Translation by ribosome shunting on adenovirus and *hsp70* mRNAs facilitated by complementarity to 18S rRNA

Andrew Yueh[1] and Robert J. Schneider[2]

Department of Microbiology, New York University School of Medicine, New York, New York 10016 USA

Translation initiation on eukaryotic mRNAs involves 40S ribosome association with mRNA caps (m^7GpppN), mediated by initiation factor eIF4F. 40S eukaryotic ribosomes and initiation factors undergo 5′ scanning to the initiation codon, with no known role for complementarity between eukaryotic 18S rRNA and the 5′ noncoding region of mRNAs. We demonstrate that the 5′ noncoding region of human adenovirus late mRNAs, known as the tripartite leader, utilizes a striking complementarity to 18S rRNA to facilitate a novel form of translation initiation referred to as ribosome shunting, in which 40S ribosomes bind the cap and bypass large segments of the mRNA to reach the initiation codon. Related elements are also shown to promote ribosome shunting in adenovirus *IVa2* intermediate phase mRNA during virus infection and in human heat shock protein 70 (*hsp70*) mRNA for selective translation during heat shock. The importance of mRNA complementarity to 18S rRNA suggests that ribosome shunting may involve either specific RNA structural features or a prokaryotic-like interaction between mRNA and rRNA.

[*Key Words*: Translation; ribosome shunting; adenovirus; *hsp70*; rRNA]

Received October 20, 1999; revised version accepted December 23, 1999.

It is thought that the majority of eukaryotic mRNAs initiate translation by a scanning mechanism, in which 40S ribosome subunits are assembled at the 5′ cap by translation initiation factors, which then promote a 5′ to 3′ nucleotide-by-nucleotide search for the initiating AUG codon (Jackson 1998). Some mRNAs initiate translation internally in the absence of a cap-dependent scanning process, which is directed by a poorly understood internal ribosome entry site (IRES; Jackson 1998). A small group of mRNAs identified to date, initiate translation by ribosome shunting, which combines features of both scanning and internal initiation (Jackson 1996). In shunting, ribosomes are loaded onto mRNA by a cap-dependent process but then shunt or bypass large segments of the mRNA before initiating translation at a downstream AUG (Jackson 1996). Ribosome shunting has been described for Cauliflower mosaic virus (CaMV) 35S mRNA (Futterer et al. 1993), Sendai virus Y protein mRNA (Curran and Kolakofsky 1988; Latorre et al. 1998), the family of adenovirus late mRNAs (Yueh and Schneider 1996), and papillomavirus E1 mRNA (Remm et al. 1999).

There are likely two functions for ribosome shunting. First, as described for CaMV 35S mRNA (Futterer et al. 1993), shunting can expand the coding capacity of an mRNA by permitting ribosomes to bypass upstream ORFs, directing ribosomes to an internal coding region that would not ordinarily be translated. Second, as described for adenovirus late mRNAs (Yueh and Schneider 1996), shunting can confer selective translation, despite inhibition of cell protein synthesis. For instance, during adenovirus late infection, or heat shock of cells, translation of most capped mRNAs is inhibited, and this inhibition is associated with dephosphorylation of translation initiation factor eIF4E (Huang and Schneider 1991; Zhang et al. 1994; Duncan 1996; Feigenblum and Schneider 1996; Schneider 1996). eIF4E is a component of the cap-binding initiation factor complex (eIF4F), which includes eIF4A, an ATP-dependent RNA helicase, and eIF4G, a large adapter protein upon which the complex assembles at the cap (Hentze 1997). Phosphorylation of eIF4E correlates positively with increased stability of the complex on the capped mRNA, increased cap-dependent RNA unwinding activity, and, therefore, increased ribosome loading onto mRNA (Rozen et al. 1990; Haghighat et al. 1995). Thus, when eIF4F–cap association is impaired, eukaryotic mRNAs that translate by either internal or shunting processes continue to initiate, probably because little or no eIF4F unwinding activity is required (Yueh and Schneider 1996).

[1]Present address: Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York USA.
[2]Corresponding author.
E-MAIL schner01@mcrcr6.med.nyu.edu; FAX (212) 263-8276.

The mechanism of ribosome shunting has not been described in molecular detail. CaMV 35S and late adenovirus mRNAs can switch between scanning and shunting mechanisms of initiation, which are thought to be mutually exclusive due to disruption of RNA shunting elements by scanning ribosomes (Yueh and Schneider 1996; Hohn et al. 1998). In CaMV, a short ORF (sORF) followed by one or more stable hairpin structures in the 5′ noncoding region (NCR) comprises a minimal shunting element (Hemmings-Mieszczak et al. 1997, 1998; Dominguez et al. 1998; Pooggin et al. 1998). This configuration is thought to provide 40S ribosome subunits to the shunting elements for nonlinear downstream translation. Ribosome shunting is directed in late adenovirus mRNAs by the tripartite leader, a highly conserved 200-nucleotide 5′ NCR found on most late adenovirus mRNAs (Yueh and Schneider 1996). The tripartite leader is thought to contain a 25- to 44-nucleotide unstructured 5′ conformation, followed by a complex group of stable hairpin structures (Zhang et al. 1989; Dolph et al. 1990; Yueh and Schneider 1996), but no sORF. The internal hairpins are essential components of the shunting element (Yueh and Schneider 1996). The tripartite leader provides preferential translation to late adenovirus mRNAs after viral inhibition of cell protein synthesis (Huang and Schneider 1990; Logan and Shenk 1984) in heat-shocked cells and in growth-arrested cells (Huang and Schneider 1990; Feigenblum and Schneider 1996; Yueh and Schneider 1996), which have been shown to have impaired eIF4F activity, possibly resulting from dephosphorylation of eIF4E. When phosphorylated eIF4E is abundant, and, therefore, cap-dependent eIF4F complex activity is high, the tripartite leader directs translation initiation by both 5′ scanning and shunting mechanisms. When eIF4E is dephosphorylated, the tripartite leader exclusively and efficiently directs initiation by shunting (Yueh and Schneider 1996).

We have investigated the molecular mechanism by which the tripartite leader directs efficient initiation by ribosome shunting. We show that the tripartite leader promotes a high level of ribosome shunting through the use of three conserved sequences that are complementary to the stem of the 3′ hairpin of 18S rRNA. Ribosome shunting was also found to be directed by the adenovirus *IVa2* 5′ NCR, which is also translated during the late phase of infection, and contains a related comlementarity to 18S rRNA. Moreover, the human heat shock protein 70 (*hsp70*) 5′ NCR contains one element related to the tripartite leader 18S rRNA complementarity, which is shown to promote ribosome shunting on this mRNA during heat shock, when eIF4F–cap-dependent protein synthesis is blocked. These results highlight the novel role of complementarity to 18S rRNA in some forms of ribosome shunting.

Results and Discussion

Ribosome shunting facilitated by adenovirus tripartite leader complementarity to 18S rRNA

Ribosome shunting directed by the adenovirus late tripartite leader occurs with high efficiency at roughly similar levels to that of scanning initiation (Yueh and Schneider 1996). This efficiency contrasts with an ~10-fold lower level of shunting found in CaMV, Sendai virus, and papillomavirus mRNAs (Futterer et al. 1993; Latorre et al. 1998; Remm et al. 1999). A feature present in the tripartite leader that is not found in these other mRNAs is a group of three elements of split (bipartite) complementary to both RNA strands of the stem in the 3′ hairpin of 18S rRNA (Fig. 1A). 18S rRNA is located in the 40S (small) ribosome subunit. There is no known role for the 18S rRNA 3′ hairpin in translation although it is highly conserved in prokaryotes and eukaryotes and is located on the outside surface of the 40S subunit (Green and Noller 1997) where it is accessible for interactions with other RNAs (Moore 1996). Tripartite leader complementarity to the 18S rRNA 3′ hairpin is conserved in human and mammalian viral serotypes (Dolph et al. 1990; Yueh and Schneider 1996).

We carried out mutational analyses of the tripartite leader to identify critical RNA elements that facilitate a high level of ribosome shunting. Specific deletions were introduced into a cDNA copy of the tripartite leader by oligonucleotide-directed PCR-mediated mutagenesis. The position of tripartite leader complementarities to 18S rRNA (*C1*, *C2*, and *C3*) are shown, as are the boundaries of the nucleotide segments deleted within a cDNA copy of the 220-nucleotide tripartite leader (Fig. 1B). To determine its ability to direct scanning and shunting initiation, wild-type and mutant forms of tripartite leader cDNAs were fused to the coding region of the hepatitis B virus surface antigen (*HBsAg*) gene as a reporter. The level of *HBsAg* secreted into the medium was then precisely quantitated by ELISA. Two sets of 5′ NCRs were constructed, with and without a stable hairpin at the 3′ end of the tripartite leader. Insertion of the stable hairpin at this position was shown previously to block scanning but not shunting ribosome initiation, providing a direct measure of the level of shunting translation (Yueh and Schneider 1996). HeLa cells were transiently transfected at similar efficiencies with plasmid reporter constructs, as determined by inclusion of a green fluorescent protein expression vector (data not shown). Equivalent steady-state levels of *HBsAg* reporter mRNAs were observed by Northern analysis (Fig. 1C), and translation of *HBsAg* mRNAs was quantified by ELISA (Fig. 1D).

As found previously, ribosome shunting accounts for 50%–60% of the normal translation activity of the wild-type tripartite leader (*3LDR*), shown by insertion of a stable 3′ hairpin (mutant *B202*; Fig. 1D). Translation of mRNAs was normalized to the unmodified wild-type tripartite leader (set to 100% activity). Replacement of the leader with a 50-nucleotide foreign RNA segment fully blocked shunting translation initiation, while reducing scanning initiation by only ~50% (Δ*3LDR* samples). Deletion of the majority of the tripartite leader abolished ribosome shunting [mutants D8 and D9 (±*B202*)] but only slightly reduced its ability to direct scanning initiation (two- to threefold). Collectively, these results confirm an essential requirement for the tripartite leader in

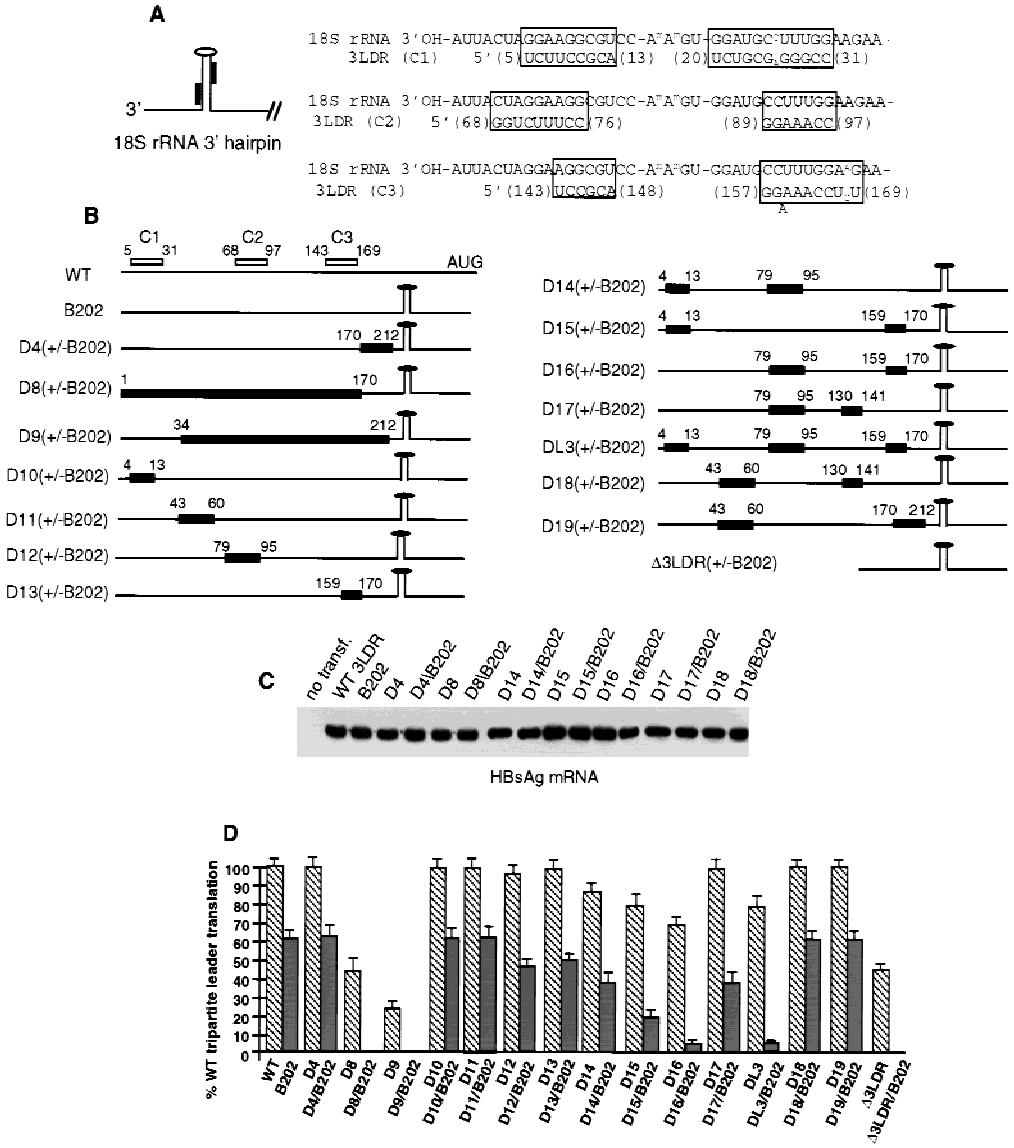

Figure 1. Scanning and shunting translation of tripartite leader mutants. (*A*) (*Left*) Diagram showing the 3′ hairpin of 18S rRNA and regions of the stem complementary to adenovirus tripartite leader elements (solid rectangles on stem). (*Right*) Bipartite sequence complementarity of the 3′ hairpin of human 18S rRNA and the human Ad5 tripartite leader. (*C1–C3*) Three complementarities in the tripartite leader (boxed), with nucleotide positions indicated in parentheses relative to the +1 start site of tripartite leader transcription. (*B*) Wild-type and deletion mutants of the tripartite leader are shown, ± insertion of a stable hairpin structure (*B202*) in the 3′ end of the NCR to block scanning-dependent initiation. Deletions are indicated by solid rectangles, with the position of junction sites indicated. (*C*) Steady-state Northern analysis of *HBsAg* reporter mRNAs obtained from equal numbers of transfected HeLa cells. Blots were hybridized to a ^{32}P-labeled probe prepared from the *HBsAg* coding region. (*D*) ELISA analysis of *HBsAg* levels obtained from five independent transfections of HeLa cells performed at similar efficiencies with the different tripartite leader 5′ NCRs. Transfection efficiencies (30%–35%) were determined by cotransfection of pGFP. Data were normalized relative to the average translational activity of the wild-type tripartite leader (WT). Mutant Δ*3LDR* contains a 50-nucleotide (*Bam*HI–*Sal*I) fragment of pBluescriptII SK polylinker in place of the tripartite leader.

ribosome shunting. Tripartite leader complementarities *C1–C3* were then mutated individually, or in combination, to assess their impact on scanning and shunting initiation. Individual deletion of complementarities *C1*, *C2*, or *C3* (mutants *D10*, *D12*, and *D13*, respectively) had a slight negative impact on the overall initiation of translation. Deletion of only *C1* had no effect on shunting initiation (*D10*/*B202*). Deletion of only *C2* (*D12*/*B202*), or of most of *C3* (*D13*/*B202*), reduced shunting initiation only slightly, by ~25% compared with *B202*. Similarly, small deletions of the tripartite leader outside the *C1–C3* complementarity regions did not diminish scanning or shunting initiation of translation (mutants *D4* and *D11*). These results implicate involvement of *C2* or *C3* com-

plementarities in ribosome shunting. Therefore, it was determined whether complementarities *C1–C3* possess functional redundancy that might mask the effects of individual deletion. Paired deletions of *C1* and *C2* (mutant *D14/B202*) reduced shunting slightly, to 60% of *B202* translation activity, and had little if any negative effect on overall (scanning) initiation. Paired deletion of *C1* and *C3* (mutant *D15/B202*) reduced shunting initiation threefold to 20% of *B202* translation activity. However, combined mutation of *C2* and *C3* (mutant *D16/B202*) strongly impaired shunting (by ~20-fold) to ~5% of *B202* translation activity. The moderate decrease in overall translation activity of mutant *D16* (by ~20%) is probably accounted for solely by the loss of shunting translation. Therefore, these results demonstrate that complementarities *C1–C3* are specifically involved in facilitating a high level of translation initiation by ribosome shunting. They also indicate that there is redundancy and a hierarchy in the importance of these elements, in which *C2* and *C3* are functionally dominant over *C1*. Collective mutation of *C1–C3* (mutant *DL3*) did not further reduce ribosome shunting or scanning initiation beyond that of the *C2–C3* mutant (*D16* and *D16/B202*). Paired mutation of similar-sized segments outside of *C1*, *C2*, or *C3* (mutants *D18* and *D19*) did not detectably reduce scanning or shunting initiation. Finally, these data also demonstrate that the tripartite leader contains a basal shunting element that promotes ~5% shunting activity in the absence of specific complementarity to 18S rRNA. The basal element remains to be identified, but it is evident that it is strongly augmented by a specific complementarity to the 3′ hairpin of 18S rRNA.

Next, we examined the importance of tripartite leader complementarities in directing ribosome shunting during late adenovirus infection, when eIF4F-dependent cell protein synthesis is inhibited. 293 cells, which are sensitive to shutoff of protein synthesis by adenovirus, were transfected with plasmids expressing *HBsAg* reporters linked to the wild-type tripartite leader, or mutant leaders, then infected 18 hr later with wild-type adenovirus. The level of reporter translation was determined prior to shutoff of cell protein synthesis (from 10 to 16 hr) or after shutoff (from 24 to 30 hr), by measurement of the amount of *HBsAg* secreted into the medium during this 6-hr period (Table 1). Inhibition of host cell protein synthesis and dephosphorylation of eIF4E at 24 hr post-infection was confirmed as described (Yueh and Schneider 1996), and Northern analysis demonstrated only a 25% decrease in cytoplasmic levels of plasmid transcribed mRNAs during the late phase of virus infection (data not shown). Translation of the wild-type tripartite leader (*3LDR*) and a tripartite leader containing the 3′ *B202* hairpin (*B202*) increased (30%–40%) during late adenovirus infection, after inhibition of cell protein synthesis as observed previously (Yueh and Schneider 1996). The increase in shunting is thought to result from reduced disruption of the RNA shunting elements by scanning ribosomes (Yueh and Schneider 1996). Specific deletion of tripartite leader complementarity to 18S rRNA also impaired shunting translation during late virus infection. Mutants *D12* and *D13*, which lack elements *C2* or *C3*, respectively, translated at levels similar to wild-type during early infection. Mutant D14, which lacks regions *C1* and *C2*, and mutant *D15*, which lacks regions *C1* and *C3*, also translated normally during early adenovirus infection. However, during late adenovirus infection, mutants *D12* and *D13* translated at about half the level of the *B202* control mRNA, and mutant *D15* translated at only ~30% the level of *B202*. Mutant *D16*, which is deleted of complementarities *C2* and *C3*, was only efficiently translated prior to viral inhibition of cell translation and was poorly translated (~5%) during late infection, following shutoff of host protein synthesis. These results are consistent with a hierarchy in functional importance of complementarities *C2* and *C3* in ribosome shunting, as determined in Figure 1 by insertion of secondary structure within the tripartite leader. These data also show that complementarity to 18S rRNA is vital for efficient tripartite leader translation by ribosome shunting during late adenovirus infection, when eIF4F activity and host cell protein synthesis are impaired.

Table 1. *Effect of Ad infection on ribosome shunting of tripartite leader and IVa2 5′ NCRs*

Construct	Translation activity (hr p.i.)	
	10–16 (%)	24–30 (%)
3LDR	100	136
B202	63	105
D12	92	54
D13	100	57
D14	85	29
D15	73	22
D16	71	5
WT *IVa2*	38	25
IVa2/B202	21	26
mIVa2	30	<2
WT *IX*	16	0

Translation activity was calculated by normalizing the average ELISA value for *HBsAg* secretion of different constructs obtained from five independent experiments against that of the wild-type tripartite leader (3LDR) at the 10- to 16-hr time point postinfection (p.i.). mRNAs are described in Figs. 1 and 2. (WT) Wild type.

Adenovirus IVa2 *mRNA is translated by shunting that is dependent on complementarity to the 18S rRNA 3′ hairpin*

Adenovirus late *IVa2* mRNA is synthesized during the intermediate phase of virus infection. It lacks the tripartite leader 5′ NCR, but nonetheless is well translated during late viral infection after inhibition of eIF4F-dependent protein synthesis (Hayes et al. 1990; Huang and Schneider 1990). Furthermore, the adenovirus late *IX* intermediate phase mRNA, which also lacks the tripartite leader, is weakly translated during the late phase of infection (Hayes et al. 1990; Huang and Schneider 1990).

Examination of these mRNAs revealed that the *IVa2* 5′ NCR sequence contains two regions strongly complementary to the 18S rRNA 3′ hairpin, which are similar to sequences found in the tripartite leader (Fig. 2A). No significant complementarity to 18S rRNA was found in the *IX* mRNA 5′ NCR. cDNA copies of the *IVa2* and *IX* 5′ NCRs were generated by synthetic oligonucleotide-mediated reverse transcription of late adenovirus mRNA, and the DNA sequence was verified. 5′ NCRs were tested for ribosome shunting activity by fusion to the *HBsAg* coding region, with and without insertion of a stable hairpin structure in the 3′ end of the NCR (Fig. 2B). The *IVa2* 5′ NCR directed strong ribosome shunting activity, averaging ~30% the level of the tripartite leader (cf. *B202* and *IVa2*/*B202*). Deletion of the majority of the *IVa2* 5′ NCR, including a region containing the two complementarities to the 18S rRNA 3′ hairpin (mutant Δ*IVa2*) specifically abolished shunting. A mutant deleted in the majority of the two *IVa2* complementarities (m*IVa2*, Fig. 2B) directed ribosome shunting very poorly (~2% of that of wild type). This mutant also displayed near normal overall translation activity, which was reduced by only ~30%, likely reflecting the loss of shunting translation. These data demonstrate that the *IVa2* 5′ NCR directs translation initiation by both ribosome shunting and scanning. They also specifically implicate complementarity to the 3′ hairpin of 18S rRNA in strong shunting activity.

Next, the ability of the *IVa2* 5′ NCR to direct translation during late adenovirus infection was investigated. 293 cells transfected with reporter expression vectors were later infected with adenovirus, and translation of *HBsAg* was examined prior to and after inhibition of cell protein synthesis during the late phase of adenovirus infection (Table 1). Prior to adenovirus shutoff of cell protein synthesis, the *IVa2* 5′ NCR directed translation at ~40% the level of the tripartite leader, which was only reduced to 25% during the late phase of infection. Insertion of the *B202* hairpin in the 3′ end of the *IVa2* 5′ NCR did not further reduce its ability to direct translation during late adenovirus infection (mutant *IVa2*/*B202*). These results indicate that the *IVa2* mRNA translates at a high level by ribosome shunting during late adenovirus infection, despite inhibition of host cell protein synthesis. The *IVa2* mRNA translated during early adenovirus infection at a level similar to that of the wild-type *IVa2* mRNA but was reduced to <2% after inhibition of cell protein synthesis during the late phase of infection. The *IX* mRNA 5′ NCR did not translate by ribosome shunting, as it was inhibited by insertion of the *B202* hairpin (Fig. 2). When expressed in transfected cells, the *IX* 5′ NCR directed translation only during the early phase of adenovirus infection (Table 1). We propose that the low translation level of the *IX* mRNA during the late phase of natural adenovirus infection results from the very large abundance of the transcript that is synthesized from the viral genome (Huang and Schneider 1990).

Translation of human hsp70 *mRNA by a ribosome shunt*

To determine whether a bipartite complementarity to the 18S rRNA 3′ hairpin might be involved in facilitating ribosome shunting in other mRNAs, we performed a database search for 5′ NCR elements related to the bipartite sequence found in the tripartite leader and *IVa2* mRNAs. The human *hsp70*-1 5′ NCR, but not *hsp70* from *Drosophila melanogaster*, contains a single element strongly related to that found in the tripartite leader (Fig. 3A). A full-length cDNA copy of the human and *Drosophila hsp70* 5′ NCRs were generated from commercial HeLa cell and *Drosophila* cDNA libraries by synthetic oligonucleotide-mediated PCR, and the DNA sequence was verified and then fused to the *HBsAg* coding region. 5′ NCRs were examined for their ability to direct translation by scanning or ribosome shunting, as

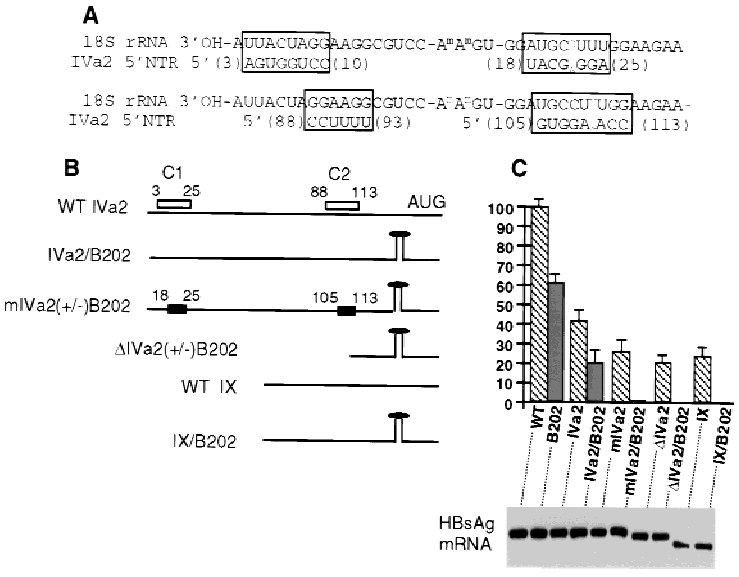

Figure 2. Scanning and shunting translation directed by the Ad5 *IVa2* 5′ NCR. (*A*) Bipartite sequence complementarity of the 18S rRNA 3′ hairpin and the 5′ NCR of human Ad5. Complementary elements *C1* and *C2* (boxed) and nucleotide positions in the *IVa2* and 5′ NCR are indicated relative to the mRNA transcription start site. (*B*) Wild-type and deletion mutants of the Ad5 *IVa2* and *IX* 5′ NCRs are shown, ± insertion of the *B202* stable hairpin structure. The boundary of deletions are shown in cDNA copies of the 5′ NCRs are shown above solid rectangles. (*C*) ELISA analysis of *HBsAg* reporter mRNAs represent an average of five independent transfections of HeLa cells at similar efficiencies. The Δ*IVa2* mutant is deleted from position 1 to position 112 of the *IVa2* 5′ NCR. Steady-state Northern analysis of cytoplasmic reporter *HBsAg* mRNAs for different constructs was performed with HeLa cells transfected at similar efficiencies.

measured by insertion of a stable hairpin in the distal end of the 5′ NCR (Fig. 3B). Both the wild-type human and *Drosophila hsp70* 5′ NCRs directed strong translation relative to the tripartite leader in transiently transfected cells (~80% and 110%, respectively). Scanning-dependent initiation was fully blocked by insertion of a stable hairpin into the 3′ end of the *Drosophila* 5′ NCR, which lacks 18S rRNA complementarity. However, the human *hsp70* 5′ NCR continued to direct translation at ~30% the wild-type level, despite insertion of the hairpin. These results indicate that the human *hsp70* 5′ NCR directs translation by both scanning and ribosome shunting.

Studies were carried out to independently confirm shunting in the human *hsp70* 5′ NCR by measurement the ability to direct translation in transiently transfected cells following heat shock and inhibition of translation. Transfected HeLa cells were subjected to heat shock at 44°C for 4 hr. These conditions block eIF4E phosphorylation and protein synthesis by 1 hr of heat treatment (Yueh and Schneider 1996; Laroia et al. 1999; data not shown). Translation of *HBsAg* from 1 to 4 hr after heat shock of cells was then determined (Table 2), by measurement of the level of protein secreted into medium during that time. Heat shock had no detectable affect on the cytoplasmic steady-state level of mRNAs (data not shown). Heat shock inhibition of eIF4F-dependent protein synthesis was evident in the translational inhibition of the Δ*3LDR* construct, which lacks the tripartite leader, and the inhibition of the *Drosophila hsp70/B202* 5′ NCR. The human *hsp70* 5′ NCR, however, continued

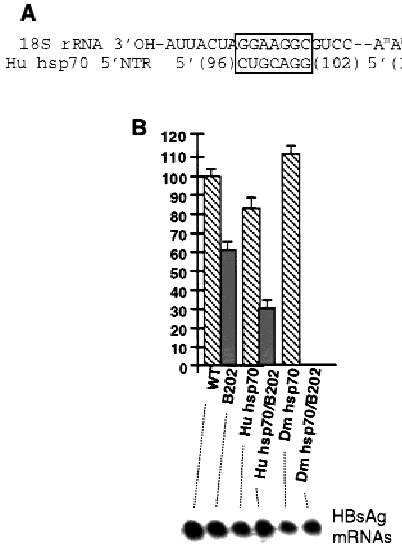

Figure 3. Translation activity of human *hsp70*-1 5′ NCR. (*A*) Bipartite complementarity of the human *hsp70* 5′ NCR with the 3′ hairpin of 18S rRNA. Complementary regions are boxed and indicated by nucleotide positions relative to the natural start of transcription in the 5′ NCR. (*B*) ELISA analysis of *HBsAg* reporter mRNAs represented by three independent transfections of HeLa cells at similar efficiencies. Northern analysis was performed on cytoplasmic mRNAs obtained from HeLa cells transfected at similar efficiencies.

Table 2. *Effect of heat shock on hsp 70 5′ NCR translation activity*

Construct	Translation activity (°C)	
	37 (%)	44 (%)
3LDR	100	145
B202	59	113
Δ3LDR	43	0
Hu hsp70	82	37
Hu hsp70/B202	33	40
Dm hsp70/B202	0	0
HuΔhsp70	27	0
Hu mhsp70	73	12

Translation activity was calculated by normalizing the average ELISA value for *HBsAg* secretion of different constructs obtained from three independent experiments against that of the wild-type tripartite leader (*3LDR*) at 37°C. *HuΔhsp70* contains a deletion of the majority of the *hsp70* 5′ NCR, from position 96 (*Pst*I) to 215 (the 3′ end). *Hu mhsp70* contains an internal deletion of the *hsp70* 5′ NCR between position 150 and 215.

to translate strongly following heat shock regardless of whether the *B202* stable hairpin was inserted in the 3′ end of the NCR. The human *hsp70* 5′ NCR did not function as an IRES when inserted internally in a dicistronic mRNA (data not shown), excluding the possibility of internal ribosome entry. A mutant deleted of the majority of the human *hsp70* 5′ NCR (Hu*Δhsp70*) was translated at about one-third the level of the wild-type 5′ NCR at normal temperature, but failed to translate during heat shock. To determine whether complementarity to 18S rRNA promotes shunting translation on the human *hsp70* 5′ NCR, a mutant was developed that is deleted of region 150–215, which encompasses the 3′ 18S rRNA complementarity (*Hu mhsp70*). Mutant *Hu mhsp70* directed efficient translation at normal temperature but was specifically impaired during heat shock, reduced in translation activity by threefold. These results demonstrate that the human *hsp70* 5′ NCR translates by both scanning and ribosome shunting mechanisms, and the shunting mechanism is used exclusively by the *hsp70* mRNA during heat shock. They also implicate elements complementary to the 3′ hairpin of 18S rRNA and related to those found in the tripartite leader and *IVa2* 5′ NCRs in promoting a stronger level of ribosome shunting in the human *hsp70* mRNA.

The molecular mechanism by which complementarity to 18S rRNA promotes ribosome shunting in the 5′ NCRs is not known. It is unlikely that these complementary elements singularly comprise the entire RNA shunting element, particularly because detectable ribosome shunting activity is retained after deletion of these elements in the tripartite leader, *IVa2*, and human *hsp70* 5′ NCRs. In this regard, tripartite leader mutants devoid of *C2* and *C3* complementarities direct ribosome shunting at a low level (~5%), similar to that of CaMV, Sendai virus, and papillomavirus 5′ NCRs (Curran and Kolakofsky 1988; Futterer et al. 1993; Remm et al. 1999), all of which lack a complementarity to the 3′ hairpin of 18S rRNA. On the basis of secondary structure mapping of

the tripartite leader (Zhang et al. 1989) and structure prediction analysis tripartite leader complementarities *C1*, *C2*, and *C3* may be partially or predominantly found in unstructured regions and single-stranded loops. This situation is reminiscent of the placement of Shine–Dalgarno elements in prokaryotic mRNAs. For example, bacteriophage MS2 mRNA translation was impaired if complementarity to 16S rRNA was embedded within secondary structure (de Smit and Van Duin 1994). There is no direct evidence, however, that complementarity to the 3′ hairpin of 18S rRNA within 5′ NCRs actually promotes rRNA–mRNA interaction. Although a direct interaction between 18S rRNA and the tripartite leader is potentially possible as a result of cooperative processes such as transient on/off base-pairing, technically, this interaction will be very difficult to detect at the molecular level. The 5′ NCR of S15 ribosomal protein mRNA and the Gtx homeodomain protein mRNA 5′ NCR, possess a striking complementarity to the 3′ hairpin of 18S rRNA that largely coincides with the *C2* and *C3* elements of the tripartite leader (Tranque et al. 1998; Hu et al. 1999). In these mRNAs, which initiate translation by ribosome scanning, the complementarity actually impairs translation initiation, possibly because 40S ribosome subunits are tethered to the mRNA (Tranque et al. 1998; Hu et al. 1999). Deletion of the element enhances scanning-mediated initiation. Therefore, it is possible that shunting involves stable association of 40S ribosome subunits to shunting elements containing complementarity to 18S rRNA. Studies now need to determine whether shunting involves direct 18S rRNA–mRNA interaction, or a specific RNA conformation directed by the complementarity to 18S rRNA, and whether binding of 40S subunits or initiation factors are then selectively recruited to the mRNA.

Materials and methods

Molecular cloning and site-directed mutagenesis

Deletions, point mutations, and inversions were introduced in the human Ad5 serotype tripartite leader cDNA in vector pSV–HBV (Dolph et al. 1988, 1990; Yueh and Schneider 1996). Deletions were generated by overlap-extension PCR with complimentary oligonucleotides. The boundaries of retained sequences are indicated in Figure 1 and 2 diagrams. Nucleotide positions are shown relative to the +1 position (transcription start site) of the tripartite leader cDNA (Yueh and Schneider 1996). Inversions of the tripartite leader cDNA fragments are indicated according to the junction of the retained and inverted sequence. Inversions were generated by cloning the complimentary orientation by use of available restriction enzyme cleavage sites. Point mutations were introduced into the tripartite leader cDNA by overlap-extension PCR mutagenesis, by use of complementary oligonucleotides containing the altered sequence. All mutations were verified by DNA sequence analysis. Point mutations of the tripartite leader complementarity to 18S rRNA in each of the three elements (*C1–C3*) generated the following sequences relative to wild type (shown in Fig. 1): mutant *C1* (nucleotide 5-AGAAUUCGU); mutant *C2* (nucleotide 68-UCGCGUCUU); mutant *C3* (nucleotide 157-CGUUUAGCU-CU). Mutations were combined as indicated in the text. A stable hairpin was developed previously (Yueh and Schneider 1996) by linking five copies of a 12-nucleotide *Bam*HI linker. The hairpin was inserted at position 220 in the tripartite leader cDNA. Full-length cDNA copies of the adenovirus *IVa2* and *IX* 5′ NCRs were prepared by reverse-transcription of viral mRNA with specific oligonucleotide primers. Human and *Drosophila* 5′ NCRs were prepared by oligonucleotide-primed PCR from commercial HeLa cell and *Drosophila* cDNA libraries. Mutation of the *IVa2* 5′ NCR (m*IVa2*) involved site-directed mutagenesis by PCR and excision of fragments to the boundaries shown in Figure 2. The *IVa2* 5′ NCR deletion mutant (Δ*IVa2*) was generated by deletion of sequences downstream of position 113. Mutation of the human *hsp70* 5′ NCR involved deletion of the sequence downstream of position 96 (*Pst*I; *Hu*Δ*hsp70*) or deletion of sequences between positions 150 and 215 (*Hu mhsp70*).

Transient transfections, and RNA and protein analyses

HEK 293 cells were grown in 100-mm dishes to ~50% confluence, transfected by the calcium phosphate procedure with 2 μg of reporter plasmid DNA and 0.5 μg of pGFP, which expresses a humanized green fluorescent protein to maintain transfection efficiencies. To determine the level of *HBsAg* synthesized and secreted, medium was removed at 40–48 hr following transfection and analyzed by commercial ELISA, as described by the manufacturer (Abbott Labs). The affect of adenovirus infection on *HBsAg* was determined by infection with 25 pfus per cell of adenovirus *dl 309* 18 hr post-transfection, medium was replaced at either 10 hr (prior to shutoff of cell protein synthesis) or 24 hr postinfection (after shutoff), followed by collection of medium 6 hr later and ELISA of *HBsAg*. The affect of heat shock on *HBsAg* synthesis was determined by replacement of medium at 36 hr post-transfection and heat shock of cells at 44°C for 4 hr, followed by collection of medium and ELISA of *HBsAg*. Data represent the average of five independent assays, with calculated standard errors shown. Northern blot RNA analysis was performed with total RNA purified from equal numbers of transfected cells. RNA was resolved by formaldehyde–agarose gel electrophoresis, transferred to blotting membrane and hybridized to ^{32}P-labeled DNA probes prepared against the *HBsAg* coding region.

Acknowledgments

We thank R. Cuesta of this laboratory for careful reading of the manuscript. This work was supported by grant CA 42357 to R.J.S. from the National Institutes of Health.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Curran, J. and D. Kolakofsky. 1988. Scanning independent ribosomal initiation of the Sendai virus X protein. *EMBO J.* **7:** 2869–2874.

de Smit, M.H. and J. Van Duin. 1994. Translational initiation on structured messengers. *J. Mol. Biol.* **235:** 173–184.

Dolph, P.J., V. Racaniello, A. Villamarin, F. Palladino, and R.J. Schneider. 1988. The adenovirus tripartite leader eliminates the requirement for cap binding protein during translation initiation. *J. Virol.* **62:** 2059–2066.

Dolph, P.J., J. Huang, and R.J. Schneider. 1990. Translation by the adenovirus tripartite leader: Elements which determine

independence from cap-binding protein complex. *J. Virol.* **64:** 2669–2677.

Dominguez, D.I., L.A. Ryabova, M.M. Pooggin, W. Schmidt-Puchta, J. Futterer, and T. Hohn. 1998. Ribosome shunting in cauliflower mosaic virus. Identification of an essential and sufficient structural element. *J. Biol. Chem.* **273:** 3669–3678.

Duncan, R.F. 1996. Translational control during heat shock. In *Translational control* (ed. J.W.B. Hershey, M.B. Mathews, and N. Sonenberg), pp. 271–294. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Feigenblum, D. and R.J. Schneider. 1996. Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. *Mol. Cell. Biol.* **16:** 5450–5457.

Futterer, J., Z. Kiss-Laszlo, and T. Hohn. 1993. Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. *Cell* **73:** 789–802.

Green, R. and H.F. Noller. 1997. Ribosomes and translation. *Annu. Rev. Biochem.* **66:** 679–716.

Haghighat, A., S. Mader, A. Pause, and N. Sonenberg. 1995. Repression of cap-dependent translation by 4E-binding protein 1: Competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* **14:** 5701–5709.

Hayes, B.W., G.C. Telling, M.M. Myat, J.F. Williams, and S.J. Flint. 1990. The adenovirus L4 100 kilodalton protein is necessary for efficient translation of viral late mRNA species. *J. Virol.* **64:** 2732–2742.

Hemmings-Mieszczak, M., G. Steger, and T. Hohn. 1997. Alternative structures of the cauliflower mosaic virus 35 S RNA leader: Implications for viral expression and replication. *J. Mol. Biol.* **267:** 1075–1088.

———. 1998. Regulation of CaMV 35S RNA translation is mediated by a stable hairpin in the leader. *RNA* **4:** 101–111.

Hentze, M.W. 1997. eIF4G: A multipurpose ribosome adapter? *Science* **275:** 500–501.

Hohn, T., D.I. Dominguez, N. Scharer-Hernandez, M.M. Poogin, W. Schmidt-Puchta, M. Hemmings-Mieszczak, and J. Futterer. 1998. Ribosome shunting in eukaryotes: What the viruses tell me. In *A look beyond transcription: Mechanisms determining mRNA stability and translation in plants.* (ed. J. Bailley-Serres and D.R. Gallie), pp. 84–95. American Society of Plant Physiologists, New York, NY.

Hu, M.C., P. Tranque, G.M. Edelman, and V.P. Mauro. 1999. RRNA complementarity in the 5′ untranslated region of mRNA specifying the Gtx homeodomain protein: Evidence that base-pairing to 18S rRNA affects translational efficiency. *Proc. Natl. Acad. Sci.* **96:** 1339–1344.

Huang, J. and R.J. Schneider. 1990. Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine. *Proc. Natl. Acad. Sci.* **87:** 7115–7119.

———. 1991. Adenovirus inhibition of cellular protein synthesis involves inactivation of cap binding protein. *Cell* **65:** 271–280.

Jackson, R.J. 1996. A comparative view of initiation site selection mechanisms. In *Translational control* (ed. J.W.B. Hershey, M.B. Mathews, and N. Sonenberg), pp. 71–112. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

———. 1998. Translation. Cinderella factors have a ball. *Nature* **394:** 829–831.

Laroia, G., R. Cuesta, and R.J. Schneider. 1999. Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. *Science* **284:** 499–502.

Latorre, P., D. Kolakofsky, and J. Curran. 1998. Sendai virus Y proteins are initiated by a ribosomal shunt. *Mol. Cell. Biol.* **18:** 5021–5031.

Logan, J. and T. Shenk. 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. *Proc. Natl. Acad. Sci.* **81:** 3655–3659.

Moore, P.B. 1996. In *Ribosomal RNA* (ed. A. Dahlberg and R.A. Zimmermann), pp. 199–236. CRC Press, Boca Raton, FL.

Pooggin, M.M., T. Hohn, and J. Futterer. 1998. Forced evolution reveals the importance of short open reading frame A and secondary structure in the cauliflower mosaic virus 35S RNA leader. *J. Virol.* **72:** 4157–4169.

Remm, M., A. Remm, and M. Ustav. 1999. Human papillomavirus type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism. *J. Virol.* **73:** 3062–3070.

Rozen, F., I. Edery, K. Meerovitch, T.E. Dever, W.C. Merrick, and N. Sonenberg. 1990. Bidirectional RNA helicase activity of eukarotic transaltion initiation factor 4A and 4F. *Mol. Cell. Biol.* **10:** 1134–1144.

Schneider, R.J. 1996. Adenovirus and vaccinia virus translational control. In *Translational control* (ed. J.W.B. Hershey, M.B. Mathews, and N. Sonenberg), pp. 575–605. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tranque, P., M.C. Hu, G.M. Edelman, and V.P. Mauro. 1998. rRNA complementarity within mRNAs: A possible basis for mRNA-ribosome interactions and translational control. *Proc. Natl. Acad. Sci.* **95:** 12238–12243.

Yueh, A., and R.J. Schneider. 1996. Selective translation by ribosome jumping in adenovirus infected and heat-shocked cells. *Genes & Dev.* **10:** 1557–1567.

Zhang, Y., P.J. Dolph, and R.J. Schneider. 1989. Secondary structure analysis of adenovirus tripartite leader. *J. Biol. Chem.* **264:** 10679–10684.

Zhang, Y., D. Feigenblum, and R.J. Schneider. 1994. A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis. *J. Virol.* **68:** 7040–7050.

Translation by ribosome shunting on adenovirus and *hsp70* mRNAs facilitated by complementarity to 18S rRNA

Andrew Yueh and Robert J. Schneider

Genes Dev. 2000, **14:**
Access the most recent version at doi:10.1101/gad.14.4.414