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# Lower concentration of La protein required for internal ribosome entry on hepatitis C virus RNA than on poliovirus RNA

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**Translation initiation of poliovirus and hepatitis C virus (HCV) RNA occurs by entry of ribosomes to the internal RNA sequence, called the internal ribosomal entry site (IRES). Both IRES bind to the La protein and are thought to require the protein for their translation initiation activity, although they are greatly different in both the primary and predicted secondary structures. To compare the La protein requirement for these IRES, we took advantage of I-RNA from the yeast *Saccharomyces cerevisiae*, which has been reported to bind to La protein and block poliovirus IRES-mediated translation initiation. In a cell-free translation system prepared from HeLa cells, yeast I-RNA inhibited translation initiation on poliovirus RNA as expected, but did not significantly inhibit translation initiation on HCV RNA. However, the translation initiation directed by either IRES was apparently inhibited by I-RNA in rabbit reticulocyte lysates, in which La protein is limiting. I-RNA-mediated inhibition of HCV IRES-dependent translation in rabbit reticulocyte lysates was reversed by exogenous addition of purified recombinant La protein of smaller amounts than necessary to reverse poliovirus IRES-dependent translation. These results suggest that HCV IRES requires lower concentrations of La protein for its function than does poliovirus IRES. Immunofluorescence studies showed that HCV infection appeared not to affect the subcellular localization of La protein, which exists mainly in the nucleus, although La protein redistributed to the cytoplasm after poliovirus infection. The data are compatible with the low requirement of La protein for HCV IRES activity.**

## Introduction

Hepatitis C virus (HCV), known to be a main causative agent of non-A, non-B hepatitis (Choo *et al.*, 1989), is classified in a separate genus of the *Flaviviridae* family. The genome of HCV consists of a single-stranded, positive-sense RNA of approximately 9500 nucleotides (nt) (Kato *et al.*, 1990; Takamizawa *et al.*, 1991). Translation initiation of HCV RNA has been shown to occur by entry of ribosomes to the internal RNA sequence (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993), and an internal ribosomal entry site (IRES) has been identified in the 5′ portion of the RNA (Reynolds *et al.*, 1995; Lu *et al.*, 1996; Kamoshita *et al.*, 1997) that has the potential to form extensive secondary structures (Brown *et al.*, 1992; Le *et al.*, 1995; Honda *et al.*, 1996).

Internal initiation of translation for eukaryotic mRNAs was first described for picornavirus RNAs (Pelletier & Sonenberg, 1988; Jang *et al.*, 1988). IRES have been discovered on some cellular mRNAs (Macejak & Sarnow, 1991; Oh *et al.*, 1992; Iizuka *et al.*, 1994) as well as other viral RNAs (Tsukiyama-Kohara *et al.*, 1992; Thiel & Siddell, 1994; Berlioz & Darlix, 1995; Poole *et al.*, 1995; Ivanov *et al.*, 1997). Nucleotide sequences that serve as IRES, so far discovered, have a variety of lengths and predicted secondary structures, although all of them have a similar function in translation initiation. Existence

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of the variety of IRES suggests that they have different functional structures, and that different sets of *trans*-acting cellular factors are required for individual IRES. In fact, the translation of poliovirus does not occur efficiently in a cell-free translation system prepared from rabbit reticulocyte lysates (RRL) (Dorner *et al.*, 1984). The poor translation in RRL, however, is markedly improved by the addition of factors from HeLa cells (Brown & Ehrenfeld, 1979). Other IRES, such as the IRES of encephalomyocarditis virus (EMCV) RNA (Jang *et al.*, 1988) and HCV RNA (Tsukiyama-Kohara *et al.*, 1992), are highly functional in the RRL system. Furthermore, it has been suggested that cellular factor requirements are altered by the introduction of mutations that affect IRES structures (Shiroki *et al.*, 1997; Kamoshita *et al.*, 1997). These observations strongly support the notion that cellular factors required for IRES activities are quantitatively and/or qualitatively different in individual IRES.

The initiation events directed by the IRES probably require most of the same set of initiation factors that are utilized by typically capped mRNAs (Scheper *et al.*, 1992). In addition to the standard initiation factors, other *trans*-acting proteins mediate IRES-dependent translation initiation, although the 40S ribosome particle interacts with HCV and classical swine fever virus RNAs in the absence of noncanonical initiation factors (Pestova *et al.*, 1998; Sizova *et al.*, 1998). La protein (Meerovitch *et al.*, 1993; Svitkin *et al.*, 1994; Craig *et al.*, 1997), polypyrimidine tract-binding protein (PTB) (Borman *et al.*, 1993; Hellen *et al.*, 1993) and poly(rC)-binding protein-2 (Blyn *et al.*, 1997) have been reported as possible host factors for IRES-dependent translation initiation of picornaviruses. The La protein was originally identified as an autoantigen that was recognized by antibodies from patients with autoimmune disorders such as systemic lupus erythematosus and Sjögren's syndrome (Tan, 1989). The La autoantigen, called SS-B, is an RNA-binding protein, belonging to the RNA recognition motif superfamily, that is involved in initiation and termination of RNA polymerase III transcription (Gottlieb & Steitz, 1989*a*, *b*; Maraia *et al.*, 1994; Maraia, 1996). Subcellular immunolocalization demonstrated that the La protein is mainly in the nucleus, but the protein redistributed to the cytoplasm after poliovirus infection (Meerovitch *et al.*, 1993). It has recently been reported that the La protein also interacts with the 5′ portion of HCV RNA and enhances the translation efficiency (Ali & Siddiqui, 1997).

A small RNA of 60 nt, called I-RNA, has been isolated from the yeast *Saccharomyces cerevisiae* on the basis of its ability to selectively block translation initiation directed by poliovirus IRES both *in vivo* and *in vitro* (Coward & Dasgupta, 1992; Das *et al.*, 1994). The I-RNA competed with the viral RNA elements within the 5′ untranslated region (UTR) for a cellular protein with an approximate molecular mass of 52 kDa, which was immunologically identified as the La autoantigen (Das *et al.*, 1996). Moreover, I-RNA-mediated inhibition of translation directed by poliovirus IRES in a cell-free system prepared from HeLa cells (HeLa S10) can be rescued by exogenous addition of purified La protein (Das *et al.*, 1996).

In this report, we took advantage of yeast I-RNA and purified recombinant La protein to compare the La protein requirements of HCV IRES and poliovirus IRES in cell-free translation systems, and demonstrated that HCV required lower concentrations of La protein for its IRES activity as compared with poliovirus. Furthermore, subcellular localization of La protein, which exists mainly in the nucleus, was not affected by HCV infection, although the La protein redistributed to the cytoplasm after poliovirus infection. This result is compatible with the low requirement of La protein for HCV IRES activity.

## Methods

■ **Construction of plasmids.** Plasmid pC1b has previously been constructed as a template for the synthesis of dicistronic mRNA which contains the 5′UTR of HCV genotype 1b between the two cistrons (Kamoshita *et al.*, 1997). The first cistron is a coding sequence for chloramphenicol acetyltransferase (CAT), and the second encodes the intact HCV (genotype 1b) polyprotein composed of core, E1 and the N-terminal part of E2/NS1. Plasmid pC1b was digested with *Sac*I and *Xba*I to remove the nucleotide sequence encoding CAT, and the *Sac*I–*Xba*I fragment was replaced by that (20 nt) from the multi-cloning site of pBluescript KS(+) (Stratagene). The plasmid thus constructed was designated pK1b. The *Eco*RI–*Xba*I DNA fragment that contained sequences of the T7 promoter and the first 2546 nt of the genome of the Mahoney strain of poliovirus type 1 was inserted into the corresponding site of pUC118, and the plasmid was designated pEXM1(T7)0 (Toyoda *et al.*, 1994). Plasmid pSDIR, which was used for preparation of yeast I-RNA, was constructed as previously described (Das *et al.*, 1994). A cDNA encoding the La protein was prepared from a cDNA library of HeLa S3 cells by PCR using 5′ GATCGAATTCATGGCTGAAAATGGTGA-TAATG 3′ and 5′ GATCTCGAGCTACTGGTCTCCAGCACC 3′ as sense and antisense primers, respectively, where *Eco*RI and *Xho*I sites are indicated by underlining, and translation initiation and termination codons by double underlining. The PCR product was digested with *Eco*RI and *Xho*I, and inserted into the corresponding site of pGEX-4T-1 (Pharmacia Biotech). The plasmid thus constructed was designated pGEX-La.

■ ***In vitro* transcription.** For preparation of RNAs for cell-free translation systems, plasmids pK1b and pEXM1(T7)0 were linearized by digestion with *Bam*HI (corresponding to nt 1357/1358 of the HCV genome) and *Xba*I (nt 2546/2547 of the poliovirus genome), respectively, and used as templates for run-off RNA synthesis by T7 RNA polymerase (Kaminski *et al.*, 1990). For preparation of IRES, plasmids pK1b and pEXM1(T7)0 were linearized by digestion with *Bsp*HI (nt 344/345) and *Msc*I (nt 629/630), respectively, and used as templates. The IRES portions of the viral RNAs thus obtained were used for UV cross-linking analysis. Yeast I-RNA and the control non-specific RNA were prepared by RNA transcription as above from plasmids pSDIR and pBluescript KS(+) that had been linearized by digestion with *Hin*dIII and *Eco*RI, respectively.

■ **UV cross-linking and immunoprecipitation.** RNA probes were labelled by RNA transcription reaction in the presence of [$\alpha$-$^{32}$P]CTP. The RNA probes ($1 \times 10^7$ c.p.m., $1 \times 10^7$ c.p.m./μg HCV RNA and $3 \times 10^7$ c.p.m./μg poliovirus RNA) were incubated with 100 μg of HeLa S10 or RRL in a binding buffer for 20 min at 30 °C and then irradiated with a UV

lamp for 30 min as described (Toyoda *et al*., 1994). For competition UV cross-linking assay, HeLa S10 or RRL were incubated with or without I-RNA of indicated amounts in a binding buffer at 30 °C for 5 min, and then mixed with the labelled RNA probes. After UV irradiation, the mixture was treated with 20 μg RNaseA (Sigma) and 20 U RNaseT1 (Boehringer Mannheim) at 37 °C for 30 min, and protein–nucleotidyl complexes were analysed by 12·5% PAGE in Laemmli's buffer system (Laemmli, 1970). In some cases, protein–nucleotidyl complexes were mixed with anti-La (SS-B) serum (30 μl) of a patient with autoimmune disease that had been preincubated with 5 mg protein A–sepharose CL-4B (Pharmacia Biotech) at 37 °C for 15 min followed by further incubation at 4 °C for 1 h. The mixtures were incubated in RIPA buffer (10 mM Tris–HCl, pH 7·5, 150 mM NaCl, 1% Triton X-100, 0·1% SDS and 1% sodium deoxycholate) at 37 °C for 10 min and then at 4 °C for 2 h, and centrifuged. The precipitates were washed with RIPA buffer several times, and analysed by PAGE as above. The gels were fixed, dried and subjected to autoradiography.

■ **Purification of La protein.** Purification of La protein essentially follows the method of Craig *et al*. (1997). *Escherichia coli* BL21 cells were transformed with plasmid pGEX-La to express a fusion protein of glutathione *S*-transferase (GST) and La protein, and grown at 37 °C in the presence of ampicillin. At an optical density at 600 nm of 0·5, IPTG was added to the culture at a final concentration of 1 mM, and further cultured at room temperature for 20 h. The cells were harvested by centrifugation, resuspended in PBS (140 mM NaCl, 2·7 mM KCl, 10 mM $Na_2HPO_4$ and 1·8 mM $KH_2PO_4$) and disrupted by sonication. The cell lysates were mixed with Triton X-100 of a final concentration of 1%, and kept at 4 °C for 30 min. The fusion protein was purified by using glutathione sepharose 4B according to the manufacturer's instruction (Pharmacia Biotech). Purified fusion protein was treated with thrombin protease (Pharmacia Biotech) to detach the GST moiety from La protein. Thrombin was removed by using benzamidine sepharose 6B (Pharmacia Biotech). Purified La protein was adjusted to a final concentration of 1 mg/ml using a filter of ULTRAFREE (Millipore). The purified protein was detected as one band on a polyacrylamide gel by silver staining and Western blotting (data not shown).

■ **Cell-free translation.** HeLa S10 was prepared from suspension-cultured HeLa S3 cells as previously described (Iizuka *et al*., 1991). RRL was purchased from Promega. For each reaction mixture of 12·5 μl, 0·5 μg RNA as a template, 170 mM potassium acetate, 1·5 mM magnesium acetate and 10 μCi [$^{35}$S]methionine (1000 Ci/mmol) were used. Reaction with 50 μg of HeLa S10 or 8·75 μl (200 μg) of RRL was carried out for 1 h at 37 °C or 30 °C, respectively. Reaction conditions including RNA and salt concentrations were adjusted such that maximum inhibition by I-RNA of poliovirus RNA translation in the HeLa S10 extract was ascertained. Translation products were analysed by gel electrophoresis as described above. The gels were treated with ENLIGHTNING (Dupont), dried and subjected to autoradiography.

■ **Virus infection and immunofluorescence.** IMY cells (Ito *et al*., 1996; Kohara *et al*., 1998; T. Ito & K. Yasui, unpublished results) were grown on chamber slides (Falcon) in Dulbecco's modified Eagle's medium containing HAT (100 μM hypoxanthine, 0·4 μM aminopterin and 16 μM thymidine) supplemented with 10% foetal calf serum. IMY cells were established from a human hepatocyte obtained from a needle biopsy which had been fused with a HepG2 cell deficient in hypoxanthine–guanine phosphoribosyl transferase activity. The cells were infected with the Mahoney strain of poliovirus type 1 at an m.o.i. of 100 or HCV at 5 genomes per cell, and incubated at 37 °C. Poliovirus- and HCV-infected cells were fixed in cold methanol 7 h and 7 days post-infection, respectively, and subjected to indirect immunofluorescence studies. The cells were incubated with a mouse anti-La monoclonal antibody (MAb) La4B6 (a generous gift from M. Bachmann) and rabbit hyperimmune sera against poliovirus type 1 or HCV core protein at 37 °C for 2 h. After washing with PBS several times, the samples were reacted with sheep anti-mouse IgG conjugated with FITC (Biosys) and goat anti-rabbit IgG conjugated with Texas red (Leinco Technologies) at 37 °C for 1 h, and then treated with TOTO-3 iodide (Molecular Probes), which has a high affinity to nucleic acids, at 37 °C for 15 min. Almost 100% of IMY cells infected with poliovirus and 20–30% of the cells infected with HCV were stained by using rabbit sera against poliovirus type 1 and HCV core protein, respectively. The cells were mounted with 80% (v/v) glycerol after washing several times, and analysed with a confocal laser scanning microscope (Bio-Rad).

## Results

### Inhibition of La protein binding to IRES by I-RNA

The La protein has been shown to bind both to poliovirus IRES (Meerovitch *et al*., 1989, 1993) and HCV IRES (Ali & Siddiqui, 1997). UV cross-linking studies were performed using HeLa S10 and RRL with $^{32}$P-labelled poliovirus IRES (nt 1–629) and HCV IRES (nt 1–344) as probes. The results are shown in Fig. 1 (lanes 2 and 8). These bands are competed by the addition of an excess amount of cold homologous RNAs (data not shown) (Das *et al*., 1994). As expected, protein–nucleotidyl complexes with an approximate molecular mass of 52 kDa are observed in any patterns obtained. To confirm that these bands include the La protein, protein–nucleotidyl complexes, after RNase digestion, were immunoprecipitated with anti-La (SS-B) serum of a patient as described in Methods. The protein of 52 kDa appeared to react with anti-La antibodies (Fig. 1, lanes 1 and 7). A similar result was obtained when rabbit anti-serum to human La protein was used instead of the patient serum, and preimmune rabbit serum did not react with the protein of 52 kDa (data not shown). The materials immunoprecipitated migrated slightly faster than the 52 kDa protein, indicated by arrows. This phenomenon seemed to be due to a patient serum that contained a large amount of immunoglobulins. These results suggest that the 52 kDa protein corresponds to La protein. The intensity of the 52 kDa protein band immunoprecipitated with a patient serum is higher in the experiments involving HeLa S10 than RRL using either of the IRES as a probe (Fig. 1, lanes 1 and 7). It is possible that this phenomenon results from the nature of the anti-serum used, which may bind more strongly to human La protein than to rabbit La protein.

Yeast I-RNA has been shown to bind to the La protein (Das *et al*., 1996). To examine if the I-RNA competes with poliovirus IRES and HCV IRES for La protein, competition UV cross-linking studies were performed using HeLa S10 or RRL as described in Methods (Fig. 1, lanes 3, 4, 9 and 10). Proteins of 52 kDa of HeLa S10 and RRL that bind to poliovirus IRES are reduced in amount by the addition of I-RNA, whereas other bands are not (Fig. 1*a*, lanes 3, 4, 9 and 10). Similar phenomena are observed for a band of 52 kDa protein bound to HCV IRES (Fig. 1*b*, lanes 3, 4, 9 and 10). Reduction in intensity of the 52 kDa protein band seems to be dependent on the increasing

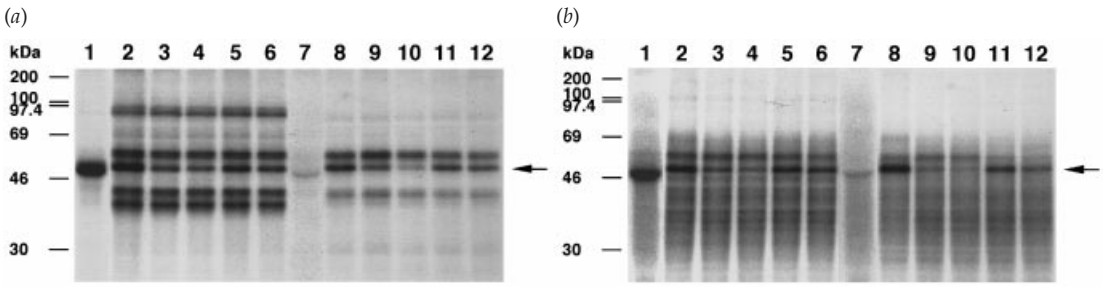


Fig. 1. (*a*) UV cross-linking studies with a $^{32}$P-labelled poliovirus IRES (nt 1–629) probe ($1 \times 10^7$ c.p.m.) were performed with HeLa S10 (lanes 1–6) and RRL (lanes 7–12) in the absence (lanes 1, 2, 7 and 8) or presence (lanes 3–6 and 9–12) of unlabelled competitor RNAs. A 100-fold (lanes 3, 5, 9 and 11) or 200-fold (lanes 4, 6, 10 and 12) molar excess of yeast I-RNA (lanes 3, 4, 9 and 10) or non-specific RNA (lanes 5, 6, 11 and 12) was used as competitor RNA. Protein–nucleotidyl complexes in the reaction mixtures were directly analysed by SDS–PAGE (lanes 2–6 and 8–12). Among the complexes shown in lanes 2 and 8, materials immunoprecipitated with anti-La serum of a patient were applied on lanes 1 and 7, respectively. Amounts of samples applied on lanes 1 and 7 were about 45-fold equivalent vols of reaction mixtures of those applied on other lanes. A protein band of 52 kDa is indicated by an arrow. Positions of protein size markers are shown on the left of each figure in kDa. (*b*) UV cross-linking studies with a $^{32}$P-labelled HCV IRES (nt 1–344) probe ($1 \times 10^7$ c.p.m.) were performed. Other conditions were the same as in (*a*). Experiments were carried out independently four times, and similar results were obtained.

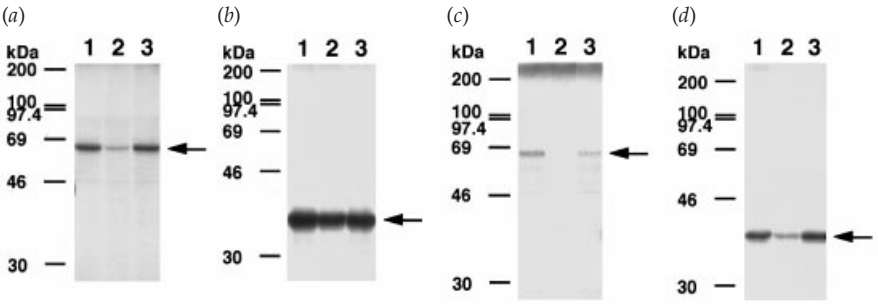


Fig. 2. *In vitro* translation in the presence of competitor RNAs. HeLa S10 (*a* and *b*) or RRL (*c* and *d*) were used as an *in vitro* translation system. Poliovirus RNA (nt 1–2546) (*a* and *c*) or HCV RNA (nt 1–1357) (*b* and *d*) was translated in the absence (lane 1) or presence of a 25-fold molar excess of I-RNA (lane 2) or non-specific RNA (lane 3) as described in Methods. Translation products from these RNAs are indicated by arrows. Positions of molecular mass markers are shown on the left of each figure in kDa.

amount of I-RNA (Fig. 1). The addition of similar amounts of unlabelled non-specific RNA appeared not to compete well with either of the IRES for any proteins cross-linked to these IRES (Fig. 1, lanes 5, 6, 11 and 12). These data suggest that I-RNA has an affinity specific to La protein among these proteins.

### Inhibition of translation initiation by I-RNA

Yeast I-RNA was reported to specifically block poliovirus IRES-dependent translation initiation, but not EMCV IRES- and cap-dependent translation initiations (Coward & Dasgupta, 1992; Das *et al*., 1994). As expected, translation in HeLa S10 directed by poliovirus IRES was efficiently inhibited by the addition of I-RNA under the conditions used (Fig. 2*a*). This inhibition of poliovirus translation has been considered to be mainly due to blocking of the La protein activity by I-RNA (Das *et al*., 1996). Since the La protein appears to be required for internal ribosome entry on HCV RNA (Ali & Siddiqui, 1997), it is possible that I-RNA also shows an inhibitory activity to the translation directed by HCV IRES. Accordingly, a similar experiment was carried out using HCV RNA (Fig. 2*b*). The amount of translation product, however, appeared not to be significantly reduced by the addition of I-RNA. This observation may suggest that the La protein concentration required for the activity of HCV IRES is lower than that of poliovirus IRES, and that La protein activity in HeLa S10 reduced by I-RNA is still sufficient to support HCV IRES function, although it is possible that some proteins present in this system compensate for La function in translation.

We thus employed RRL, in which the La protein was limiting (Meerovitch *et al*., 1989,1993), as an *in vitro* translation system. In RRL, translation initiations directed both by poliovirus IRES and HCV IRES were inhibited by the addition of I-RNA at the same molar ratio to mRNA as used for translation initiation in HeLa S10, whereas no significant inhibition by non-specific RNA was observed (Fig. 2*c* and *d*). These data support the previous observation that HCV IRES requires La protein for its activity (Ali & Siddiqui, 1997), and

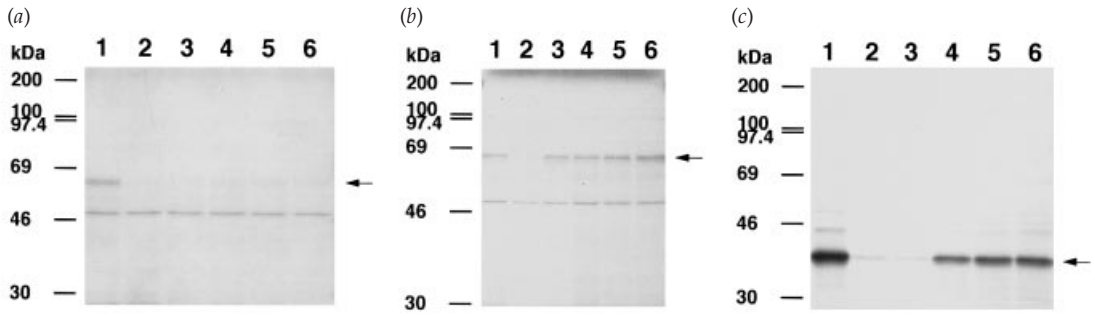


Fig. 3. Effect of exogenous addition of La protein on IRES-dependent translation in RRL with or without I-RNA. Poliovirus RNA (nt 1–2546) (*a* and *b*) or HCV RNA (nt 1–1357) (*c*) was translated in RRL that had been preincubated at 30 °C for 10 min with no I-RNA (lane 1 of each panel), a 25-fold molar excess of I-RNA (lanes 2–6 of *a* and *c*) or a 12·5-fold molar excess of I-RNA (lanes 2–6 of *b*). Translation reactions were performed with 0 μg (lanes 1 and 2), 0·25 μg (lane 3), 0·5 μg (lane 4), 0·75 μg (lane 5) and 1 μg (lane 6) of purified recombinant La protein. Translation products are indicated by arrows. Positions of molecular mass markers are shown on the left of each figure in kDa.

the data also support the view that a lower concentration of La protein is required for HCV IRES function as compared with that of the poliovirus IRES.

### Reversion of I-RNA-mediated translation inhibition by La protein

It has been reported that inhibition of poliovirus IRES-dependent translation by I-RNA in HeLa S10 is rescued by the exogenous addition of La protein (Das *et al.*, 1996). Accordingly, we examined whether I-RNA-mediated inhibition of HCV translation was rescued by the addition of purified La protein. For this purpose, the La protein was expressed in *E. coli* and purified as described in Methods.

I-RNA-mediated inhibition of HCV IRES-dependent translation in RRL was restored by the exogenous addition of increasing amounts of purified recombinant La protein (Fig. 3*c*), but not by BSA (data not shown). The data suggest that inhibition by I-RNA is mainly due to a deficiency in La protein in the *in vitro* translation system. In contrast, poliovirus IRES-dependent translation in RRL inhibited by I-RNA appeared to be only slightly rescued by exogenous addition of the La protein under similar conditions (Fig. 3*a*), although poliovirus translation inhibition by I-RNA in HeLa S10 was rescued completely by exogenous addition of the La protein (data not shown) (Das *et al.*, 1996). It is possible that the molar ratio of La protein to I-RNA is not enough to rescue the translation activity. Indeed, the poliovirus translation activity was almost recovered by the addition of La protein when the amount of I-RNA added was reduced to 50% (Fig. 3*b*). These data support the notion that La protein requirement of HCV IRES is lower than that of poliovirus IRES.

### Immunolocalization of La protein in HCV-infected cells

Subcellular localization of the La protein is mainly in the nucleus, although it has been reported that the La protein shuttles between the nucleus and the cytoplasm (Bachmann *et al.*, 1989*a*, *b*). A time-course of immunostaining of La protein in CV-1 cells after poliovirus infection has demonstrated that the nuclear staining intensity is decreased to some extent 1 h after the infection and is dramatically decreased 3 h after the infection (Meerovitch *et al.*, 1993). At the same time, the La protein concentration increases in the cytoplasm. This kinetics of La distribution after poliovirus infection appear to roughly parallel the switch from cellular translation to poliovirus-specific translation.

In IMY cells infected with poliovirus, the La protein was redistributed to the cytoplasm (compare Fig. 4*d*, *e*, *f* and *g* with *a*, *b* and *c*) as observed in CV-1 cells infected with poliovirus (Meerovitch *et al.*, 1993). An immunolocalization study of La protein in the HCV-infected IMY cells was carried out 7 days after the infection (Fig. 4*h*, *i*, *j* and *k*), when HCV core antigens were detected both in the cytoplasm and the nucleus (Fig. 4*h*) of 20–30% of IMY cells, suggesting that 20–30% of the cells were infected with HCV. Indeed, the antigens were not detected in mock-infected IMY cells by the method employed (data not shown). PCR titres of both the positive- and negative-strand HCV RNA in the cells increased until 14 days after infection (Ito *et al.*, 1996). In these cells, the La antigens are detected mainly in the nucleus (Fig. 4*i*). A merged image (Fig. 4*k*) of Fig. 4 (*h*, *i* and *j*) shows that subcellular localization of the La protein in HCV-infected cells is not different from that in mock-infected cells (Fig. 4 compare *k* with *c*). The results indicate the possibility that subcellular redistribution of IRES-related host factors occurs differently in infected cells, depending on the virus. The reason for the difference in La protein distribution between poliovirus- and HCV-infected cells is not clear at present. The lack of redistribution of La protein to the cytoplasm in HCV-infected cells could reflect inefficiency of HCV replication as compared to poliovirus replication. Our recent experiments suggested that the poliovirus 3C protease cleaved a part of the La protein, that the truncated La protein distributed to the cytoplasm, and that the truncated La protein retained the ability to stimulate translation

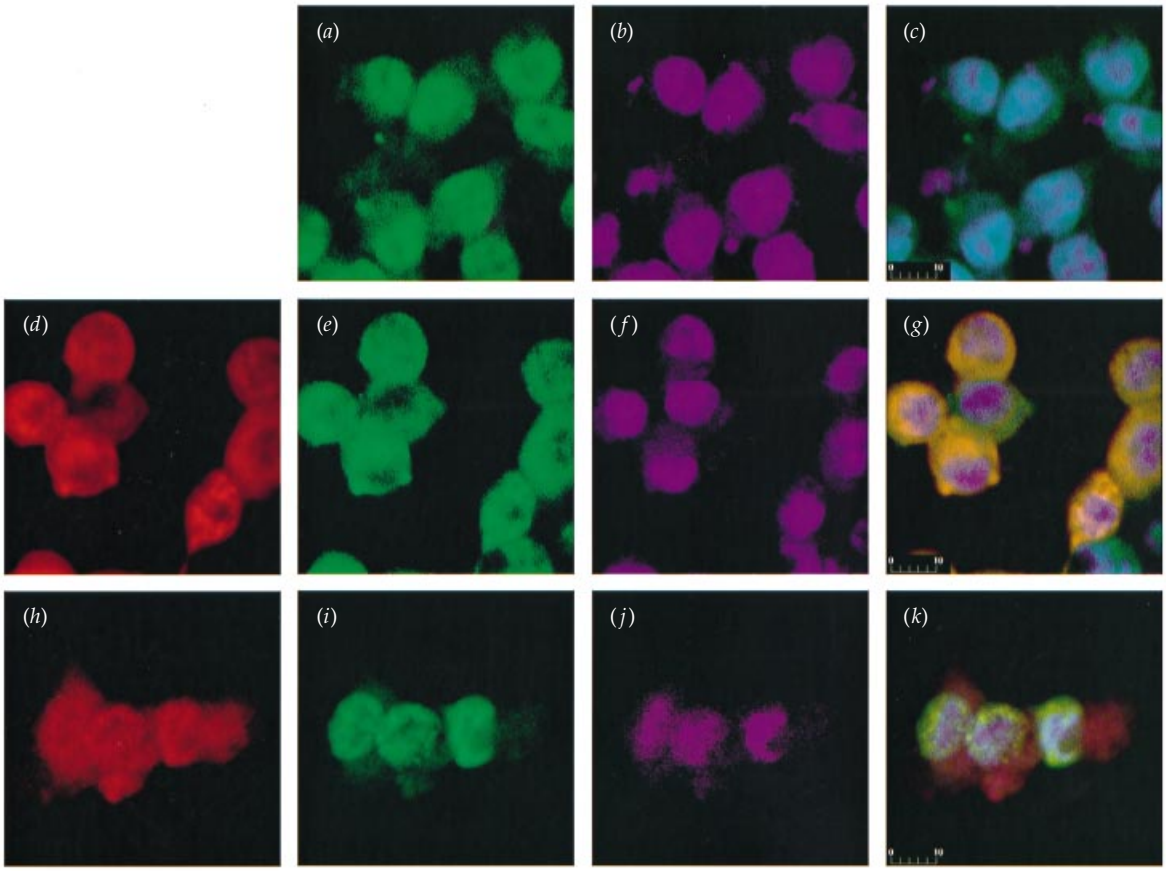


Fig. 4. Immunofluorescence studies on subcellular localization of the La protein. Subcellular localization of La protein was examined in IMY cells infected with poliovirus (*d*, *e*, *f* and *g*) or HCV (*h*, *i*, *j* and *k*). Mock-infected IMY cell culture (*a*, *b* and *c*) was used as a control. Infected cells were fixed 7 h after poliovirus infection or 7 days after HCV infection, and subjected to indirect immunofluorescence studies as described in Methods. Poliovirus proteins (*d*), HCV core protein (*h*) and La protein (*a*, *e* and *i*) were stained by using rabbit anti-poliovirus type 1 serum, rabbit anti-HCV core protein serum and mouse anti-La MAb, respectively. Staining with TOTO-3 iodide is shown in (*b*), (*f*) and (*j*). (*c*) is the merged image of (*a*) and (*b*); (*g*) is of (*d*), (*e*) and (*f*); and (*k*) is of (h), (*i*) and (*j*). Scale bars shown in (*c*), (*g*) and (*k*) represent 10 μm.

initiation driven by poliovirus IRES (Shiroki *et al.*, 1999). This kind of alteration of cellular metabolism may not be induced by HCV infection, and HCV translation may proceed with the low concentration of La protein existing in the cytoplasm.

## Discussion

The La protein requirements of poliovirus IRES and HCV IRES were compared in cell-free translation systems, by using yeast I-RNA and purified recombinant La protein. We employed HeLa S10 and RRL as cell-free systems, in which La protein (p52) is abundant and limiting, respectively (Meerovitch *et al.*, 1989, 1993). Poliovirus IRES activity was reduced by I-RNA in the HeLa S10 system (Fig. 2*a*) (Das *et al.*, 1996), and this reduction of poliovirus IRES activity was significantly recovered by the addition of recombinant La protein (data not shown) (Das *et al.*, 1996). Similar observations were obtained for HCV IRES activity in the RRL system (Figs 2*d* and 3*c*). These results support the previous reports that La protein is required for the IRES activities of poliovirus (Das *et al.*, 1996) and HCV (Ali & Siddiqui, 1997). Our study, however, demonstrated here that the La protein concentration required for HCV IRES is lower than that for poliovirus IRES.

I-RNA-mediated inhibition of poliovirus IRES activity in RRL is hardly restored by the addition of La protein (Fig. 3*a*). As mentioned above, however, poliovirus IRES activity was significantly restored by La protein when the amount of I-RNA added was reduced to 50% (Fig. 3*b*). This may indicate that I-RNA preferentially inactivates La protein even if I-RNA also has an ability to inactivate an additional host factor(s) required for poliovirus IRES. Indeed, I-RNA appeared not to have an affinity for PTB, which is also considered to be involved in translation initiation activities of both poliovirus and HCV IRES (data not shown). These results support the view that I-RNA-mediated translation inhibition in RRL is also mainly due to lowered levels of La protein activity.

In the RRL system, HCV IRES activity was reported to be greatly enhanced by the exogenous addition of La protein (Ali & Siddiqui, 1997). In our case, however, the activity was not enhanced significantly in a similar experiment (data not shown). Ali and Siddiqui used an mRNA which consisted of the HCV 5′UTR (nt 1–341) and the coding region of luciferase mRNA. We used an intact HCV RNA of nt 1–1357 as an mRNA. Since the interaction of La protein with HCV RNA may occur in the context of the initiator AUG (Ali & Siddiqui, 1997), it is possible that the discrepancy results from different structures of mRNAs used. In fact, our preliminary data indicated that the stimulatory effect of exogenous La protein on translation initiation is less evident when longer HCV core coding sequences are inserted at the junction of the HCV 5′UTR and the *β*-galactosidase mRNA coding sequence (data not shown). In addition, the different data might result from, in part, the different assay conditions such as salt concentrations.

Only a part of the nucleotide sequence of mRNA of the rabbit homologue of the human La protein has been elucidated (Chan *et al*., 1989). The alignment of deduced amino acid sequences of La proteins between human and rabbit shows 85·7% identity. The genetic variation of the two La proteins may result in different affinities to I-RNA and IRES. Furthermore, these La proteins may have different activities as IRES-related factors. Thus, it may be impossible to compare directly the data obtained from HeLa S10 and RRL. In any event, both sets of data independently suggest that HCV IRES has a lower La protein requirement than does poliovirus IRES.

Observed differences in La protein requirements between poliovirus IRES and HCV IRES support the notion that individual IRES differ in cellular factor requirements for their activity. Since distribution of the IRES-related cellular factors may differ in tissues and cells, expression of individual IRES may also have specific tissue- and cell-tropisms. This may, in turn, have an affect on the tropism of viruses that have an IRES-dependent translation initiation mechanism. Indeed, IRES-dependent virus tropism has been strongly suggested for poliovirus neurotropism, that is, some of the determinants for neurovirulence (virus replicating capacity in the central nervous system) have been mapped within the IRES region of poliovirus RNA (Evans *et al*., 1984; Kawamura *et al*., 1989; Macadam *et al*., 1991; Horie *et al*., 1994).

La protein was mainly detected in the nucleus of IMY cells producing HCV core antigen (Fig. 4*h*, *i*, *j* and *k*), and was mainly in the cytoplasm of the cells infected with poliovirus (Fig. 4*d*, *e*, *f* and *g*). It is of interest to know that subcellular distribution of an IRES-related cellular factor varies in infected cells, depending on viruses. Redistribution of La protein to the cytoplasm may be advantageous to poliovirus translation and replication. In addition to La protein, cellular factors supporting poliovirus replication that usually exist mainly in the nucleus may also be redistributed to the cytoplasm – the site of poliovirus replication. Subcellular localization of cellular factors for virus replication may influence the efficiency of virus production in infected cells. Therefore, virus-induced alteration of the subcellular localization of cellular factors should be taken in consideration when studying virus tropism.

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