JOURNAL OF VIROLOGY, Feb. 1994, p. 941–950
0022-538X/94/$04.00+0

Vol. 68, No. 2

# The Cellular Polypeptide p57 (Pyrimidine Tract-Binding Protein) Binds to Multiple Sites in the Poliovirus 5′ Nontranslated Region

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Received 8 June 1993/Accepted 9 November 1993

**Initiation of translation of poliovirus RNA by ribosomal entry into an internal segment of the 742-nucleotide (nt)-long 5′ nontranslated region involves *trans*-acting factors, including p57, a 57-kDa polypeptide which has been identified as the pyrimidine tract-binding protein (PTB). A UV cross-linking assay was used to compare the RNA-binding properties of the p57 present in various mammalian cytoplasmic extracts with those of purified murine p57 and recombinant human PTB. Three noncontiguous p57-binding sites were located within the poliovirus 5′ nontranslated region, between nt 70 and 288, and 443 and 539 (domain V), and 630 and 730. With the same assay, a novel 34-kDa polypeptide was identified that bound nt 1 to 629 specifically. A single A→G substitution of nt 480 which attenuates poliovirus did not alter UV cross-linking of p57 to domain V. Although UV cross-linking of p57 to the internal ribosome entry site was specifically reduced by competition with poly(U) but not by competition with poly(C), poly(G), and poly(A) homoribopolymers, the presence of a polyuridine tract was not a sufficient determinant for binding of RNA to the p57 present in cytoplasmic extracts, nor was the polypyrimidine tract downstream of domain V necessary for binding to this site.**

Initiation of translation of poliovirus mRNA is cap independent and results from entry of ribosomes into an internal segment of the 5′ nontranslated region (NTR) (45). This internal ribosomal entry site (IRES) encompasses nucleotides (nt) 135 to 585 of the 742-nt-long 5′ NTR (40) and is highly structured (Fig. 1; 33, 49, 53, 56). Encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus are picornaviruses of the *Cardiovirus* and *Aphthovirus* genera, respectively, and their genomes contain similar IRES elements (e.g., see references 23 and 30).

Poliovirus translation is efficient in HeLa and Krebs 2 cells (46, 47, 60) but is restricted in neural cells in vivo and in rabbit reticulocyte lysate (RRL) in vitro (1, 10, 32). The translation defect can be alleviated either by deletion of the IRES (41) or by supplementation of RRL with ribosomal salt wash (RSW) fractions from HeLa or Krebs cells (10, 61). Poliovirus translation thus depends on the interaction of *trans*-acting factors with the IRES. This interaction may also be an important determinant of poliovirus pathogenesis, since a major determinant of the attenuation phenotype of poliovirus vaccine strains is a substitution in domain V (38) which impairs poliovirus translation in a tissue-specific manner (58, 60, 61). We have begun to identify factors that interact with the poliovirus IRES to elucidate the mechanism of internal ribosomal entry and have thus implicated a 57-kDa RNA-binding protein (p57) in poliovirus translation (19, 24, 46). p57 was first described as binding to the EMCV and foot-and-mouth disease virus IRES elements in a manner which correlated with their ability to direct efficient translation in vitro (3, 4, 25, 34). p57 has recently been found to be identical to the pyrimidine tract-binding protein (PTB) (2a, 19), a member of the heterogeneous nuclear ribonucleoprotein family. cDNAs of four human PTB isoforms and of murine and rat homologs have been sequenced (5, 14, 16, 43).

In this study, we further investigated the role of p57 by purifying it from murine ascites carcinoma Krebs II cells and analyzing some aspects of its RNA-binding properties and by mapping p57-binding sites within the poliovirus 5′ NTR. The biochemical properties of p57 and the nature of its interaction with picornavirus IRES elements are discussed in light of current models for the initiation of translation by internal ribosomal entry.

## MATERIALS AND METHODS

**Plasmid construction and site-directed mutagenesis.** Excision of *Kpn*I-*Esp*I and *Kpn*I-*Sty*I fragments from pBS$^+$VP0 (28) yielded plasmids pIRES(Δ286) and pIRESΔ388), containing PV1(M) nt 287 to 742 and 389 to 742, respectively, downstream of a T3 promoter. Poliovirus residues are numbered in accordance with the revised sequence (27). An *Eco*RI site was introduced at nt 439 by site-directed mutagenesis (31) with uracil-substituted single-stranded DNA derived from pBS$^+$VP0 and the oligonucleotide 3′-CGATGAATTCTTAA GAGGCCGGG-5′. An *Eco*RI-*Bam*HI fragment (corresponding to nt 439 to 670 of the 5′ NTR) was excised from this mutated plasmid and ligated between corresponding restriction sites in pBS$^+$ (Stratagene, La Jolla, Calif.), yielding pIRES(Δ438). pMN22 and pMN25 (41) contain the coding sequence of the PV1(M) capsid protein precursor downstream of nt 670 to 742 and 1 to 742 of the 5′ NTR, respectively. Plasmid pIRES(Δ629)-ΔP1 contains a truncated form of this precursor downstream of PV1(M) nt 630 to 742. It was constructed by ligating an *Msc*I-*Eco*RI fragment derived from pIRESΔP1 (19) between the *Hin*cII and *Eco*RI sites of pBlue-

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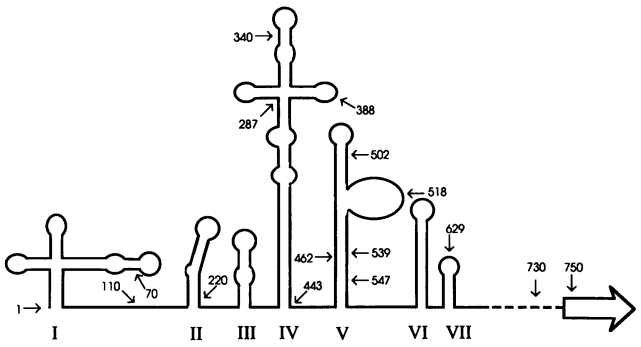


FIG. 1. Schematic representation of the secondary structure of the PV1(M) 5′ NTR (not to scale). Nucleotide residues at the 5′ and 3′ ends of the RNA transcripts used in this study are numbered.

script II (KS$^-$). pBS-βGlobin (19) contains cDNA of the complete β-globin mRNA downstream of a T3 promoter. Plasmid pBS-ECAT contains the entire EMCV IRES (22). Plasmid pE15 contains a full-length PTB-1 cDNA (16) ligated to the blunt-ended *Bam*HI site of pET-3A (Novagen, Inc., Madison, Wis.). It expresses PTB-1 and 12 upstream vector-derived amino acid residues under the transcriptional control of an inducible T7 promoter.

Manipulation of DNA was done by using standard procedures (54). Nucleotide changes were confirmed by sequence analysis using Sequenase in accordance with the manufacturer's directions (U.S. Biochemicals). Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs. Mutagenic oligonucleotides and primers for sequencing, transcription, and PCR amplification were synthesized on an Applied Biosystems 380B DNA synthesizer.

**RNA transcription from PCR-derived DNA templates.** RNAs corresponding to all or part of domain V were synthesized by a PCR technique (46). Oligonucleotides 5′ TTGTAA TACGACTCACTATAGGGTCCTCCGGCCCCTGAA TG-3′ (no. 1) and 5′-CCGGGATCCAAAAGGAAACACG GACACCC-3′ (no. 2) (in which PV-specific residues are underlined) were used to copy the cDNA sequence of PV1(M) from nt 443 to 567 by PCR. Oligonucleotides 1 and 5′-CGGTTCCGCCAC-3′ were used to copy the cDNA sequence of PV1(M) from nt 443 to 539 by PCR. To transcribe an RNA corresponding to nt 443 to 518, the PCR product amplified with primers 1 and 2 was digested with *Bst*UI. Gel-purified restriction fragments were transcribed with T7 polymerase. Equivalent PV1(S) and PV2(S) RNAs were synthesized in the same manner, with plasmids pVS(1)IC-O(T) (29) and p2/T7/SAB2 (51).

**UV cross-linking of RNAs.** UV cross-linking reactions were done as previously described (46). A panel of [$^{32}$P]UTP-labelled RNA probes corresponding to segments of the PV1(M) 5′ NTR were transcribed from the plasmids described above that had been digested with *Bam*HI (nt 220), *Esp*I (nt 286), *Bsm*I (nt 462), *Pfl*MI (nt 502), *Msc*I (nt 629), *Mun*I (nt 730), or *Bsi*HKAI (nt 751) as described in the figure legends. An RNA probe $N_{44}$-$U_{45}$-$N_{91}$ was transcribed with T7 polymerase from pBSβ-globin that had been digested with *Bgl*II. The $U_{45}$ tract is the complementary sequence to the poly(A) tail of β-globin mRNA. An EMCV IRES-specific probe (nt 260 to 488) was transcribed with T7 polymerase from pBS-ECAT. Cytoplasmic S10 extracts were prepared from HeLa S3, Krebs 2 ascites carcinoma, and human SKNSH and NGP neuroblastoma cells (2, 7) as previously described (46). Samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (41) followed by autoradiography; labelling of polypeptides was quantitated by scanning X-ray films with an LKB laser densitometer. Immunoprecipitation of p57 was done as previously described (19).

**Purification of murine p57.** Crude polysomes were prepared from a cytoplasmic S10 extract of Krebs 2 cells (59–61) by centrifugation through a discontinuous sucrose gradient (55). The pellet was resuspended in buffer A (0.25 M sucrose, 5 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 0.1 mM EDTA), and KCl (4 M) was then added slowly with stirring at 0°C to a final concentration of 0.2 M and a minimum final optical density at 260 nm of 100 U/ml. Stirring was continued at 0°C for 30 min. After centrifugation for 2.5 h at 60,000 rpm in a Beckman Ty65 rotor, the pellet of salt-washed ribosomes was resuspended in buffer A and washed with salt as described above, except that the final KCl concentration was 0.5 M. After centrifugation, both this and the previous supernatant (crude ribosomal low- and high-salt wash fractions, respectively) were made 40 mM in Tris-HCl (pH 7.6). Powdered $(NH_4)_2SO_4$ was added slowly to each with constant stirring at 0°C to 25% saturation (17). Stirring was continued at 0°C for 30 min, and a 0 to 25% $(NH_4)_2SO_4$ saturation precipitate was collected by centrifugation for 10 min at 10,000 rpm in a Sorvall SS-34 rotor, dissolved in buffer B (10% [vol/vol] glycerol, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 1 mM dithiothreitol, 0.2 mM EDTA) with 100 mM KCl, and dialyzed overnight at 0°C against the same buffer. The supernatants were used to prepare 25 to 40% and 40 to 70% $(NH_4)_2SO_4$ saturation precipitates in a similar manner.

The 25 to 40% $(NH_4)_2SO_4$ saturation fraction of the 0.2 M KCl RSW resuspended in buffer B with 100 mM KCl was put on a column of DEAE-52 equilibrated with the same buffer. Nonabsorbed protein (DEAE void fraction) and proteins eluted by washing with 2 column volumes of the same buffer were pooled, and step elution was done with buffer B with 0.5 M KCl. The pooled 0.1 M KCl elution fraction was concentrated by ultrafiltration with a Centricon 30 filter (Amicon) and then applied to a phosphocellulose column. Nonabsorbed protein (P-11 void fraction) and proteins eluted by washing with 2 column volumes of the same buffer were pooled, and step elution was done with buffer B, first with 0.25 M KCl and then with 0.4 M KCl. All three pooled fractions were dialyzed overnight against buffer B with 0.1 M KCl and then concentrated by Amicon pressure ultrafiltration. Purification was monitored by determining the protein concentrations of all fractions by the Bradford assay (6) and by determining the optical density at 280 nm; the presence of p57 and other RNA-binding proteins was assayed in all fractions after dialysis against buffer B by UV cross-linking to a [$^{32}$P]UTP-labeled EMCV IRES-specific probe.

**Purification of recombinant human PTB.** Recombinant PTB-1 was overexpressed in *Escherichia coli* BL21 (DE3) that had been transformed with plasmid pE15 and was grown in Luria broth at 37°C to an optical density at 600 nm of 0.8. PTB expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, and incubation was continued with shaking for 3 h at 37°C. PTB-1 was purified as previously described (15), except that the *E. coli* cell paste was lysed by passage three times through a French pressure cell at 10,000 lb/in$^2$. The cell lysate was mixed with NaCl to a final concentration of 0.5 M and centrifuged for 1 h at 30,000 rpm in a Beckman 50.2 Ti fixed-angle rotor at 4°C. The supernatant was dialyzed against buffer B with 100 mM KCl for 4 h at 4°C and put on a DEAE-52 column. Nonadsorbed protein (DEAE void fraction) was pooled, concentrated by Amicon pressure ultrafiltra-

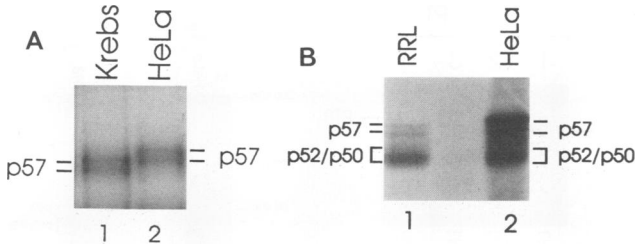


FIG. 2. UV cross-linking of p57 in ascites carcinoma (lanes 1) and HeLa S3 (lane 2) cytoplasmic extracts (A) and RRL (lane 1) and HeLa cytoplasmic extract (lane 2) (B) to [$^{32}$P]UTP-labelled EMCV (nt 260 to 488) RNA transcripts. Proteins were separated by SDS-PAGE on a 10 to 20% gradient gel. p57, p52, and p50 species are indicated.

tion, and put on a poly(U) Sepharose column. The column was washed with buffer B in 0.25 M KCl, and bound PTB-1 was eluted with the same buffer in 0.5 M KCl.

## RESULTS

**Purification of murine p57.** p57 occurs in mammalian tissues such as rabbit reticulocytes, rat liver, and HeLa, baby hamster kidney, and murine ascites carcinoma cells, as well as in human SKNSH and NGP neuroblastoma cells (3, 25, 34; see below). p57 refers collectively to polypeptides that are resolved by SDS-PAGE as a set of two to four closely migrating bands: the major p57 doublets from RRL and murine ascites cells were slightly smaller than the corresponding HeLa doublet (Fig. 2). A UV cross-linking assay indicated that the abundance of p57 was lower in RRL than in HeLa cells (Fig. 2B) but similar in HeLa S3 and ascites carcinoma cells (Fig. 2A).

Purification of p57 from ascites cells was monitored by UV cross-linking to a [$^{32}$P]UTP-labelled EMCV RNA probe and thus involved a functional RNA-binding assay. Cytoplasmic p57 occurs mostly in the RSW, and there is little in the postribosomal fraction (25, 46). Isolation of polysomes from the S10 fraction removed most cytoplasmic proteins and was thus a simple and efficient first step in purification. Similar amounts of p57 were detected in the 0.2 M and 0.5 M KCl RSW fractions (Fig. 3A, lanes 1 to 3 and 4 to 6, corresponding to fractions Ia-Ic and IIa-IIc, respectively), but p57 constituted a higher proportion of both total and RNA-binding proteins found in the 0.2 M KCl RSW fraction, which was therefore used for further purification. Most of the p57 was precipitated at a lower ammonium sulfate saturation percentage (0 to 25%) from 0.5 M KCl RSW fraction IIa (lane 4) than from the 0.2 M KCl RSW fraction Ib (25 to 40%; lane 2), probably because fraction IIa has both higher KCl and protein concentrations than fraction Ib. Chromatography of fraction Ib (Fig. 3B, lane 1) on a DEAE-52 column separated most of the p57 (fraction IIIa; lane 3) from many other RNA-binding proteins (fraction IIIb; lane 2). Some of these proteins were not apparent in fraction Ib prior to this chromatographic step (compare lanes 1 and 2); their appearance could be related to the relative absence of p57 in fraction IIIb, since the high affinity of p57 for the EMCV IRES might normally competitively inhibit binding of proteins that have a lower affinity for the same RNA probe and thereby mask their presence. A higher KCl concentration (0.4 M) was required to elute p57 from a phosphocellulose column than to elute other RNA-binding proteins present in fraction IIIa (lanes 5 and 6). p57 was purified as a doublet of proteins that had very similar mobilities (lane 4). It was the predominant moiety detected by silver staining (data not shown) and was the only distinct RNA-binding protein detected by UV cross-linking to EMCV IRES-specific (Fig. 3b, lane 4; see Fig. 5A, lane 1) and poly(U)-containing RNA probes (see Fig. 5A, lane 2).

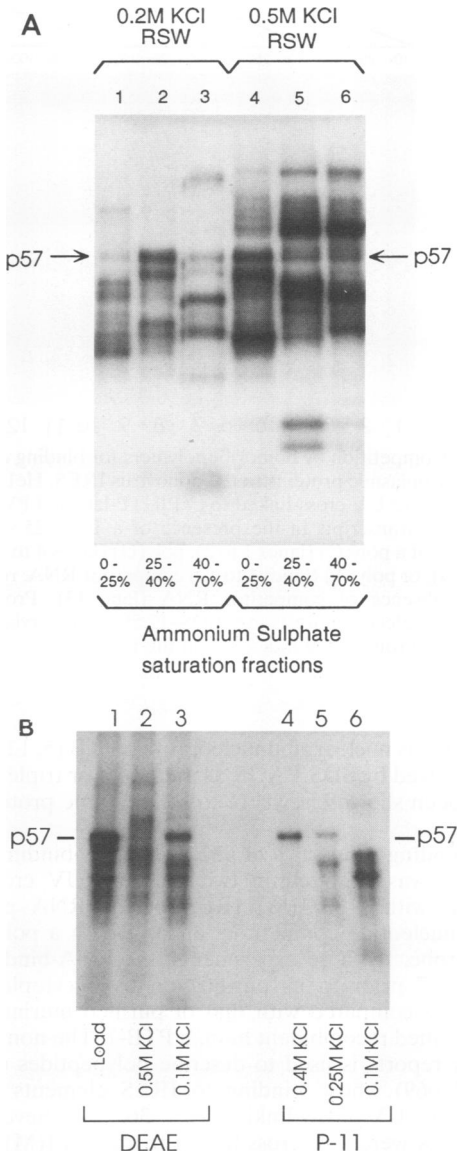


FIG. 3. Purification of p57 from murine ascites carcinoma cells. UV cross-linking of RNA-binding proteins in fractions of ascites carcinoma cells to [$^{32}$P]UTP-labelled EMCV (nt 260 to 488) RNA transcripts. (A) Proteins in the 0 to 25% $(NH_4)_2SO_4$ saturation fraction (lanes 1 and 4), in the 25 to 40% $(NH_4)_2SO_4$ saturation fraction (lanes 2 and 5), and in the 40 to 70% $(NH_4)_2SO_4$ saturation fraction (lanes 3 and 6) of the 0.2 M KCl RSW (lanes 1 to 3) and of the 0.5 M KCl RSW (lanes 4 to 6). (B) Proteins in the 25 to 40% $(NH_4)_2SO_4$ saturation fraction of the 0.2 M KCl RSW prior to loading on a DEAE-52 column (lane 1) and in the 0.5 M KCl eluate (lane 2) and the 0.1 M KCl eluate (lane 3) from this column and in the flowthrough of the 0.1 M KCl eluate from a phosphocellulose column (lane 6) and in the 0.25 M KCl eluate (lane 5) and the 0.4 M KCl eluate (lane 4) from this column. Proteins were separated by SDS-PAGE on 10 to 20% gradient gels. p57 species are indicated.

The elution profiles of p57 from DEAE-52 and phosphocellulose matrices described above closely resemble those of

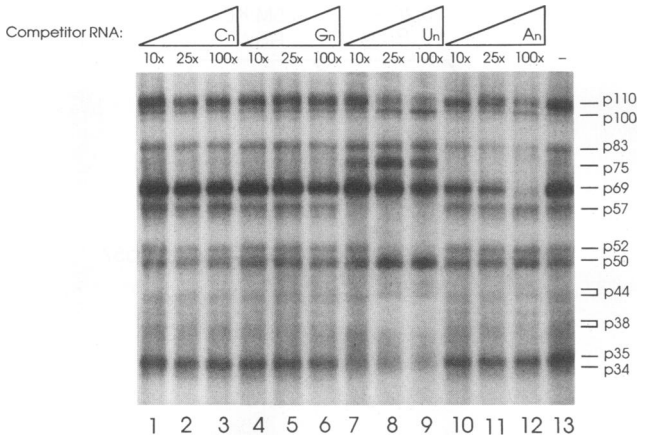


FIG. 4. Competition by homoribopolymers for binding of p57/PTB and other cytoplasmic proteins to the poliovirus IRES. HeLa cytoplasmic extracts were UV cross-linked to [$^{32}$P]UTP-labelled PV1(M) (nt 1 to 629) RNA transcripts in the presence of a 10×, 25×, or 100× molar excess of a poly(C) (lanes 1 to 3), poly(G) (lanes 4 to 6), poly(U) (lanes 7 to 9), or poly(A) (lanes 1 to 12) competitor RNA, respectively, or in the absence of competitor RNA (lane 13). Proteins were separated by electrophoresis on SDS–12.5% polyacrylamide gels. RNA-binding proteins are indicated on the right.

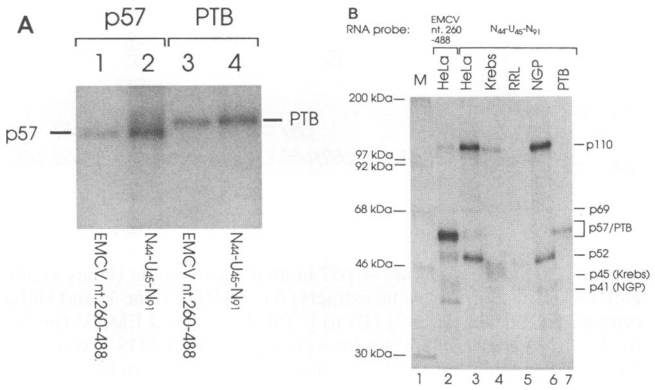


FIG. 5. Binding of p57 and PTB to EMCV IRES-specific and polyuridine-containing RNAs. (A) UV cross-linking of purified murine p57 (lanes 1 and 2) and recombinant human PTB-1 (lanes 3 and 4) to [$^{32}$P]UTP-labelled RNAs EMCV nt 260 to 488 (lanes 1 and 3) and $N_{44}$-$U_{45}$-$N_{91}$ (lanes 2 and 4). (B) UV cross-linking of purified recombinant PTB (lane 7) and RNA-binding proteins present in RRL (lane 5) and cytoplasmic lysates of HeLa S3, Krebs 2 ascites carcinoma, and NGP neuroblastoma cells (lanes 3, 4, and 6, respectively) to a [$^{32}$P]UTP-labelled ($N_{44}$-$U_{45}$-$N_{91}$) probe. HeLa cytoplasmic proteins UV cross-linked to EMCV nt 260 to 488 were in lane 2; $^{14}$C-labelled molecular size (M) markers were in lane 1. Proteins were separated by electrophoresis on an SDS–10 to 20% polyacrylamide gradient gel (A) and an SDS–13.5% polyacrylamide gel (B). p57, PTB, and RNA-binding proteins p110, p69, p52, p45, and p41 are indicated.

heterogeneous nuclear ribonucleoprotein PTB (5, 12, 43). PTB is also resolved by SDS-PAGE as a doublet or triplet of bands and has been shown elsewhere to be the same protein as p57 (2a, 19).

**RNA-binding properties of p57.** The RNA-binding specificity of p57 was analyzed in two different UV cross-linking assays: (i) with a PV1(M) IRES-specific RNA probe and oligoribonucleotide competitors and (ii) with a poly(U)-containing probe. In these experiments, the RNA-binding specificity of p57 prior to its purification from cytoplasmic S10 extracts was compared with that of purified murine p57 and that of purified recombinant human PTB-1. The nomenclature of earlier reports is used to describe polypeptides (p50, p52, p57, and p69) whose binding to IRES elements has been detected by UV cross-linking (25, 36, 46). Several other polypeptides were UV cross-linked to the PV1(M) probe in addition to these four species. They included 34-, 35-, 38-, 44-, 75-, 83-, 100-, and 110-kDa moieties, which we have termed p34, p35, p38, p44, p75, p83, p100, and p110, respectively (Fig. 4).

UV cross-linking of p57 in a HeLa S10 extract to PV1(M) nt 70 to 629 was strongly reduced by inclusion of a 10-fold molar excess of poly(U) in the preincubation mixture (Fig. 4, lane 7) and was further reduced by increasing molar excesses of this competitor (lanes 8 and 9). UV cross-linking of p110, p52, the larger component of the p44 doublet, p38, p35, and p34 to this probe was also reduced by competition with poly(U) (lanes 7 to 9). This result is consistent with identification of p52 as the La autoantigen (37) which binds oligo(U) tracts (57). We confirmed the identity of p52 and La by immunoprecipitation and found that neither p50 nor any of the other polypeptides described above is immunologically related to La (data not shown). Competition with poly(U) reduced binding of p57 more than binding of p52 or p110 to the IRES. The prominence of a 75-kDa species increased in the presence of the poly(U) competitor, which may be the result of alleviation of a masking phenomenon of the type discussed above. Cross-linking of p57 was not significantly influenced by competition with poly(C), poly(G), or poly(A), even when present in 100-fold molar excess (lanes 1 to 3, 4 to 6, and 10 to 12, respectively). However, p69 was barely detectable and UV cross-linking of p83 was reduced in the presence of a 100-fold molar excess of poly(A) (lane 12). UV cross-linking of purified recombinant PTB to the same RNA probe was strongly reduced by competition with both poly(U) and poly(C) (data not shown), which is consistent with its known RNA-binding properties (43). The strong reduction in UV cross-linking of PTB in the presence of a poly(C) competitor contrasts markedly with the minor effect of this competitor on UV cross-linking of the p57 in cytoplasmic extracts to the same probe. This result suggests that cytoplasmic extracts contain a factor whose affinity for poly(C) exceeds that of p57.

The RNA-binding specificities of purified recombinant PTB and purified murine p57 were indistinguishable in a UV cross-linking assay. Both proteins were labelled more intensely after UV cross-linking to a [$^{32}$P]UTP-labelled RNA transcript containing a poly(U) ($U_{45}$) tract than after UV cross-linking to an equivalent input of an EMCV probe with a similar length (nt 260 to 488) (Fig. 5A). This difference is probably due to the greater specific radioactivity of the former probe. Labelled p57 has a greater electrophoretic mobility than PTB-1, reflecting both the authentic differences between human and murine p57/PTB (Fig. 1B) (5) and the presence of 12 vector-derived amino acid residues at the amino terminus of recombinant PTB. The same $U_{45}$-containing RNA probe was UV cross-linked to the p52 and p110 present in RRL and in cytoplasmic extracts of HeLa, NGP, and ascites carcinoma cells (Fig. 5B, lanes 2 to 5). Less p52 and p110 was detected in RRL than in these other cell types. Additional 45- and 41-kDa species were detected in extracts of ascites carcinoma and NGP cells, respectively. Significantly, little or none of the p57 detected in these four extracts by UV cross-linking to an EMCV RNA probe was UV cross-linked to the $U_{45}$-containing probe, although the identical probe had previously been shown to be

readily UV cross-linked to purified p57 from murine ascites carcinoma cells. These results indicate that p57 has a greater binding specificity for poly(U) than for other homoribopolymers but that the presence of a polyuridine tract in an RNA molecule is not a sufficient determinant for its binding to p57. The ease with which p57 was displaced from the PV1(M) IRES by competition with poly(U) is consistent with earlier reports of its relatively low affinity for this element (34, 46).

**Binding of p57 to the poliovirus 5′ NTR.** p57 is one of several cytoplasmic proteins detected by UV cross-linking to PV1(M) nt 70 to 629 (46). A panel of overlapping [$^{32}$P]UTP-labelled RNAs was used in UV cross-linking assays to localize the p57-binding site(s) within the PV1(M) 5′ NTR. p57 was cross-linked to nt 70 to 629 (Fig. 6A, lane 7) and more weakly to some fragments of this probe, such as nt 70 to 286, 389 to 629 (lanes 3 to 5), and 437 to 629 (data not shown). The identity of this polypeptide was confirmed by immunoprecipitation with affinity-purified polyclonal PTB antiserum (Fig. 6B). p57 was not labelled by UV cross-linking to nt 1 to 220 (Fig. 6A, lane 2), 542 to 629, 559 to 624, or 670 to 751 (36, 46), and although polypeptides of about 57 kDa were faintly labelled after cross-linking to nt 287 to 460 (lane 5), they were not immunoprecipitated with PTB antiserum. Some other polypeptides bound specifically to fragments of the 5′ NTR. For example, p34/p35 (which were not resolved in this gel) were labelled by cross-linking to nt 70 to 629 and 389 to 629 probes (Fig. 6A, lanes 4, 5, and 7) but not to probes derived from nt 1 to 460 of the 5′ NTR (lanes 2, 3, and 6).

The specificity of binding of p57 to these IRES fragments was confirmed with purified recombinant PTB-1 and equimolar amounts of some of the RNA probes described above. PTB was not UV cross-linked to nt 1 to 220, 70 to 220, 287 to 462, and 287 to 502 probes (Fig. 6C, lanes 2, 3, 7, and 8). However, it bound to nt 70 to 286, 70 to 386, 70 to 630, 389 to 629, 389 to 670, and 437 to 629 probes (lanes 4 to 6 and 9 to 11). PTB was also cross-linked to two probes corresponding to residues downstream of the IRES (nt 630 to 730 and 630 to 750; lanes 12 and 13).

The relative prominence of p57 and p34 increased when RNA transcripts were extended from nt 1 to 629 to nt 1 to 730 (Fig. 7A, lanes 1 and 2). UV cross-linking of p34 and p57 was virtually abolished, and the prominence of several other proteins (including p83, p69, p52, and p50) was strongly reduced by inclusion of a fivefold molar excess of an unlabelled nt 1 to 629 transcript in the preincubation mixture (lane 4). The prominence of p110 was least affected by this competitor. Conversely, competition with 3 μg of rRNA strongly reduced cross-linking of all of the polypeptides except p57 and p34 to the nt 1 to 751 probe (lane 5). p34 thus binds transcripts corresponding to nt 1 to 629, 1 to 730, and 1 to 751 of PV1(M) but not those corresponding to nt 630 to 730 or 630 to 751 (see below). Binding of p34 to the IRES is specific, since it is reduced by competition with RNA containing a cognate binding site but not by a heterologous competitor such as rRNA. The enhanced UV cross-linking of p57 that resulted from an increase in RNA transcript length from nt 1 to 629 to nt 1 to 730 suggested that nt 630 to 730 influences binding of p57 to the 5′ NTR, even though they are downstream of the IRES. p57 was labelled by UV cross-linking to nt 630 to 730 and 630 to 751 probes (Fig. 7, lanes 6 and 7), albeit weakly. p34 was not cross-linked to these two probes, but p110, p83, p69, and p52 and 50 all became strongly labelled (lanes 6 to 9). UV cross-linking of p57 to the nt 630 to 751 probe was readily reduced by competition with increasing concentrations of unlabelled PV1(M) (nt 1 to 629) transcripts (5- and 10-fold molar excesses in lanes 8 and 9, respectively). Similar reductions in the prominence of all of the other polypeptides except p110 were caused by the presence of this competitor (lane 9). UV cross-linking of p57 to the nt 630 to 751 probe was unaffected by competition with 2 μg of rRNA, whereas that of the other proteins (except p110) was strongly reduced by such

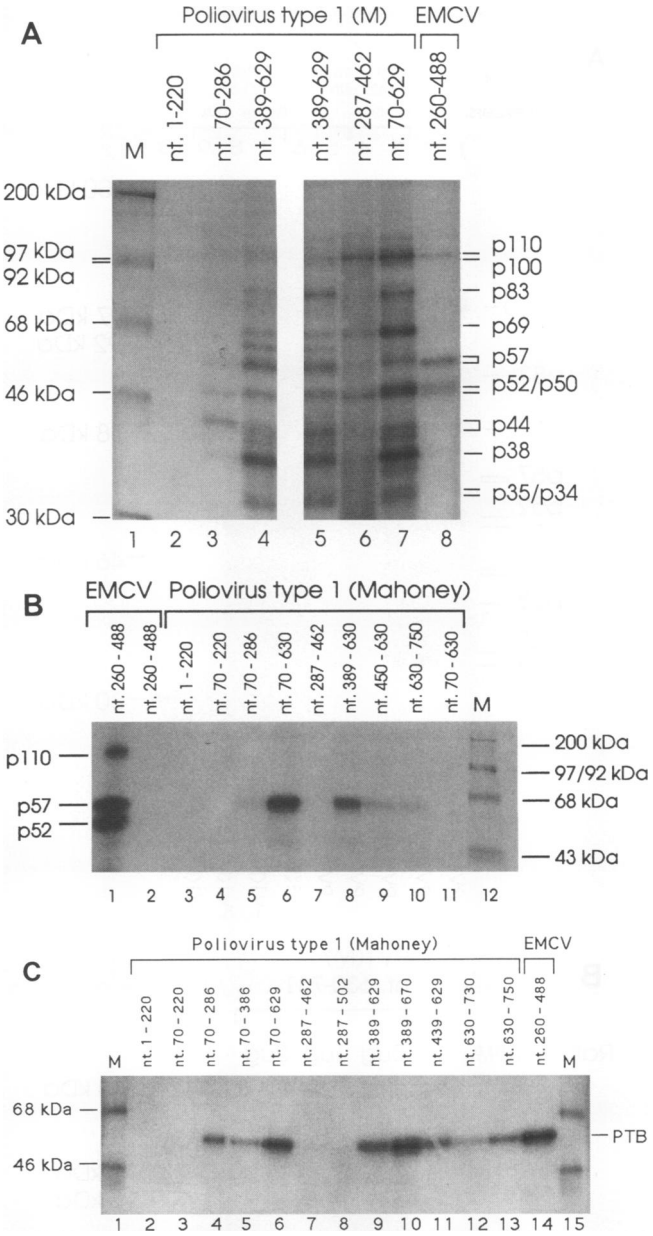


FIG. 6. UV cross-linking of a panel of [$^{32}$P]UTP-labelled RNA probes derived from the IRES elements of EMCV and PV1(M) to cytoplasmic HeLa proteins (A and B) and purified recombinant human PTB-1 (C) in the presence of 3 μg of rabbit rRNA. Labelled cytoplasmic HeLa proteins were immunoprecipitated with affinity-purified PTB antibodies (B; lanes 2 to 10) or with preimmune serum (lanes 2 and 11). Proteins were separated by SDS-PAGE on a 10 to 20% polyacrylamide gradient gel (A and C) and a 12.5% polyacrylamide gel (B). The molecular sizes of protein markers (M) (A, lane 1; B, lane 12; C, lanes 1 and 15) and labelled RNA-binding proteins are indicated on all three panels.

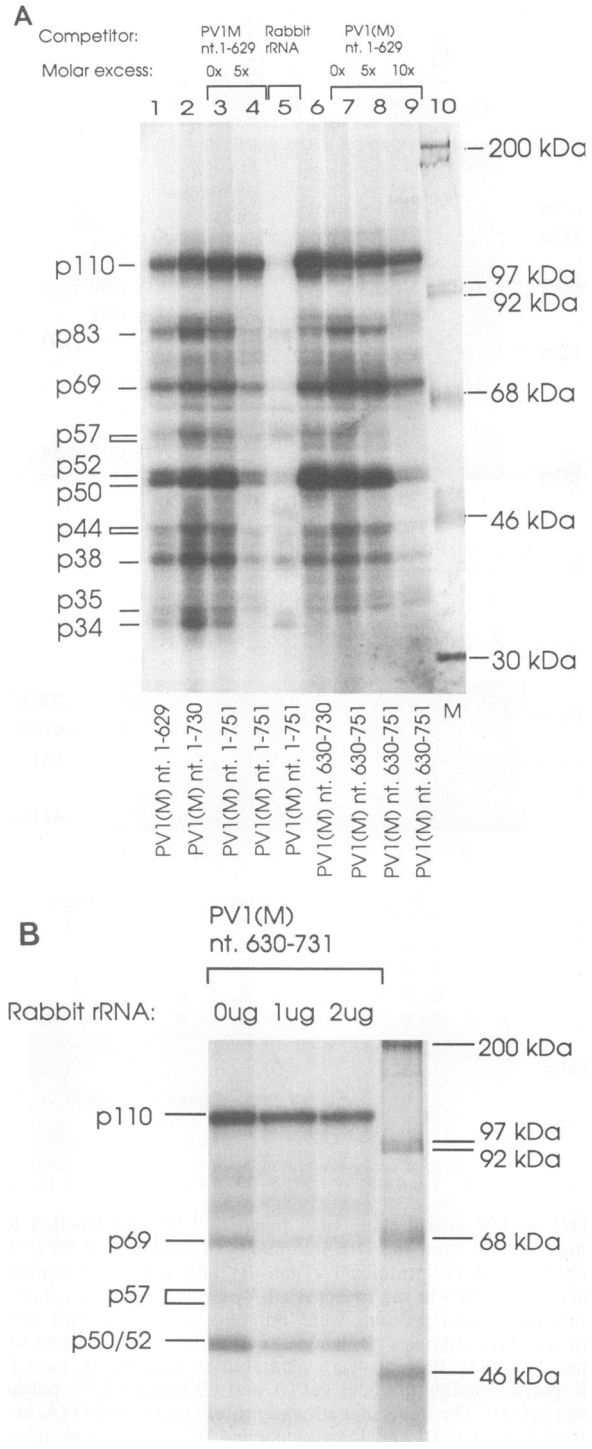


FIG. 7. Binding of p34 and p57 to segments of the poliovirus 5′ NTR. UV cross-linking of HeLa cytoplasmic proteins to [$^{32}$P]UTP-labelled RNA transcripts corresponding to nt 1 to 629 (lane 1), 1 to 730 (lane 2), 1 to 751 (lanes 3 to 5), 630 to 730 (lane 6), and 630 to 751 (lanes 7 to 9) in the absence of competitor RNA (lanes 1, 2, 3, 6, and 7), in the presence of 5-fold (lanes 4 and 8) and 10-fold (lane 9) molar excesses of unlabelled PV1(M) (nt 1 to 629) RNA, or in the presence of 3 μg of rabbit rRNA (lane 5).

competition (Fig. 7B). UV cross-linking of p57 to nt 630 to 730 of the PV1(M) 5′ NTR is therefore specific.

In summary, three p57-binding sites have been identified within the pV1(M) 5′ NTR. The central binding site encompasses domains V and VI of the 5′ NTR, the 5′-proximal site is located between nt 70 and 286, and the 5′-distal site lies between nt 630 and 730.

**Binding of p57 to domain V of the PV1(M) 5′ NTR.** PV1(M) domains V and VI contain the central p57-binding site. Three 5′ coterminal RNA probes (nt 443 to 567, 443 to 539, and 443 to 518; Fig. 8A) were used to define the borders of this site more precisely. The longest transcript (nt 443 to 567) bound p57 to the virtual exclusion of other proteins (Fig. 8B, lane 1). The pattern of protein binding to an equivalent PV2(S) transcript was similar, although p44, p50, p52, p69, and p110 were more prominent (lane 3). Domain V and flanking regions thus contain the central p57-binding site, and domain VI is not required for p57 binding. Truncation of both the PV1(M) and PV2(S) probes (to nt 518 and 524, respectively) virtually eliminated their ability to be UV cross-linked to p57 and concomitantly increased labelling of p44, p50, p52, p69, and p110 (Fig. 8B, lanes 2 and 4). The loss of nt 519 to 567 deleted the 3′ basal segment and most of the large internal loop of domain V, as well as part of the downstream pyrimidine-rich tract (Fig. 5A). Shorter RNAs [PV1(M) nt 443 to 539 and equivalent PV1(S) and PV2(S) transcripts] bound to p57 in cytoplasmic HeLa cell extracts (Fig. 8C, lanes 1 to 3) and in an RRL RSW fraction (lanes 4 to 6), indicating that the pyrimidine-rich tract is not essential for binding of p57 but that the integrity of the 3′ basal segment of the interrupted stem in domain V may be important. The affinity of p57 for these truncated RNAs may be reduced, since p57 constituted a smaller proportion of RNA-binding proteins labelled by UV cross-linking to the shorter (nt 443 to 539) than to the larger (nt 443 to 567) transcripts (compare Fig. 8B, lanes 1 and 3, with Fig. 8C, lanes 1 to 3).

**Binding of p57 from neuroblastoma cells to the PV1(M) IRES.** A point mutation in domain V is a major determinant of the attenuation phenotype of Sabin strains of poliovirus (38). In poliovirus type 1, the attenuating point mutation resides in an A→G substitution at nt 480 (Fig. 8A). UV cross-linking assays revealed no significant differences in the binding of p57 from HeLa cells to RNA transcripts derived from PV1(M), PV1(S), and PV2(S), but the tissue-specific expression of attenuating mutations suggested that differences might be apparent when cytoplasmic extracts from neuroblastoma cells were used. Labelled RNA transcripts corresponding to domains V of PV1(M), PV1(S), and PV2(S) were UV cross-linked to p57 from cytoplasmic S10 extracts of SKNSH cells to the same extent (Fig. 8B, lanes 5, 7, and 9). However, the prominence of p57 as a proportion of all labelled RNA-binding proteins was lower when SKNSH cells were used as a source of cytoplasmic proteins than when HeLa cells were used. Neuroblastoma cells may contain less p57 than do HeLa cells; alternatively, p57 from these cells may have a lower affinity for domain V than does p57 from HeLa cells. Binding of the p57 in the neuroblastoma cell extract to truncated RNA transcripts [nt 443 to 518 in PV1(M) and equivalent residues in PV1(S) and PV2(S)] was strongly reduced (Fig. 8B, lanes 6, 8, and 10), exactly as in HeLa cells (Fig. 8C).

## DISCUSSION

RNA-binding polypeptide p57 has been implicated in the initiation of picornavirus translation (2a, 3, 4, 19, 25, 34, 46). In the present study, p57 was purified from the cytoplasm of

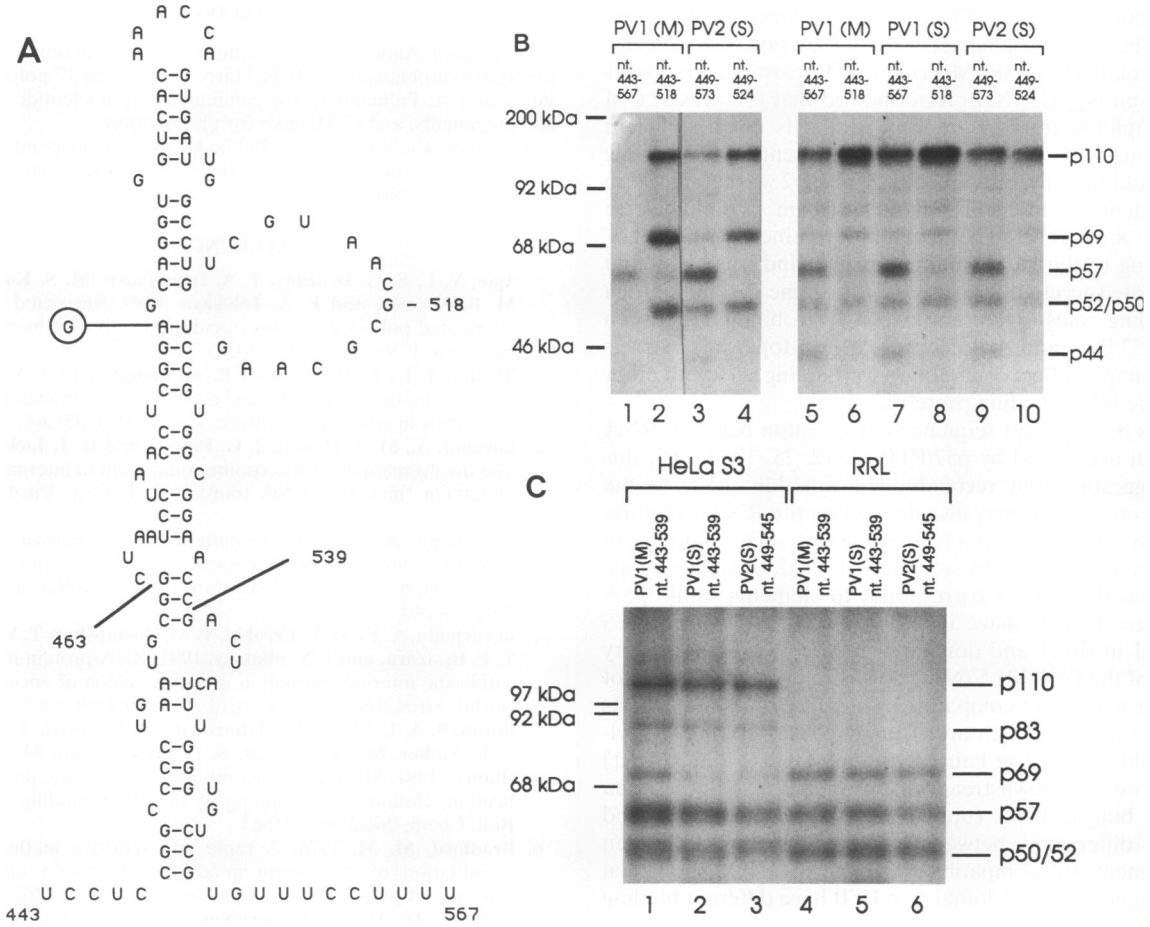


FIG. 8. Binding of p57 to domain V of the 5′ NTRs of neurovirulent and attenuated strains of poliovirus. (A) Schematic representation of the secondary structure of domain V of the PV1(M) 5′ NTR. (B) UV cross-linking of cytoplasmic proteins from HeLa S3 (lane 1 to 4) and human neuroblastoma SKNSH (lanes 5 to 10) cells to [$^{32}$P]UTP-labelled RNA transcripts that correspond to nt 443 to 567 (lanes 1 and 5) or 443 to 518 (lanes 2 and 6) of PV1(M), to nt 443 to 567 (lane 7) or 443 to 515 (lane 8) of PV1 (S), or to nt 449 to 573 (lanes 3 and 9) or 449 to 524 (lanes 4 and 10) of PV2 (S). (C) UV cross-linking of cytoplasmic proteins from HeLa S3 cells (lane 1 to 3) and from the RSW fraction of RRL (lanes 4 to 6) to [$^{32}$P]UTP-labelled RNA transcripts that correspond to nt 443 to 539 (lanes 1 and 4) of PV1(M), to nt 443 to 539 (lanes 2 and 5) of PV1 (S), or to nt 449 to 545 (lanes 3 and 6) of PV2 (S). Proteins were separated by SDS-PAGE on a 13% polyacrylamide gel. Cellular RNA-binding proteins p110, p83, p69, p57, p52, p50, and p44 are indicated on the right, and $^{14}$C-labelled molecular size markers are indicated on the left.

murine ascites carcinoma cells and its RNA-binding properties were investigated.

UV cross-linking to labelled RNA probes suggests that the poliovirus 5′ NTR contains three noncontiguous p57-binding sites, comprising all or part of nt 70 to 286 (site 1, which encompasses domains II and III), 443 to 539 (site 2; domain V), and 630 to 730 (site 3, downstream of the 3′ border of the IRES). The poliovirus IRES therefore resembles other picornavirus IRES elements in containing multiple p57-binding sites (4, 34, 46) which could either be bound to different p57 molecules or be brought into close proximity by secondary or tertiary interactions within the IRES and bound to a single p57 moiety. The latter mode of interaction is feasible, since biochemical and phylogenetic evidence indicates that IRES elements contain extensive secondary structure (11, 33, 48, 49, 52, 53, 56) and genetic evidence supports the possibility of a tertiary interaction between sites 1 and 2 in the PV1(S) 5′ NTR (29). Moreover, p57 has been identified as PTB (2a, 19) and sequence analysis of this polypeptide has revealed four repeated domains which can be aligned with RNA recognition motif consensus sequences found in other RNA-binding proteins (26). Proteins containing multiple RNA-binding domains can interact with several RNA sequences simultaneously, and individual domains can have distinct binding specificities (8, 42). An interaction of this type might stabilize a specific tertiary conformation of an IRES element. Numerous proteins have been identified that can be UV cross-linked to the poliovirus IRES (13, 46; this report). Competition with rabbit rRNA readily reduced UV cross-linking of most of these polypeptides, but p34 appeared to bind nt 1 to 630 of the PV1(M) 5′ NTR specifically.

PTB was initially identified by UV cross-linking to the polypyrimidine tract upstream of the 3′ splice site in pre-mRNAs, although it is now apparent that its binding to pre-mRNAs cannot be predicted solely on the basis of pyrimidine content (12, 39, 43). Purified p57 was efficiently UV cross-linked to poly(U)-containing RNA (Fig. 5A), and binding of both cytoplasmic p57 and purified recombinant PTB to the poliovirus IRES was specifically reduced by competition with a small molar excess of poly(U) (Fig. 4). Greater molar excesses of polypurine homoribopolymers did not compete with p57 for binding to the RNA. These results confirm that

the interaction between p57 and the poliovirus IRES is specific, but the facile displacement of p57 by poly(U) indicates that it is relatively weak. Moreover, UV cross-linking assays done with an $N_{44}$-$U_{44}$-$N_{91}$ probe showed that the presence of a polypyrimidine tract in an RNA probe is not a sufficient determinant for its binding to the p57 present in cytoplasmic extracts. Additionally, binding of p57 to site 2 was found not to be dependent on the adjacent downstream pyrimidine-rich tract. p52 and p110 were labelled prominently after UV cross-linking to the $U_{44}$-containing probe, and it is likely that they compete successfully against p57 for some pyrimidine-rich RNA-binding sites. Differences in RNA binding between purified p57/PTB and the p57 present in cytoplasmic extracts may thus simply reflect competition for binding sites with other cytoplasmic RNA-binding proteins.

There is no apparent sequence conservation between RNA probes that are bound by p57/PTB (4, 12, 25, 39, 43, 65; this work), suggesting that recognition is not due solely to the primary sequence but may also depend on the RNA structure. It has been suggested that a p57-binding site may comprise an RNA helix interrupted by an internal single-stranded bulge (4), and this description corresponds to elements within p57-binding sites that we have identified in the poliovirus IRES (domain II in site 1 and domain V in site 2). The secondary structure of the PV1(M) 5′ NTR downstream of nt 620 has not been determined, but comparative sequence analysis suggests that nt 622 to 638 (which are strongly conserved in enteroviruses) could form a 7-nt hairpin with a UGGCC loop (Fig. 1) and that residues downstream of this element could form an internally bulged helix (unpublished data). Sequence and structural differences between p57-binding sites within an IRES element are compatible with the idea that individual RNA recognition motif domains in PTB have different binding specificities.

Mutations within domains II, III, and V have detrimental effects on poliovirus translation and viability, suggesting that residues within these domains are important either for interaction with *trans*-acting factors or to maintain secondary or tertiary structures and thus to ensure their recognition by such factors (9, 18, 35, 44, 50, 63, 64). These domains constitute two p57-binding sites, and the effects of mutations within them on p57 binding are being investigated. A major determinant of the attenuation phenotype of Sabin strains of poliovirus is located within domain V, but differences in UV cross-linking with p57 between RNA probes derived from neurovirulent and attenuated poliovirus strains were not apparent.

The finding that nt 630 to 730 of the 5′ NTR constitute a p57-binding site is the first indication of a specific function for this segment of the 5′ NTR. It is unexpected, for although the length of this spacer is conserved in polioviruses (52, 62), these residues can be deleted without detrimental effect on the ability of the 5′ NTR to promote translation initiation by internal ribosomal entry (40, 45), do not have an equivalent in the closely related rhinovirus 5′ NTRs (24), and are absent in two independently isolated pseudorevertant polioviruses (18, 50). However, retention of this spacer may confer a selective advantage on poliovirus in some circumstances. Replication of type 1 Sabin and Mahoney polioviruses lacking nt 600 to 727 is slightly impaired, since they formed slightly (ca. 20%) smaller plaques than did the wild-type virus in monkey kidney cells and were significantly less neurovirulent than the parental strains (20). Similar results have been reported for coxsackievirus type B1 (21).

## ACKNOWLEDGMENTS

We thank J. Almond and A. Nomoto for gifts of plasmids, B. Semler for NGP neuroblastoma cells, K. Harris for purified T7 polymerase, T. Pöyry and A. Palmenberg for communicating nucleotide sequences and alignments, and C. Helmke for photography.

This work was supported by Public Health Service grants AI 32100 and AI 15122 from the National Institute of Allergy and Infectious Diseases of the NIH.

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