Unr Is Required In Vivo for Efficient Initiation of Translation from the Internal Ribosome Entry Sites of both Rhinovirus and Poliovirus

Oréda Boussadia,[1] Michael Niepmann,[2] Laurent Créancier,[3] Anne-Catherine Prats,[3] François Dautry,[4] and Hélène Jacquemin-Sablon[5]*

Nucleis, Parc Technologique des Capucins, 49033 Angers,[1] INSERM U397, Institut Fédératif de Recherche Louis Bugnard, CHU Rangueil, 31403 Toulouse Cedex 04,[3] CNRS UPR 1983, Institut André Lwoff, 94801 Villejuif Cedex,[4] and CNRS UMR 5540, Laboratoire de Pharmacologie des Agents Anticancéreux, Institut Bergonié, 33076 Bordeaux,[5] France, and Institute of Biochemistry, Justus-Liebig-University, Giessen, Germany[2]

Translation of picornavirus RNAs is mediated by internal ribosomal entry site (IRES) elements and requires both standard eukaryotic translation initiation factors (eIFs) and IRES-specific cellular *trans*-acting factors (ITAFs). Unr, a cytoplasmic RNA-binding protein that contains five cold-shock domains and is encoded by the gene upstream of N-*ras*, stimulates translation directed by the human rhinovirus (HRV) IRES in vitro. To examine the role of Unr in translation of picornavirus RNAs in vivo, we derived murine embryonic stem (ES) cells in which either one (−/+) or both (−/−) copies of the *unr* gene were disrupted by homologous recombination. The activity of picornaviral IRES elements was analyzed in *unr*$^{+/+}$, *unr*$^{+/-}$, and *unr*$^{-/-}$ cell lines. Translation directed by the HRV IRES was severely impaired in *unr*$^{-/-}$ cells, as was that directed by the poliovirus IRES, revealing a requirement for Unr not previously observed in vitro. Transient expression of Unr in *unr*$^{-/-}$ cells efficiently restored the HRV and poliovirus IRES activities. In contrast, the IRES elements of encephalomyocarditis virus and foot-and-mouth-disease virus are not Unr dependent. Thus, Unr is a specific regulator of HRV and poliovirus translation in vivo and may represent a cell-specific determinant limiting replication of these viruses.

After infection of a susceptible cell, translation of the picornavirus plus-strand RNA genome is controlled by the internal ribosome entry site (IRES). This *cis*-regulatory RNA element of about 450 nucleotides folds into complex and highly conserved secondary structures and facilitates translation by direct binding of ribosomes to an internal site of the viral RNA. IRES elements have been found in various viral RNAs and also in cellular mRNAs (for reviews, see references 1 and 16).

Internal initiation of picornavirus translation seems to require all canonical eukaryotic initiation factors (eIFs) also involved in cellular cap-dependent translation (28, 33), except for the actual cap-binding protein eIF4E. For the majority of eukaryotic mRNAs that are translated by a ribosome-scanning mechanism (13, 18, 38), the initiation of translation is the most important point of regulation in the overall process of protein synthesis (29). Modulation of the activity of these eIFs alters the general rate of protein synthesis, and the signal transduction routes leading to the eIFs are becoming clear now (36), supporting the idea that translational control substantially contributes to the regulation of gene expression in eukaryotes.

For picornaviruses, the initiation of translation also appears to be a major point of control. In vitro studies revealed that additional noncanonical translation initiation factors are involved in picornavirus IRES-mediated translation (1, 41). Accordingly, there is significant genetic evidence that picornavirus IRES elements contain determinants of cell specificity.

Analysis of poliovirus IRES mutants showed that translation defects could be cell type specific, since a decreased translation capacity of mutant templates was evident in cell extracts of neuronal origin, but not in HeLa cells (26). Cell-specific determinants were recently demonstrated to exist in the poliovirus 5′-untranslated region (UTR) by experiments using viruses with chimeric genomes. When the poliovirus IRES was replaced with that of human rhinovirus (HRV), neuropathogenicity in a mouse model was abrogated (15). The differential translation of wild-type and attenuated Sabin vaccine strains of poliovirus in different cell types indicates that cellular factors influencing translational activity may be differentially expressed, consistent with the idea that factors distinct from the standard initiation factors determine picornavirus translation efficiency (for review, see reference 1). The general idea that IRES elements may allow fine-tuning of gene expression was also supported for cellular IRES elements. The ornithine decarboxylase and p58^{PISTLRE} IRES elements specifically function during the G_2/M period (8, 37), the Ultrabithorax and Antennapedia IRES activities exhibit a high degree of developmental regulation in transgenic *Drosophila* (46), and stage- and tissue-specific modulation of FGF2 and c-myc IRES activities was observed in transgenic mice (9, 10). Although the molecular basis for cell-specific activity of IRES elements is not known, it is likely that cell type-specific proteins are involved in modulation of both picornaviral and cellular IRES-directed translation.

Five cellular *trans*-acting factors (ITAFs) specific for picornaviral IRES elements have been identified, four of which are RNA-binding proteins: the polypyrimidine tract-binding pro-

* Corresponding author. Mailing address: CNRS UMR 5540, Institut Bergonié, Bordeaux, France. Phone: (33) 5 56 33 04 28. Fax: (33) 5 56 33 04 21. E-mail: Jacquemin-h@bergonie.org.

tein (PTB) (24, 25, 32); the poly(rC)-binding protein 2 (PCBP2) (3, 12, 44); the autoantigen La (30); the Unr protein (20), an RNA-binding protein with five cold shock domains that is encoded by the gene upstream of N-*ras* (11, 23, 27); and ITAF$_{45}$, a cell-cycle-dependent protein homologous to Mpp1 (murine proliferation-associated protein) (34). Interestingly, functional in vitro assays revealed that some picornavirus IRES elements require a specific combination of two or three of these ITAFs for an efficient translational activity: PTB plus ITAF$_{45}$ for the IRES of foot-and-mouth disease virus (FMDV) (34), PTB plus PCBP2 for the poliovirus IRES (21), and PTB plus Unr plus PCBP2 for the HRV IRES (20). An attractive hypothesis is that specific translation initiation factors assembled on IRES elements regulate translation initiation in the same way as specific transcription factors bound to promoters regulate transcription (40). An exception is the encephalomyocarditis virus (EMCV) IRES, which appears to depend only on PTB in vitro. PTB is an abundant and ubiquitously expressed protein and is not expected to confer cell-specific translational regulation alone. Accordingly, the EMCV IRES displays a broad range of activities in different cell lines (5) and in transgenic mice (10).

Very few studies have focused on the in vivo role of ITAFs in the regulation of viral or cellular IRES activities. In this study, we have developed an in vivo approach to evaluate the significance of *unr*'s contribution to IRES-mediated translation. We generated mouse embryonic stem (ES) cell lines with gene-targeted deletion of *unr* and examined whether the *unr*$^{-/-}$ cells are defective in internal initiation of translation directed by picornaviral IRES elements. The advantage of this strategy is that primary cells are used, providing a physiological environment for the analysis of regulatory interactions that is as close as possible to the natural conditions. Thus, this strategy relies on the effect of endogenous cellular *unr*, but not on *unr* overexpressed in cell lines. We report here that the type I IRES-dependent translation of HRV and poliovirus RNA is strongly dependent on Unr expression in ES cells. In contrast, the activities of the type II IRES elements of EMCV and FMDV IRES elements were not affected by the absence of Unr expression. These results indicate that Unr functions as a specific regulator of HRV and poliovirus IRES-directed translation in vivo.

MATERIALS AND METHODS

ES cell culture. ES cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 150 μM monothioglycerol (Sigma), and 1,000 U of leukemia inhibitory factor per ml (ESGRO).

Generation of *unr*$^{-/-}$ cell clones. Heterozygous *unr*$^{+/-}$ mice were intercrossed, and ES cell lines were established from individual blastocyst-stage embryos as described previously (39). Briefly, blastocysts were cultured on irradiated feeders in a 24-well tissue culture well in ES media. After 4 to 6 days blastocysts were picked, disaggregated, and redispersed onto fresh feeders (1 well of a 24-well dish). Undifferentiated colonies that grew were picked 1 to 2 weeks later and replated on fresh feeders. Exponentially growing cells were passaged, grown to near confluency, and frozen. The genotype was determined by Southern blotting with a *unr* probe corresponding to exon 1. This probe detects 4.3- and 1.6-kb bands representing the 5′ ends of the wild-type and mutant alleles, respectively.

RNA isolation and analysis. To eliminate possible contamination of the ES cells with feeder cells, ES cells were trypsinized, seeded on gelatinized plates, and incubated for 60 min at 37°C. Nonadherent ES cells were then harvested for RNA extraction. Total RNA was prepared according to the guanidium-thiocya-

nate-CsCl protocol (45). RNase protection assays were carried out as previously described (6), with the murine *unk* probe (*unr*, N-*ras*, Ki-*ras*). Northern blot analysis was performed as described previously (42). Briefly, 15 μg of total RNA per lane was electrophoresed through 1.2% agarose gels containing formaldehyde and transferred in 150 mM NH$_4$Ac onto an uncharged nylon membrane (Amersham Hybond N). Hybridization was performed overnight in 50% formamide at 42°C for DNA probes and at 60°C for RNA probes. Signals were detected and quantified with a Fuji Bioimager (BAS 1000). The Rluc probe was a 960-bp *Hind*III-*Bam*HI fragment corresponding to the *Renilla* luciferase coding sequence. The Fluc probe was a 600-bp *Nco*I-*Eco*RI fragment corresponding to the 5′end of the firefly luciferase coding sequence. ^{32}P-radiolabeled probes were generated with the Stratagene random primer labeling kit Prime-It II. The β-actin probe was the 600-bp *Pst*I-*Taq*-I fragment corresponding to the 5′ end of the murine cDNA cloned into the Bluescript plasmid. Linearized plasmid was used as template for in vitro transcription to generate ^{32}P-radiolabeled probe.

Western blot analysis. For Western blots, cell lysates were prepared from ES cells made devoid of feeders by incubation on gelatinized plates as described above. Samples were fractionated on 10% denaturing gels, transferred to Immobilon-P membranes (Millipore), and probed with antibodies. Anti-*unr* rabbit polyclonal antibodies (23) were used at a 1:350 dilution, and anti-Flag M5 and anti-α-tubulin mouse monoclonal antibodies (Sigma) were used at 1:600 and 1:10,000 dilutions, respectively. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Amersham) were used as the secondary antibodies. Blots were developed with the Amersham ECL enhanced chemiluminescence system.

Plasmids. Most of the dicistronic constructs used in this study have already been described previously (9, 10, 19). The two luciferase open reading frames (ORFs) coding for *Renilla* luciferase (Rluc) and firefly luciferase (Fluc) are controlled by the cytomegalovirus immediate-early (CMV-IE) promoter and are separated by either a hairpin (pCRHL), the EMCV IRES, or the poliovirus IRES element (pCREL or pCRpolL, respectively). The plasmid pHRVL was derived from pCREL by replacing the EMCV IRES with the HRV-2 IRES between the two luciferase genes. To generate pHRVL, the full-length 5′ UTR sequence of HRV-2 (nucleotides 1 to 612) was excised from pGEM2HRV2 as a *Sac*I-*Nco*I fragment, and the HRV-2 *Sac*I (blunt ended)-*Nco*I fragment was ligated together with the *Nco*I-*Xho*I firefly luciferase fragment in pCREL previously digested with *Bam*HI (blunt ended)-*Xho*I. Plasmid pD128 (32) contains downstream of the CMV-IE promoter a dicistronic expression unit composed of the chloramphenicol acetyltransferase (CAT) ORF, the FMDV IRES, and the Fluc ORF. In the control plasmid pDmt, a hairpin was inserted between CAT and Fluc. pSGluc contains the firefly luciferase coding sequence inserted at the *Bgl*II site of pSG5 (Stratagene).

To construct pSGFlag-unr, an *Eco*RI-*Bcl*I (blunt ended) Unr cDNA fragment (nucleotides 1008 to 2750) was ligated to *Eco*RI (blunt ended)-*Bam*HI sites of pSG5 to generate plasmid pSG3′unr. A *Bam*HI-*Eco*RI fragment, containing the 5′ Unr cDNA (nucleotides 414 to 1008) in which the ATG initiating codon was substituted for by a *Bam*HI site, was isolated from pRSET-His*unr* (43). The *Eco*RI-*Bam*HI Unr fragment was ligated, along an *Eco*RI-*Bam*HI Flag epitope tag, to the *Eco*RI site of pSG3′unr to generate the final construct, pSGFlag-unr. From this plasmid, a full-length *unr* gene (−exon 5) with a Flag tag added at the amino terminus is expressed.

DNA transfection. ES cells (10^5 cells) in six-well pregelatinized petri dishes were routinely transfected with 1.5 μg of plasmid and 4.5 μl of Fugene 6 reagent (Boehringer-Roche) according to the manufacturer's recommendations. Twenty-four hours following transfection, cells were assayed for reporter gene activity. Adherent ES cells were washed with phosphate-buffered saline (PBS), trypsinized, and mixed with the nonadherent cells. Cells were pelleted, washed in PBS, and homogenized in 50 μl of passive lysis buffer (Promega). Luminescence was measured with 5- and 10-μl samples of extract. For cotransfection experiments, 1.5 μg of reporter plasmid and 1.0 μg of *unr* expression vector (or empty vector) were mixed with 9 μl of Fugene 6 reagent. For dicistronic RNA analysis, 3×10^6 cells were transfected with 10 μg of plasmids and 30 μl of Fugene 6 reagent. Twenty-four hours following transfection, RNA was isolated and subjected to Northern blot analysis.

Luciferase and CAT analysis. Firefly and *Renilla* luciferase activities were determined by using the dual-luciferase reporter system (Promega). Luciferase activities were measured with a Lumat LB9501 luminometer (Berthold), and the IRES activity was determined by calculating the Rluc/Fluc ratios. CAT activity in cell extracts was determined by the liquid scintillation method as described previously (7).
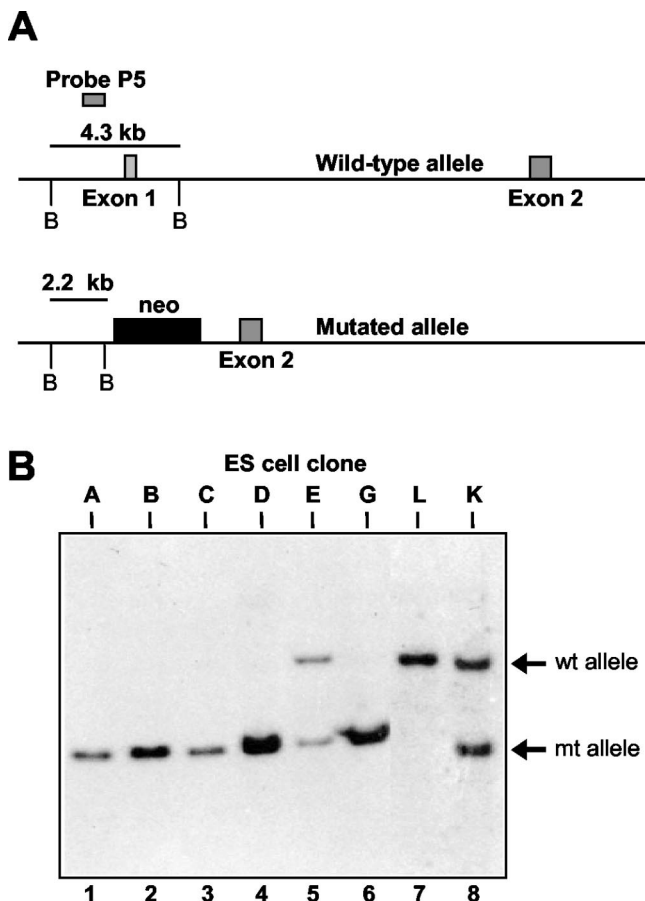
A

B

ES cell clone

FIG. 1. Genotype of ES cell clones mutated at the *unr* locus. (A) Schematic representation of the 5′ end of the wild-type *unr* allele and of the mutated allele generated by homologous recombination; *Bam*HI sites are indicated by the letter B. (B) Southern blot analysis of ES cell clones. Genomic DNA from eight independent ES cell clones (A, B, C, D, E, G, L, and K [lanes 1 to 8, respectively]) was digested with *Bam*HI and hybridized with the *unr* probe P5 indicated in panel A. This probe detects a 4.3-kb fragment and a 2.2-kb fragment from the wild-type (wt) and mutated (mt) alleles, respectively.

RESULTS

Generation of *unr*$^{-/-}$ ES cell lines. We previously reported the targeted disruption of the *unr* gene in mouse ES cells and the generation of mice heterozygous for the *unr* locus (6). Briefly, the targeting vector was designated to encompass the promoter and 5′ end of the *unr* gene, leading to inactivation of *unr* transcription. To generate *unr*$^{-/-}$ ES cell lines, we intercrossed *unr*$^{+/-}$ mice and isolated individual blastocysts (39). Eight ES cell lines were generated and genotyped by Southern blot analysis. The probe, located in the 5′ end of the *unr* gene, allowed us to distinguish the wild-type from the mutated *unr* alleles (Fig. 1A). Five ES clones were *unr*$^{-/-}$, two were *unr*$^{+/-}$, and one was *unr*$^{+/+}$ (Fig. 1B). The absence of *unr* expression in three *unr*$^{-/-}$ clones, designated A, B, and D, was confirmed by an RNase protection assay. The probe (Fig. 2A) was designed to simultaneously detect the *unr*, N-*ras* and Ki-*ras* messages (the amount of Ki-*ras* mRNA was used for normalization of the results) (6). As expected, no *unr* transcripts were detected in the homozygous *unr*$^{-/-}$ ES clones A, B, and D (Fig. 2A),

whereas in the heterozygous *unr*$^{+/-}$ clone K, *unr* expression was about 60% of the *unr*$^{+/+}$ clone L (after normalization). N-*ras* expression was increased two- to three-fold in *unr*$^{-/-}$ clones compared with the *unr*$^{+/+}$ clone L (Fig. 2A), in agreement with the observation of an increased expression of N-*ras* in tissues of heterozygous *unr*$^{+/-}$ mice (6). The absence of *unr* expression in two *unr*$^{-/-}$ ES clones (A and B) was further confirmed by Western blotting (Fig. 2B) with anti-*unr* rabbit polyclonal antibody (23).

When cultured on mouse embryonic fibroblast (MEF) feeder cells, *unr*$^{-/-}$ cells were morphologically indistinguishable from wild-type ES cells. In addition, *unr*$^{-/-}$ cells grew normally on MEF feeders (with doubling times of 12 h), suggesting that *unr* is not necessary for the growth of undifferentiated ES cells.

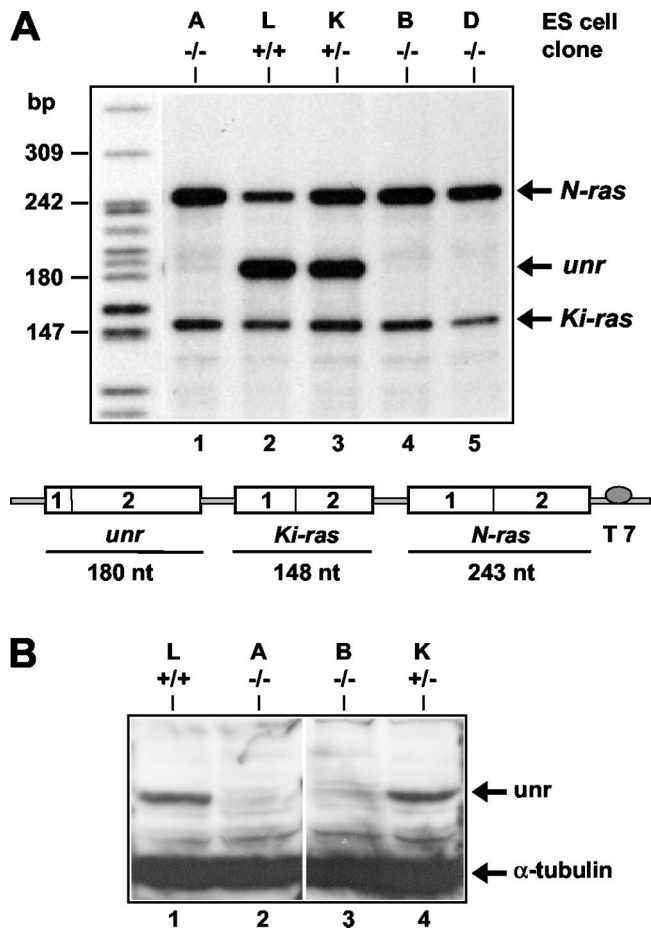**The rhinovirus and poliovirus IRESs work efficiently in wild-**

A

B

FIG. 2. Expression of *unr* in ES cell clones. (A) RNase protection analysis of RNA isolated from wild-type, *unr*$^{+/-}$, and *unr*$^{-/-}$ ES cells. Probe UNK comprises *unr*, N-*ras* and Ki-*ras* sequences. Ten micrograms of total RNA from five ES clones of each genotype as shown in Fig. 1 was analyzed with probe UNK. The molecular size marker on the left was generated with end-labeled *Msp*I fragments of pBR322. The protected fragments are indicated on the right. (B) Immunoblot of wild-type, heterozygous *unr*$^{+/-}$, and homozygous *unr*$^{-/-}$ ES cells. Lysates (25 μg) prepared from genotyped ES cell clones were analyzed by Western blotting with anti-*unr* and anti-α-tubulin antibodies. Arrows indicate *unr* (85 kDa) and α-tubulin (55 kDa).

type ES cells. The dicistronic plasmids pCRPolL, pHRVL and pCREL contain the poliovirus, HRV, and EMCV IRESs, respectively, inserted between Rluc and Fluc. Translation of RLuc, the first cistron, is cap dependent, whereas expression of the second cistron, Fluc, depends on the activity of the IRES located between the two reporter genes. As a negative control, plasmid pCRHL, which contains a hairpin but no IRES between the two cistrons, was used. The dicistronic plasmid pD128 contains the FMDV IRES inserted between CAT (upstream cistron) and Fluc (downstream cistron). For this plasmid, translation of CAT is cap dependent, whereas Fluc expression depends on FMDV IRES activity. As a control, plasmid pDmt, which contains a hairpin between CAT and Fluc, was used.

These plasmids were first transfected into wild-type ES cells that had been depleted of feeders prior to transfection. We determined that the contribution of residual feeders to the Rluc and Fluc activities was less than 1% and therefore negligible. We then determined the poliovirus, HRV, EMCV, and FMDV IRES activities in $unr^{+/+}$ (clone L) and $unr^{+/-}$ (clone K) ES cells. In $unr^{+/+}$ cells (Fig. 3A, black bars), the relative IRES activities were 80-fold for poliovirus, 30-fold for HRV, 120-fold for EMCV, and 90-fold for FMDV those of the negative controls. For comparison, the relative EMCV IRES activity in Cos-7 cells transiently transfected with the same dicistronic construct was 14.5 (19).

These results show that ES cells have the capacity to sustain efficient picornaviral IRES-directed initiation of translation. In the heterozygous $unr^{+/-}$ cells (clone K, dark gray bars) the four viral IRES elements were as efficient as in $unr^{+/+}$ cells, indicating that a twofold decrease in unr expression had no effect on these IRES activities.

Rhinovirus and poliovirus IRES-directed translation is impaired in $unr^{-/-}$ ES cells. We next examined the effect of unr disruption on the internal initiation of translation directed by picornaviral IRES elements. In $unr^{-/-}$ cells (clone A, Fig. 3A, upper panel, light gray bars), both the HRV and poliovirus IRES activities were severely impaired, their activities being about 10-fold lower than those in $unr^{+/+}$ or $unr^{+/-}$ cells. In contrast, the EMCV and FMDV IRES activities did not significantly differ between the $unr^{+/+}$, $unr^{+/-}$, and $unr^{-/-}$ cells. These results were confirmed after transfection in another $unr^{-/-}$ ES clone (clone B, data not shown). A decrease in IRES activity (Fluc/Rluc ratio) could result from a decrease in Fluc (second cistron) translation, an increase in Rluc (first cistron) translation, or a combination of both. As shown in Fig. 3A (lower panel), the Rluc activity did not vary significantly in $unr^{+/+}$, $unr^{+/-}$, and $unr^{-/-}$ cells, independently of the transfected plasmid. In contrast, the Fluc activity directed by the HRV and poliovirus IRES elements was specifically decreased in $unr^{-/-}$ cells. Therefore, the reduction in HRV and poliovirus IRES activities in $unr^{-/-}$ cells was the consequence of a decrease in Fluc activity and not of an increase in Rluc activity. Thus, in ES cells, unr is required for efficient translation directed by the HRV and poliovirus IRES elements, but not for the internal initiation of translation directed by the EMCV and FMDV IRESs.

To exclude that the results obtained above could be due to differential RNA degradation of the two cistrons, we checked the integrity of the dicistronic HRV mRNA in $unr^{+/+}$ and
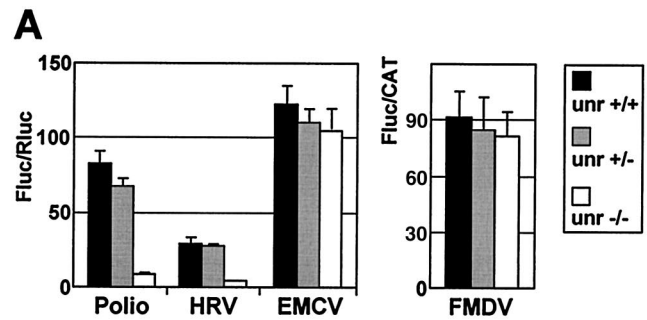
|	L (+/+)			K (+/-)			A (-/-)		
	Rluc	Fluc	Fluc/Rluc	Rluc	Fluc	Fluc/Rluc	Rluc	Fluc	Fluc/Rluc
control	194590	1206	0.0062	239161	1458	0.0061	311130	1804	0.0058
Polio	163901	83590	0.51	235800	99070	0.42	199047	10270	0.0516
HRV	145200	27590	0.19	186371	31683	0.17	194310	3497	0.018
EMCV	164450	120046	0.73	229640	149266	0.65	208788	123184	0.59
	CAT	Fluc	Fluc/CAT				CAT	Fluc	Fluc/CAT
FMDV	664	19008	28.62				1808	29911	16.54
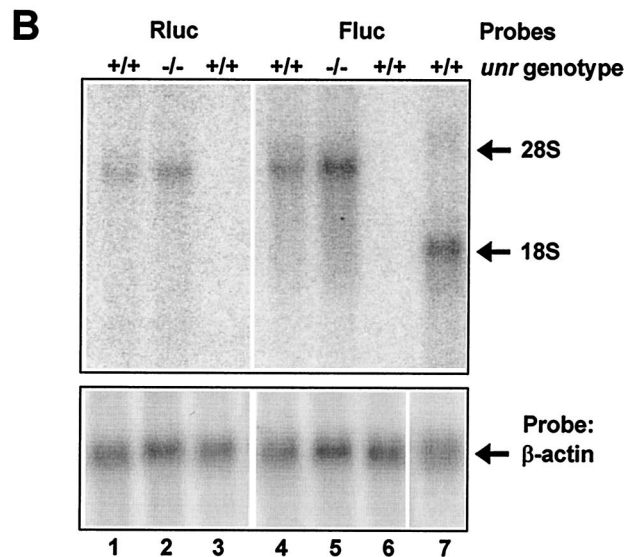
FIG. 3. Effect of unr expression on the activity of picornaviral IRES elements. (A) The dicistronic constructs pCRpolL, pHRVL, pCREL, and pD128 containing the poliovirus, HRV, EMCV, and FMDV IRESs, respectively, and the control construct pCRHL were transfected in $unr^{+/+}$, $unr^{+/-}$, and $unr^{-/-}$ ES cells. Twenty-four hours after transfection, cells were analyzed for Fluc, Rluc, and CAT activities. The ratio of the enzymatic activities of the second cistron to the first cistron are presented by histograms for $unr^{+/+}$ (clone L, black bars), $unr^{+/-}$ (clone K, gray bars), and $unr^{-/-}$ (clone A, light gray bars) cells. Fluc/Rluc ratios (left) or Fluc/CAT ratios (right) were normalized to the values obtained with control plasmids (pCRHL or pDmt, respectively). Three independent experiments were performed, each time in triplicate. Rluc and Fluc activities (relative light units) and CAT (cpm) activities are presented in the lower panel of the figure. (B) Northern blot analysis of total RNA extracted from transfected ES cells. Total cellular RNA was isolated from $unr^{+/+}$ and $unr^{-/-}$ ES cells transfected with pHRVL (lanes 1, 2, 4, and 5) or pSGluc (lane 7), or from untransfected ES cells (lanes 3 and 6). Fifteen micrograms of total RNA was analyzed by Northern blotting with the Rluc and Fluc probes (top) and with a β-actin probe as a loading control (bottom). The migration of 28S and 18S rRNA is indicated on the right.

$unr^{-/-}$ cell lines. Total RNA was extracted from transfected ES cells and analyzed by Northern blotting with Rluc and Fluc probes. In both in $unr^{+/+}$ and $unr^{-/-}$ cells, a single band of the size expected (~3.5 kb) for the dicistronic mRNAs was detected (Fig. 3B). As controls, RNA isolated from nontransfected cells was analyzed and showed no specific hybridization to the Rluc and Fluc probes (Fig. 3B, lanes 3 and 6), while after transfection of the monocistronic pSGluc plasmid, a single band of greater mobility (1.7 kb) was detected (Fig. 3B, lane 7). Thus, the integrity of the dicistronic HRV mRNA was not affected in transfected $unr^{-/-}$ cells. Moreover, quantification of the Rluc, Fluc, and β-actin (used for loading control) signals revealed that the Fluc/β-actin and Fluc/β-actin ratios differed by ~1.5-fold in the $unr^{+/+}$ and $unr^{-/-}$ cells, excluding a major change of the dicistronic mRNA metabolism in the absence of Unr.

Altogether, these results show that *unr* is required in vivo for internal initiation of translation directed by the picornaviral type I IRES elements of both HRV and poliovirus, but is dispensable for the activity of the type II IRES elements of EMCV and FMDV.

Rhinovirus and poliovirus IRES activities are complemented by Unr cDNA. We then tested whether reintroduction of Unr in *trans* into Unr-deficient cells would restore HRV and poliovirus IRES activities. In a calibration experiment, dicistronic plasmids containing the poliovirus or the EMCV IRES were cotransfected into $unr^{-/-}$ ES cells with increasing amounts of a *unr*-expressing vector (pSGFlag-unr) or an empty vector (pSG5). We observed a specific stimulation of poliovirus IRES activity when 0.3 to 1 μg of PSGFlag-unr was added, while a larger amount of plasmid (3 μg) led to an inhibition of cap-dependent and IRES-dependent translation initiation for both dicistronic plasmids (data not shown).

We then performed cotransfections by using 1 μg of pSG5 or pSGFlag-unr, respectively, along with 1.5 μg of dicistronic plasmids (Fig. 4A). pSFlag-unr protein expression was confirmed by Western blotting with an anti-Flag monoclonal antibody (Fig. 4B). In $unr^{+/+}$ cells (Fig. 4A, left panel), the activities of the poliovirus, HRV, and EMCV IRESs were similar when the control vector pSG5 and the *unr*-expressing vector pSGFlag-unr were cotransfected. In contrast, in $unr^{-/-}$ cells (Fig. 4A, right panel), the activities of the HRV and poliovirus IRESs were markedly reduced. Transfection of pSGFlag-unr then rescued poliovirus as well as HRV IRES activities to about 66 and 69%, respectively, of their activities in $unr^{+/+}$ cells. In contrast, EMCV IRES activity was not markedly reduced in $unr^{-/-}$ cells and was not significantly affected by transfection of pSG5 or pSGFlag-unr. These results thus confirmed that the defect in HRV and poliovirus IRES activities resulted from the absence of Unr expression.

DISCUSSION

Previous in vitro evidence indicated that the cellular RNA-binding protein Unr is required for efficient translation of HRV RNA (20). To analyze in vivo the role of Unr in the internal initiation of translation driven by picornaviral and cellular IRESs, we have generated $unr^{-/-}$ ES cells.

Generating isogenic cell lines deficient in specific genes is a powerful tool for analysis of the biochemical functions of the
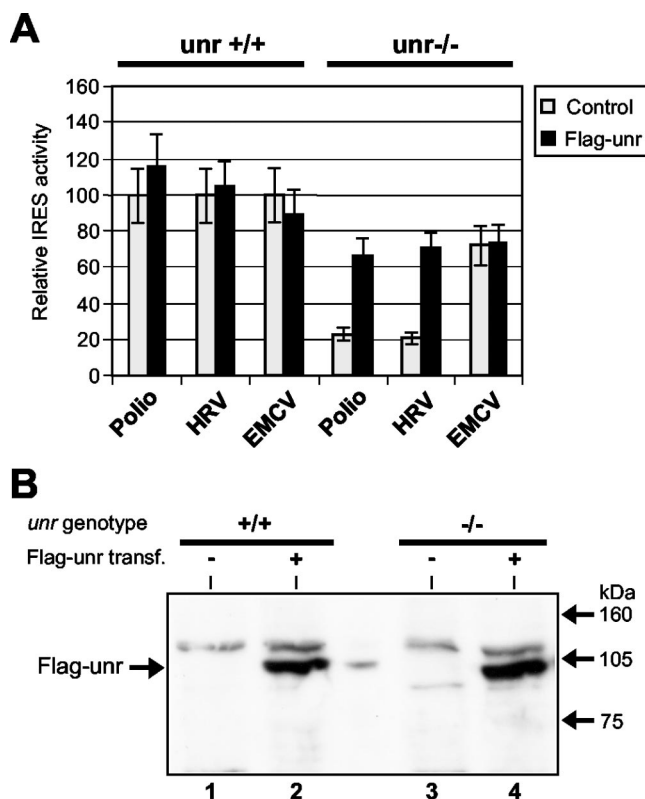

FIG. 4. Rescue of HRV and poliovirus IRES activity by *unr*. $unr^{+/+}$ and $unr^{-/-}$ ES cells were transfected with 1.5 μg of dicistronic plasmids containing the poliovirus, HRV, and EMCV IRESs, respectively. Each of the above plasmids was transfected together with 1 μg of either pSG5 (control) or pSG Flag-*unr* plasmids, respectively. Twenty-four hours after transfection, cells were analyzed for Fluc and Rluc activity (A) or Flag-*unr* protein expression (B). (A) The IRES activities in $unr^{+/+}$ and $unr^{-/-}$ cells were determined as described in the legend to Fig. 3; the values were taken as 100 for each dicistronic plasmid cotransfected with pSG5 in $unr^{+/+}$ cells. (B) Immunoblot analysis with anti-Flag antibody to detect the expression of Flag-*unr* protein. Lysates were prepared from $unr^{+/+}$ (lanes 1 and 2) or $unr^{-/-}$ (lanes 3 and 4) ES cells transfected with 1 μg of pSG5 (lanes 1 and 3) or 1 μg of pSG Flag-*unr* (lanes 2 and 4) plasmids.

encoded protein of interest in vivo. The level of *unr* expression in wild-type ES cells was similar to that observed in other cell lines (31), making these cell lines suitable to assess the consequences of *unr* inactivation on internal initiation of translation. ES cell lines were established from eight blastocysts, five of which were homozygous for the *unr* mutation. Within the limits of detection, the homozygous $unr^{-/-}$ cells were defective in *unr* expression, reflecting an essentially null mutation. Once established, the mutant cells grew with the same doubling time as wild-type cells, indicating that *unr* does not control cell growth of these undifferentiated cells, whereas development of mouse embryos was seriously affected (6; O. Boussadia and H. Jacquemin-Sablon, unpublished observations).

Here we report that HRV and poliovirus IRES-dependent translation is about 10-fold less efficient in $unr^{-/-}$ ES cells than in $unr^{+/+}$ or $unr^{+/-}$ cells. Reintroduction of a *unr* expression vector into $unr^{-/-}$ ES cells efficiently restores HRV and poliovirus IRES activities. In contrast, the IRES elements from EMCV and FMDV are as effective or decreased by less than

twofold in $unr^{-/-}$ ES cells compared with $unr^{+/+}$ cells. Altogether, our results demonstrate that *unr* functions as a specific regulator of translation initiation directed by the HRV and poliovirus elements in vivo.

The observed decrease in HRV and poliovirus IRES activity (Fluc/Rluc ratio) in $unr^{-/-}$ ES cells reflects the specific inhibition of translation of the second, IRES-dependent cistron (Fluc). This conclusion is based on the following observations. (i) An 8- to 12-fold decrease in Fluc activity is observed specifically after transfection of dicistronic plasmids containing HRV and poliovirus elements. (ii) Repeated transfections of all dicistronic plamids showed that in $unr^{-/-}$ cells, *Renilla* luciferase activity (the first cistron) did not change significantly compared with that in $unr^{+/+}$ cells. (iii) Northern blot analysis of the HRV dicistronic mRNA from transfected cells indicates that IRES integrity was maintained in $unr^{-/-}$ cells. Moreover, the limited variations in the amount of the Fluc/Rluc mRNA in the $unr^{-/-}$ and $unr^{+/+}$ cells indicate that *unr* expression did not significantly affect the steady-state level of the dicistronic mRNA. Thus, in agreement with previously reported in vitro data (20), our results indicate that physiological *unr* concentrations do not significantly modulate cap-dependent translation and that the relative decrease in the HRV and poliovirus IRES activities in the *unr*-knockout ES cells was not a secondary consequence of competing translation of the first cistron.

On the basis of their primary sequences and secondary structures as well as their requirements for efficient translation initiation in reticulocyte lysates, picornaviral IRES elements have been assigned to three groups: the type I IRES elements of the enterovirus and rhinovirus group, the type II IRES elements of the cardiovirus and aphthovirus group, and the type III IRES of hepatitis A virus (1). Our results establish that Unr functions in vivo as a specific regulator of translation directed by picornaviral type I, but not type II IRES elements. The direct involvement of Unr in HRV IRES-directed translation has been investigated in detail in vitro. (i) In HeLa cell extracts, Unr could be cross-linked to the HRV 5′ UTR, but not to the EMCV 5′ UTR (4). (ii) In rabbit reticulocyte lysates, Unr acts synergistically with PTB to stimulate HRV IRES activity (20), and this effect depends on the interaction of Unr with the HRV IRES, since mutations introduced in the Unr cold shock domains that knock out the RNA-binding activity of these domains impaired the Unr activity. PCBP2 enhances the activity of Unr and PTB on the HRV IRES, while PCBP2 alone has no effect on HRV IRES activity. In contrast, the stimulation of poliovirus IRES activity by Unr was uncertain, although purified Unr protein interacts both with HRV and poliovirus IRES elements. Recombinant Unr protein was unable to stimulate poliovirus IRES activity in rabbit reticulocyte lysate, whether Unr was added separately or in combination with PTB and PCBP2. Stimulation of the poliovirus IRES was only obtained with PTB, while PCBP2 had no effect. In contrast, purified native Unr protein (the so-called B-type activity) purified from HeLa cells stimulated poliovirus translation by a factor of 1.8 (20). Thus, the requirements of the HRV and the poliovirus IRES for added recombinant Unr, PTB, and PCBP2 appear to be different in reticulocyte lysates (which contain various endogenous levels of these factors). We can only speculate whether the combination of RNA-binding proteins that promotes internal initiation directed by a given IRES differs

from one tissue to another or according to the proliferation or differentiation status of the cells. Moreover, factors other than those tested in vitro, including Unr, PTB, and PCBP2 (20), that possibly contribute to the poliovirus IRES activity may be limiting in rabbit reticulocyte lysate, but not in ES cells. Nevertheless, our findings that Unr strongly regulates both HRV and poliovirus IRES activities in vivo indicate that Unr is one of the key regulators of type I IRES activity.

The mechanism by which ITAFs facilitate the recruitment of ribosomal subunits is yet unknown. One hypothesis is that ITAFs have a chaperone activity (17, 22) and help to fold the IRES into the conformation required for maximal IRES activity. This hypothesis is based mainly on the structural properties of these RNA-binding proteins. All of them have multiple-RNA-binding domains, namely cold shock domains for Unr, RNA recognition motif (RRM) domains for La, and PTB and KH domains in PCBP2. Moreover, most of them may dimerize in solution, like PTB, La, or PCBP2. Accordingly, these proteins may make several contacts with the IRES, like PTB (25). Understanding the role of Unr in IRES-mediated translation will require a detailed mapping of Unr binding sites on the HRV and poliovirus elements. The strong effect of Unr on HRV and poliovirus IRES-directed translation that we observed in ES cells should facilitate the determination of *cis* RNA sequences within the HRV and poliovirus elements required for regulation by Unr. Our system also provides a powerful experimental approach to analyze Unr domains required for stimulation of IRES activity. This could be investigated by cotransfection of constructs that express wild-type or mutant Unr proteins into $unr^{-/-}$ ES cells and analysis of their ability to restore HRV and poliovirus IRES activities.

In contrast to the large number of in vitro studies, there are only a few reports concerning the role of ITAFs in picornaviral IRES-directed translation in vivo. Transient overexpression of PTB in BSC-1 or HeLa cells was shown to stimulate cap-independent translation directed by picornaviral type I (poliovirus) and type III (hepatitis A virus) IRES elements as well as by the flaviviral hepatitis C virus IRES (14). PCBP proteins appear to be required for poliovirus IRES-directed translation, since addition of antibodies against PCBP1 and PCBP2 inhibited viral translation in *Xenopus* oocytes (12).

The results presented here show that ES cells sustain efficient internal initiation of translation directed by the four picornavirus IRES elements that we analyzed. Interestingly, it was recently reported that two cellular IRES activities, c-myc and FGF-2, are regulated during mouse embryonic development; while these IRESs are very active in 11-day-old embryos, they are inactive or poorly active in adult organs. In contrast, the EMCV IRES was found to be active in both embryonic and adult tissues (9, 10). It would be interesting to examine whether the HRV and poliovirus IRES activities depend on the differentiation state of ES cells.

Our results demonstrate that Unr is required in vivo for HRV and poliovirus IRES activity. Our approach using Unr-deficient cells implies that IRES activities were analyzed at physiological Unr concentrations. It was recently reported that PTB and nPTB, a neural-specific homologue of PTB, promote translation initiation directed by the IRES of the GVII strain of Theiler's murine encephalomyelitis virus and are critical for viral pathogenicity (35). It is tempting to speculate that Unr

could affect production of rhinovirus and of poliovirus, since at least in the latter case, a correlation between translational efficiency and neurovirulence has been established (2, 35).

REFERENCES

1. **Belsham, G. J., and R. J. Jackson.** 2000. Translation initiation on picornavirus RNA. Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
2. **Belsham, G. J., and N. Sonenberg.** 1996. RNA-protein interactions in regulation of picornavirus RNA translation. Microbiol. Rev. **60:**499–511.
3. **Blyn, L. B., J. S. Towner, B. L. Semler, and E. Ehrenfeld.** 1997. Requirement of poly(rC) binding protein 2 for translation of poliovirus RNA. J. Virol. **71:**6243–6246.
4. **Borman, A., M. T. Howell, J. G. Patton, and R. J. Jackson.** 1993. The involvement of a spliceosome component in internal initiation of human rhinovirus RNA translation. J. Gen. Virol. **74:**1775–1788.
5. **Borman, A. M., P. Le Mercier, M. Girard, and K. M. Kean.** 1997. Comparison of picornaviral IRES-driven internal initiations of translation in cultured cells of different origins. Nucleic Acids Res. **25:**925–932.
6. **Boussadia, O., F. Amiot, S. Cases, G. Triqueneaux, H. Jacquemin-Sablon, and F. Dautry.** 1997. Transcription of *unr* (*u*pstream of *N-ras*) down-modulates N-ras expression *in vivo*. FEBS Lett. **420:**20–24.
7. **Cassinotti, P., and M. Weitz.** 1994. Increasing the sensitivity of a common CAT assay. BioTechniques **17:**36–40.
8. **Cornelis, S., Y. Bruynooghe, G. Denecker, S. Van Huffel, S. Tinton, and R. Beyaert.** 2000. Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Mol. Cell **5:**597–605.
9. **Créancier, L., P. Mercier, A.-C. Prats, and D. Morello.** 2001. c-*myc* internal ribosome entry site activity is developmentally controlled and subjected to a strong translational repression in adult transgenic mice. Mol. Cell. Biol. **21:**1833–1840.
10. **Créancier, L., D. Morello, P. Mercier, and A. C. Prats.** 2000. Fibroblast growth factor 2 internal ribosome entry site (IRES) activity ex vivo and in transgenic mice reveals a stringent tissue-specific regulation. J. Cell. Biol. **150:**275–281.
11. **Doniger, J., D. Landsman, M. A. Gonda, and G. Wistow.** 1992. The product of unr, the highly conserved gene upstream of N-ras, contains multiple repeats of the cold shock domain (CSD) a putative DNA-binding motif. New Biol. **4:**389–395.
12. **Gamarnik, A. V., and R. Andino.** 1997. Two functional complexes formed by KH domain containing proteins with the 5′ noncoding region of poliovirus RNA. RNA **3:**882–892.
13. **Gingras, A. C., B. Raught, and N. Sonenberg.** 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. **68:**913–963.
14. **Gosert, R., K. H. Chang, R. Rijnbrand, M. Yi, D. V. Sangar, and S. M. Lemon.** 2000. Transient expression of cellular polypyrimidine-tract binding protein stimulates cap-independent translation directed by both picornaviral and flaviviral internal ribosome entry sites in vivo. Mol. Cell. Biol. **20:**1583–1595.
15. **Gromeier, M., L. Alexander, and E. Wimmer.** 1996. Internal ribosomal entry site substitution eliminates neurovirulence in intergenic poliovirus recombinants. Proc. Natl. Acad. Sci. USA **93:**2370–2375.
16. **Hellen, C. U., and P. Sarnow.** 2001. Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev. **15:**1593–1612.
17. **Herschlag, D.** 1995. RNA chaperones and the RNA folding problem. J. Biol. Chem. **270:**20871–20874.
18. **Hershey, J. W. B., and W. C. Merrick.** 2000. Pathways and mechanism of protein synthesis. Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Huez, I., L. Créancier, S. Audigier, M.-C. Gensac, A.-C. Prats, and H. Prats.** 1998. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol. Cell. Biol. **18:**6178–6190.
20. **Hunt, S. L., L. J. J. Hsuan, N. Totty, and R. J. Jackson.** 1999. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. Genes Dev. **13:**437–448.
21. **Hunt, S. L., and R. J. J. Jackson.** 1999. Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. RNA **5:**344–359.
22. **Jackson, R. J., and A. Kaminski.** 1995. Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA **1:**985–1000.
23. **Jacquemin-Sablon, H., G. Triqueneaux, S. Deschamps, M. Le Maire, J. Doniger, and F. Dautry.** 1994. Nucleic acid binding and intracellular localization of unr, a protein with five cold shock domains. Nucleic Acids Res. **22:**2643–2650.
24. **Kaminski, A., S. L. Hunt, J. G. Patton, and R. J. J. Jackson.** 1995. Direct evidence that polypirimidine tract binding protein (PTB) is essential for internal ribosomal entry site of encephalomyocarditis virus RNA. RNA **1:**924–938.
25. **Kolupaeva, V. G., C. U. Hellen, and I. N. Shatsky.** 1996. Structural analysis of the interaction of the pyrimidine tract-binding protein with the internal ribosomal entry site of the encephalomyocarditis virus and foot-and-mouth disease virus RNAs. RNA **2:**1199–1212.
26. **La Monica, N., and V. R. Racaniello.** 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. J. Virol. **63:**2357–2360.
27. **Landsman, D.** 1992. RNP-1, an RNA-binding motif is conserved in the DNA-binding cold shock domain. Nucleic Acids Res. **20:**2861–2864.
28. **Lomakin, I. B., C. U. T. Hellen, and T. V. Pestova.** 2000. Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. Mol. Cell. Biol. **20:**6019–6029.
29. **Mathews, M. B., N. Sonenberg, and J. W. B. Hershey.** 2000. Origins and principles of translational control. Translational control of gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Meerovitch, K., Y. V. Svitkin, H. S. Lee, F. Lejbkowicz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. D. Keene, and N. Sonenberg.** 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J. Virol. **67:**3798–3807.
31. **Nicolaiew, N., G. Triqueneaux, and F. Dautry.** 1991. Organization of the human N-*ras* locus: characterization of a gene located immediately upstream of N-*ras*. Oncogene **6:**721–730.
32. **Niepmann, M., A. Petersen, K. Meyer, and E. Beck.** 1997. Functional involvement of polypyrimidine tract-binding protein in translation initiation complexes with the internal ribosome entry site of foot-and-mouth disease virus. J. Virol. **71:**8330–8339.
33. **Pestova, T. V., C. U. Hellen, and I. N. Shatsky.** 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol. Cell. Biol. **16:**6859–6869.
34. **Pilipenko, E. V., T. V. Pestova, V. G. Kolupaeva, E. V. Khitrina, A. N. Poperechnaya, V. I. Agol, and C. U. T. Hellen.** 2000. A cell cycle-dependent protein serves as a template-specific translation initiation factor. Genes Dev. **14:**2028–2045.
35. **Pilipenko, E. V., E. G. Viktorova, S. T. Guest, V. I. Agol, and R. P. Roos.** 2001. Cell-specific proteins regulate viral RNA translation and virus-induced disease. EMBO J. **20:**6899–6908.
36. **Pyronnet, S., J. Dostie, and N. Sonenberg.** 2001. Suppression of cap-dependent translation in mitosis. Genes Dev. **15:**2083–2093.
37. **Pyronnet, S., L. Pradayrol, and N. Sonenberg.** 2000. A cell cycle-dependent internal ribosome entry site. Mol. Cell **5:**607–616.
38. **Raught, B., A. C. Gingras, and N. Sonenberg.** 2000. Regulation of ribosomal recruitment in eukaryotes. Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Robertson, E. J.** 1987. Embryo-derived stem cells, p. 71–112. *In* E. J. Robertson (ed.), Teratocarcinomas and embryonic stem cells: a practical approach. IRL Press, Oxford, United Kingdom.
40. **Sachs, A. B., and S. Buratowski.** 1997. Common themes in translational and transcriptional regulation. Trends Biochem. Sci. **22:**189–192.
41. **Sachs, A. B., P. Sarnow, and M. W. Hentze.** 1997. Starting at the Beginning, Middle, and End: translation initiation in eukaryotes. Cell **89:**831–838.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Triqueneaux, G., M. Velten, P. Franzon, F. Dautry, and H. Jacquemin-Sablon.** 1999. RNA binding specificity of UNR, a protein with five cold shock domains. Nucleic Acids Res. **27:**1926–1934.
44. **Walter, B. L., J. H. C. Nguyen, E. Ehrenfeld, and B. L. Semler.** 1999. Differential utilization of poly(rC) binding protein 2 in translation directed by picornavirus elements. RNA **5:**1570–1585.
45. **Weil, D., S. Brosset, and F. Dautry.** 1990. RNA processing is a limiting step for murine tumor necrosis factor β expression in response to interleukin-2. Mol. Cell. Biol. **10:**5865–5875.
46. **Ye, X., P. Fong, N. Iizuka, D. Choate, and D. R. Cavener.** 1997. *Ultrabithorax* and *Antennapedia* 5′ untranslated regions promote developmentally regulated internal translation initiation. Mol. Cell. Biol. **17:**1714–1721.