked to initiation solely by ribosome shunting (Fig. 7). Although it is possible that initiation by ribosome shunting may be selected for by alteration or inactivation of other cellular factors, several different experimental approaches all coincide with the inactivation of eIF-4F as a fundamental molecular event that mediates inhibition of ribosome scanning but not jumping.

There may be similarities between the shunting of ribosomes in the tripartite leader and the mechanism of internal ribosome entry described in mammalian cells. The binding of several cellular factors to the stem–loop structures of IRES elements facilitates translation by internal ribosome entry (for review, see Jackson 1996). These factors include the cellular 52-kD La protein (Meerovitch et al. 1989; Gebhard and Ehrenfeld 1992), a 57-kD protein that binds polypyrimidine tracts known as PTP (Luz and Beck 1991; Pestova et al. 1991), and possibly eIF-2 (Del Angel et al. 1989). Binding of La and PTP to IRES elements probably occurs by interaction with an unstructured pyrimidine-rich sequence (for review, see Jackson 1996). Whether any of these factors also preferentially associate with the tripartite leader is not known, although the leader does contain several polypyrimidine-rich nucleotide tracts (16/22 nucleotides in position 2–22; 13/16 nucleotides in position 69–84).

The polypyrimidine-rich tract of picornavirus IRES elements (Jackson 1996) and those of the tripartite leader (Zhang et al. 1989) occurs in largely unstructured regions of otherwise structured hairpin elements. The picornavirus polypyrimidine tract overlaps a sequence that is complementary to the 3′ end of 18S rRNA, akin to the Shine–Dalgarno sequence of prokaryotes (Nicholson et al. 1991; Pilipenko et al. 1992). Interestingly, the tripartite leader posseses a striking complementarity to the 3′ end of 18S rRNA as well (Dolph et al. 1990); two conserved repeats in the tripartite leader (GGAUCGGAAAACC; nucleotides 86–96, 154–166) are partly identical and partly complementary (underscored) to the 3′ end of 18S rRNA. It is conceivable that an interaction between the 3′ end of 18S rRNA and the tripartite leader might stabilize or dock a 40S ribosome subunit, which in combination with other signals and factors might direct translocation of the ribosome subunit to the downstream AUG. Alternatively, this region might structurally mimic 18S rRNA, perhaps promoting ribosome binding without requisite scanning, in some unknown manner. Therefore, in many respects the tripartite leader resembles picornavirus IRES elements, although it cannot promote internal initiation in a dicistronic mRNA (Jang et al. 1988; Dolph et al. 1990). Conceptually, the tripartite leader may represent half an IRES element, in that once 40S subunits are bound to the capped 5′ end of the tripartite leader, the mechanism for their nonlinear translocation to the downstream AUG may be similar to that of a picornaviral IRES. We now need to address the possible role of tripartite leader RNA regions, cellular and viral *trans*-acting factors, and the molecular mechanism for ribosome jumping in eIF-4F-independent translation and in shunting.

Materials and methods

Viruses and cells

Ad5*dl*309 is a phenotypically wild-type strain with altered restriction enzyme sites (Jones and Shenk 1979). 293 cells comprise a human embryonic kidney cell line that expresses the E1 region of Ad5. 293/large T antigen (T-Ag) cells are 293 cells that stably express SV-40 T-Ag. 293 and 293/T-Ag cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum. Virus stocks were grown and titers determined on 293 cells. Infection of cells with viruses was typically carried out for 1 hr with 25 plaque-forming units per cell (PFU/cell), unless otherwise noted.

Construction of tripartite leader mutants

Plasmids were constructed by introducing deletions, point mutations and insertions into plasmid vector pSV–HBV (Dolph et al. 1988, 1990). Point mutations were created by overlap extension PCR and verified by DNA sequence analysis. To create secondary structure insertion mutants, five copies of a 12-nucleotide *Bam*HI linker were ligated to generate a hairpin of −80 kcal/mole. Insertion mutants B34 and B202 were created by the insertion of the *Bam*HI hairpin into the *Pvu*II site at position 34 or the *Sal*I site at position 202, respectively. Mutants S85 and S174 were generated by the insertion of one copy of an *Nco*I linker containing an AUG codon in excellent context into the *Sca*I site at position 85 or the *Xho*I site at position 174, respectively. Mutant S44 was generated by introducing an AUG codon in excellent context at position 44 by site-directed mutagenesis. The upstream AUGs in constructs S85, S174, and S44 were inserted in-frame with the downstream S-Ag AUG. Mutant stem–AUG^{134} contains an AUG in a perfect stem–loop ($\Delta G = -20$ kcal/mole). Stem–AUG^{134} was constructed by overlap-extension PCR strategy. Based on the secondary structure analysis of the tripartite leader (Zhang et al. 1989), two complementary oligonucleotides were designed to convert the partial stem–loop region to a perfect duplex between nucleotides 104 and 140: 5′-CCCGTCATCTCCCTCGGTACTCCGCCACCGAGGGAGATGAGCGAG-3′ and 5′CTCGCTCATCTCCCTCGGTGGCGGAGTACDCGAGGGAGATGACGGG-3′. Mutagenesis also created one AUG in excellent context within the double-stranded stem region at position 134, in-frame with the downstream AUG for S-Ag. Mutant stem–AUG^{134}/B202 was constructed by insertion of the *Bam*HI hairpin at position 202. Mutant S38/T184 was created by insertion of a perfect context

AUG into the *Pvu*II (nucleotide 38) site of the tripartite leader, out of frame with the downstream S-Ag AUG. S84/T184 and S175/T184 were generated by inserting the AUG into the *Sca*I site (nucleotide 84) or the *Xho*I site (nucleotide 175) of the leader, respectively, both out of frame with S-Ag. A series of stop codon mutants were generated from the construct S38/T184/B202, which was produced by insertion of the *Bam*HI hairpin at position 202 of mutant S38/T184. Stop codon mutants were constructed by either site-directed mutagenesis or by overlap-extension PCR, using synthetic primers. Mutant S38/T47/B202 is similar to S38/T184/B202, except for a G → T and a C → A substitution in the tripartite leader at positions 47 and 48, respectively. These changes create a stop codon at position 47. Similarly, mutants S38/T85/B202, S38/T118/B202, and S38/T139/B202 were derived from mutant S38/T184/B202, with the exception that mutations were created by inserting a stop codon at positions 85, 118, and 139 of the leader, respectively. Mutant Wt-160 was prepared by inserting a 160-nucleotide DNA fragment from an altered HBVayw HBx gene (pX8: *Xmn*I to *Bam*HI) into the *Sal*I site of the leader. There are no AUGs in the 160-nucleotide fragment. The 160-nucleotide fragment was also inserted into the *Sal*I site of mutant B202 to create mutant B202-160. The Δ160 and the Δ160-B202 mutants were constructed by deleting the sequence between positions 33 and 202 of the tripartite leader from mutant WT-160 and B202-160, respectively.

Labeling of cells and analysis of polypeptides

Cells were labeled with [^{35}S]methionine for 2–3 hr using 100 μCi of *trans*-[^{35}S]methionine (New England Nuclear) per milliliter in DMEM lacking methionine and supplemented with 2% fetal calf serum. Cell extracts were prepared by sonication of washed cells in 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 0.25% NP-40, and 1 mM EDTA at 4°C and cleared of debris by centrifugation at 10,000*g*. Immunoprecipitation analysis was performed with equal amounts of protein in cell extracts, using specific antisera to S-Ag (Accurate Co.) and protein G–Sepharose beads (Santa Cruz Co.). Equal amounts of protein were analyzed by SDS–15% PAGE, fluorographed, and quantitated by densitometry. All experiments were repeated independently at least three times. Data represent typical results. The enzyme-linked immunosorbent assay (ELISA) was also carried out using a microdilution well system (Abbott Laboratories) to determine levels of S-Ag in cell lysates or medium for independent confirmation of immunoprecipitation results. Commercial S-Ag standards were used to quantitate the analysis.

RNA analysis

For Northern analysis, poly(A)$^+$ RNAs were prepared, and equal amounts (1.0 μg) resolved by formaldehyde–agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P-labeled DNA probes prepared from the S-Ag gene. For secondary structure analysis, wild-type and B34 tripartite leaders were transcribed in vitro using T7 polymerase at 43°C to permit extension through the hairpin, 5′ end labeled with [γ-^{32}P]ATP, and treated with increasing amounts of single-strand-specific endonulcease S1 exactly as described previously (Zhang et al. 1989). Products were resolved in denaturing urea acrylamide gels.

Construction of recombinant adenoviruses

A subgenomic Add*l*309 viral DNA fragment was prepared by digestion with *Cla*I–*Xba*I, and purified by sedimentation in a 10%–40% sucrose gradient in 1 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA for 14 hr at 36,000 rpm at 4°C in a SW41 rotor. Approximately 2 μg of Add*l*309 DNA was cotransfected into 293 cells with 2 μg of the pSV–HBV-based constructs linearized by *Kpn*I digestion. Viral recombinants were created by in vivo recombination between the defective 3′ end of the E1B gene in pSV–HBV derivatives and the E1B gene in viral genomic DNA. Viral plaques were selected under agar, expanded, analyzed, and stocks prepared as described previously (Doria et al. 1995).

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Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells.

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