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Oxidation–reduction sensitive interaction of a cellular 50-kDa protein with an RNA hairpin in the 5′ noncoding region of the poliovirus genome

(RNA gel retention assay/RNA footprinting/α-sarcin/diamide)

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ABSTRACT **Genetic and biochemical analyses of the 5′ noncoding region of poliovirus have indicated the importance of this region in both translation and amplification of the viral RNA. The role of the cellular machinery required for these events is just beginning to be revealed. Using an RNA gel retention assay, we have identified a cellular 50-kDa protein that forms a specific complex with a stable stem–loop structure present in the viral 5′ noncoding region. The formation of the RNA–protein complex is dependent on the availability of free sulfhydryl groups in the protein. The possible involvement of this RNA–protein complex in the regulation of viral gene expression is discussed.**

Poliovirus, a member of the Picornaviridae family, is a plus-stranded RNA virus that multiplies in the cytoplasm of the infected human or monkey host cell. The 7500-nucleotide (nt) RNA molecule encodes a 220-kDa polyprotein that is cleaved by virally encoded proteases to yield the processed structural and nonstructural proteins (1). The 5′ and 3′ noncoding regions of the viral RNA contain sequences that mediate efficient translation and positive-strand and negative-strand amplification. Although certain sequence elements involved in these events have been identified by site-directed mutagenesis of an infectious cDNA clone (reviewed in ref. 2), the identification of cellular and viral proteins that bind to these sequences has only recently been actively pursued. For example, a sequence element in the 747-nt 5′ noncoding region from nt 320–631 has been found to bind cellular ribosomes internally, explaining the unusual cap-independent translation of the viral RNA (3). Subsequently, cellular proteins have been identified that bind to this region and are being examined for their role in mediating internal ribosome binding (4, 5).

We have used an RNA gel retention assay to identify RNA–protein interactions between the 5′ noncoding region of the poliovirus genome and viral or cellular proteins. We report here an interaction between a cellular, membrane-associated 50-kDa protein and an RNA hairpin present between nt 186 and 221 in the 5′ noncoding region of poliovirus RNA.

MATERIALS AND METHODS

Cells and Viruses. Propagation of HeLa cells and protocol for the preparation of type 1 Mahoney poliovirus stocks have been described (6).

Construction of Recombinant Plasmids. A subclone of the 5′ noncoding region (pGEM7ZpNB42) containing poliovirus sequences nt 178–224 was constructed by first digesting plasmid pGEM3p5′M (7) with *Nci* I and repairing the 5′ protruding single-stranded termini with Klenow enzyme. Subsequently, the linear plasmid DNA was digested with *Bam*HI, and the excised 46-base-pair fragment was isolated and ligated into a pGEM7Z plasmid vector (Promega Biotec), which had been digested with *Sma* I and *Bam*HI.

Preparation of Cell Extracts. Cytoplasmic extracts were prepared from both uninfected and poliovirus-infected HeLa cells as described (8). The RNA-binding activity was partially purified from uninfected HeLa cell extracts. Briefly, solid ammonium sulfate was added to the cytoplasmic extract to a concentration of 60% (wt/vol). After centrifugation (10,000 × *g* at 4°C for 20 min) the supernatant was dialyzed against 2 liters of binding buffer [25 mM KCl/5 mM Hepes, pH 7.6/2 mM $MgCl_2$/0.1 mM EDTA/3.8% (vol/vol) glycerol/2 mM dithiothreitol] including 10% glycerol at 4°C for 12 hr. The dialyzed fraction was loaded onto a diethylaminoethyl-52 column (Whatman) and RNA-binding activity was eluted from the column with binding buffer containing 100 mM KCl.

Gel Retention Assay. A gel retardation assay was developed by a modification of the assay used by Konarska and Sharp (9). Briefly, ^{32}P-labeled RNA molecules (1 pmol; 10^8 cpm/μg), made *in vitro* by T7 RNA polymerase, were incubated in 10 μl (total volume) of binding buffer into which extracts from uninfected or poliovirus-infected HeLa cells had been added. Seventy micrograms of total protein was present in each binding reaction mixture unless otherwise specified. Poly(A,C,U) (Sigma) or *Escherichia coli* 16S and 23S ribosomal RNA (Boehringer Mannheim) was added at a 250-fold molar excess over radiolabeled RNA to adsorb nonspecific RNA binding proteins. Incubations were carried out at 30°C for 30 min. Subsequently, free RNA and RNA–protein complexes were separated by electrophoresis on 4% acrylamide/bisacrylamide (60:1) gels containing 0.5% glycerol for 2 hr at 30 mA in a Tris borate buffer (10). Gels were dried and subjected to autoradiography.

Chemical and Enzymatic Probing of Free RNA and RNA–Protein Complexes. RNA molecules were labeled at their 3′ termini with [5′-^{32}P]pCp and T4 RNA ligase to a specific activity of 10^7 cpm/μg (11), and full-length RNA molecules were purified by gel electrophoresis. Labeled RNA molecules were incubated either with binding buffer or with 4 μg of protein from the 100 mM KCl DEAE-52 eluate. For digestions with α-sarcin, the nuclease was added to the reaction mixtures to a final concentration of 0.2 μM and was incubated in the absence or presence of protein extract at 30°C for 30 and 120 min, respectively. Digestions with nuclease T1 were performed at 1.6×10^{-4} units/μl in the

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Abbreviation: nt, nucleotide(s).

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absence or presence of protein extract at 30°C for 5 and 120 min, respectively. Reactions were stopped by the addition of EDTA followed by phenol extraction and collection of the RNA by ethanol precipitation. Samples were analyzed on 8% acrylamide/7 M urea gels. Chemical probing of RNA by dimethyl sulfate was performed as described (12).

RESULTS

A 50-kDa Cellular Protein Binds to a 46-nt Sequence in the 5′ Noncoding Region of the Poliovirus RNA Genome. We have used an RNA gel retention assay to detect interactions of the 5′ noncoding region of poliovirus with cellular proteins. Small radiolabeled RNA molecules representing various regions of the 747-nt 5′ noncoding region of the poliovirus RNA were synthesized *in vitro* by T7 RNA polymerase. These RNA molecules were mixed with extracts obtained from uninfected or poliovirus-infected cells and their electrophoretic mobility was analyzed by PAGE. The formation of RNA–protein complexes was detected by a slower migration of the complexed RNA as compared to that of the unbound radiolabeled RNA species.

Using this assay, we observed an extremely stable and specific RNA–protein complex when we incubated the first 224 nt of the viral RNA with detergent-solubilized, cytoplasmic extracts from either uninfected or infected human HeLa cells (data not shown). This RNA–protein complex could be abolished by addition of RNA molecules containing sequences from nt 1 to 224, but not from nt 1 to 178, suggesting that nt 178–224 sequences are involved in the formation of this RNA–protein complex (data not shown).

To test whether nt 178–224 sequences were sufficient to bind the cellular protein, we incubated radiolabeled RNA molecules containing nt 178–224 with extracts obtained from uninfected cells. The gel retention assay (Fig. 1) showed that the RNA molecules containing nt 178–224 bound the cellular protein at protein extract concentrations identical to those required to bind the nt 1–224-containing RNA molecule (Fig. 1, lane 1). The nt 178–224 RNA–protein complex could not be abolished by addition of RNA molecules containing viral nt sequences 1–178 (lane 2), 224–380 (lane 4), 380–517 (lane 5), 517–627 (lane 6), 517–697 (lane 7), or 627–743 (lane 8). However, addition of RNAs containing nt sequences 178–224 (lane 3), 1–627 (lane 9), or the entire (1–7500) full-length viral RNA (lane 10) abolished the complex.

These findings showed that a 46-nt RNA element (nt 178–224) was sufficient to bind specifically to a cellular protein-containing factor. This RNA–protein complex could be efficiently eliminated by competition with the viral genome, implying that virion RNA as well contains functional determinants necessary for binding the cellular factor. In addition, competition experiments with other segments of the poliovirus 5′ noncoding region (Fig. 1) showed that this cellular factor is distinct from a recently identified cellular protein (p52) that binds specifically to nt 559–624 in the viral genome (4).

Next, we wanted to determine the size of the cellular protein that bound to the 46-nt viral RNA sequence element. To this end, we incubated radiolabeled nt 178–224 RNA with a partially purified cellular extract containing the RNA-binding activity. Solutions containing the RNA–protein complex were exposed to UV light to crosslink the RNA to any bound proteins. Subsequently, the RNA in irradiated and unirradiated complexes was partially digested with ribonuclease A, such that some radioactively labeled nucleotides would remain crosslinked to the bound protein. ^{32}P-labeled proteins were then visualized by SDS/PAGE and autoradiography (Fig. 2 *Left*). Proteins with molecular masses of approximately 100, 50, and 32 kDa were labeled by RNA binding in the absence of any unlabeled competitor RNA (lane 1, +UV). Addition of specific competitor RNA (nt 178–224) to the binding reaction mixture eliminated the binding of the 50-kDa and 32-kDa proteins but not the 100-kDa protein (lane 2). In contrast, addition of nonspecific competitor RNA (nt 1–178) did not inhibit the binding of the 50-kDa protein (lane 3) and had little effect on the binding of the 100-kDa protein. The enhanced binding of an 80-kDa protein in this reaction is presently not understood. Taken together, these findings indicate that the 50-kDa protein alone displayed specific binding to viral nt 178–224 sequences. Interestingly, we observed that the same polypeptides remained stably bound to the radiolabeled RNA during SDS/PAGE even without UV illumination (Fig. 2 *Left*, −UV lanes), indicating that they form extremely stable interactions with the labeled RNA.

To demonstrate that the cellular 50-kDa protein was indeed involved in the gel retention of the nt 178–224 fragment, the UV-crosslinking experiment was repeated with gel-purified complexes. Specific RNA complexes were formed and separated by PAGE. The gel was then first exposed to UV light, the RNA–protein complexes were excised from the gel matrix, treated with ribonuclease A, and subjected to SDS/PAGE to identify the size of the protein present in the complex. Fig. 2 *Right* shows that the predominant protein in retarded RNA–protein complexes was a single polypeptide. The size of the protein was ≈50 kDa, similar or identical to that of the protein that bound to the RNA in solution. Thus, the cellular 50-kDa protein binds specifically to nt 178–224 sequences and is present in the complex causing the RNA–protein complex observed in Fig. 1.

The 50-kDa Cellular Protein Interacts with a Stem–Loop Structure in the 5′ Noncoding Region of the Poliovirus RNA. Based on computer analysis and phylogenetic conservation, a stem–loop structure has been predicted between nt 185 and 221 in the 5′ noncoding region of poliovirus RNA (13, 14). We examined the potential interaction of the 50-kDa protein with the predicted RNA hairpin structure by enzymatic and chemical probing of free and protein-bound RNA molecules.

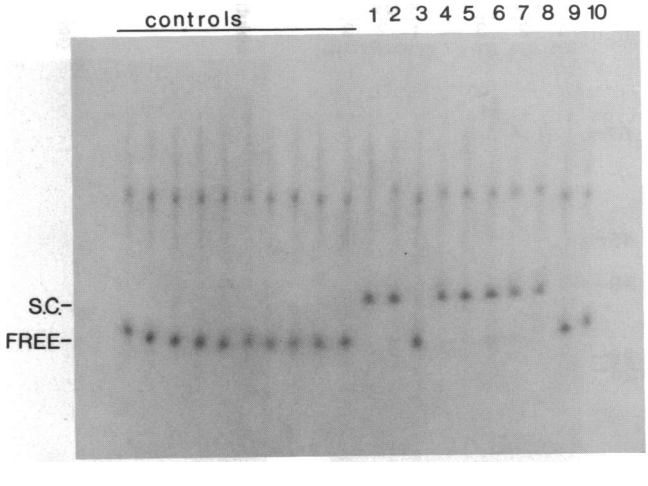

FIG. 1. Interaction of a cellular protein with nt 178–224 sequences in the poliovirus RNA. Radiolabeled RNA molecules (nt 178–224) were incubated in the absence (controls) or in the presence (lanes 1–10) of uninfected HeLa cell extracts. A 20-fold molar excess of unlabeled competitor RNA molecules (over labeled RNA) was added and the migration of unbound (free) RNA and specific RNA–protein complexes (S.C.) was analyzed by PAGE. An autoradiograph is shown. Reaction mixtures contained the following competitor RNA molecules: no competitor RNA (lane 1), nt 1–178 (lane 2), nt 178–224 (lane 3), nt 224–380 (lane 4), nt 380–517 (lane 5), nt 517–627 (lane 6), nt 517–697 (lane 7), nt 627–743 (lane 8), nt 1–627 (lane 9), nt 1–7498 (lane 10). The slower-migrating, radiolabeled RNA was derived from transcription of longer, incompletely digested DNA templates, which are retarded in a similar pattern.

The structures of free and protein-bound RNAs were first examined with nucleases T1 and α-sarcin. T1 cuts 3′ of guanidines and α-sarcin cuts on the 3′ side of most, but not all, purine and occasionally pyrimidine nucleotides present in single-stranded RNA sequences (15, 16). RNA molecules containing viral nt 178–224 sequences were 3′ end-labeled (11) and partially digested with T1 or α-sarcin in the absence or presence of partially purified 50-kDa protein. The digestion products were analyzed on urea-containing sequencing gels (10). An autoradiograph of such an experiment is shown in Fig. 3. Digestion of free (lanes 1 and 3) and protein-bound RNAs (lanes 2 and 4) with T1 (lanes 1 and 2) and α-sarcin (lanes 3 and 4) revealed that nucleotides *G-22, U-193, G-203, and G-211 are protected from nuclease cleavage upon protein binding, while nt *G-50, G-181, G-184, A-185, and A-218 displayed enhanced susceptibility to cleavage. The asterisks denote nucleotides derived from nonviral vector sequences. It is important to note that the nuclease-treated RNAs (lanes 1–4) have slightly reduced mobilities (18) compared with the corresponding RNAs in the sequencing lanes (A, G, C, U). This is due to a lack of 5′ phosphoryl residues and, as a consequence, they exhibited a reduced mobility (18) compared with RNAs that underwent the chemical sequencing reactions and therefore bear a 5′ terminal phosphate (17). Nuclease cleavage sites that were >2-fold enhanced or diminished in the presence of the RNA binding protein, as determined by densitometric scanning of several autoradiographs, are marked in Fig. 3. Cleavage by either T1 or α-sarcin at nt G-203, for example, was diminished at least 25-fold in the presence of protein.

The results of these enzymatic and additional chemical probing reactions (data not shown) are diagrammed in Fig. 4. The computer-predicted RNA hairpin (13, 14) is displayed in the bottom part of Fig. 4. The locations of the single-stranded A residues, sensitive to modification by dimethyl sulfate at the N-1 position (12) and the sites of cleavage by the single-stranded nucleases T1 and α-sarcin support this predicted secondary structure for the free RNA.

Alterations in the pattern of nuclease reactivity for the viral sequences in the presence of partial purified cellular extract are also shown in Fig. 4. Nucleotide G-203, for example, was protected from T1 and α-sarcin cleavage in the presence of extracts and is part of the loop of the hairpin structure. In summary, our studies indicated that the interaction of the cellular 50-kDa protein with the hairpin structure predominantly protected from nuclease cleavage this single nt in the loop and 2 nt in the bulge of the RNA hairpin. Furthermore,

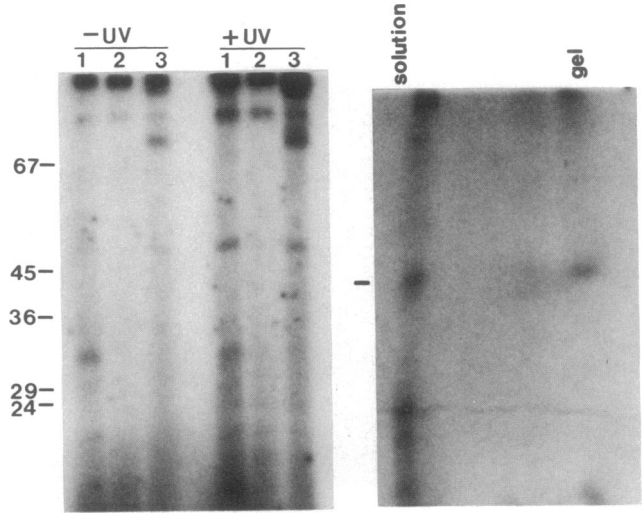

FIG. 2. (*Left*) Detection of cellular proteins tightly bound to nt 178–224 sequences in poliovirus RNA. Samples were mock illuminated (−UV) or exposed to UV light (+UV) as described in the text and were analyzed by SDS/PAGE. An autoradiograph is shown. Lanes: 1, no competitor RNA; 2, competitor RNA (nt 178–224); 3, competitor RNA (nt 1–178). The positions of molecular size markers (kDa) separated on the same gel are indicated on the left. (*Right*) UV crosslinking of a cellular 50-kDa protein to nt 178–224 in the poliovirus RNA. The experimental protocol is described in the text. An autoradiograph of an SDS gel is shown. The bar indicates the migration of a marker protein of 50 kDa. The labeled bands at the top of the autoradiographs are likely to result from nuclease-resistant aggregates that failed to enter the gels.

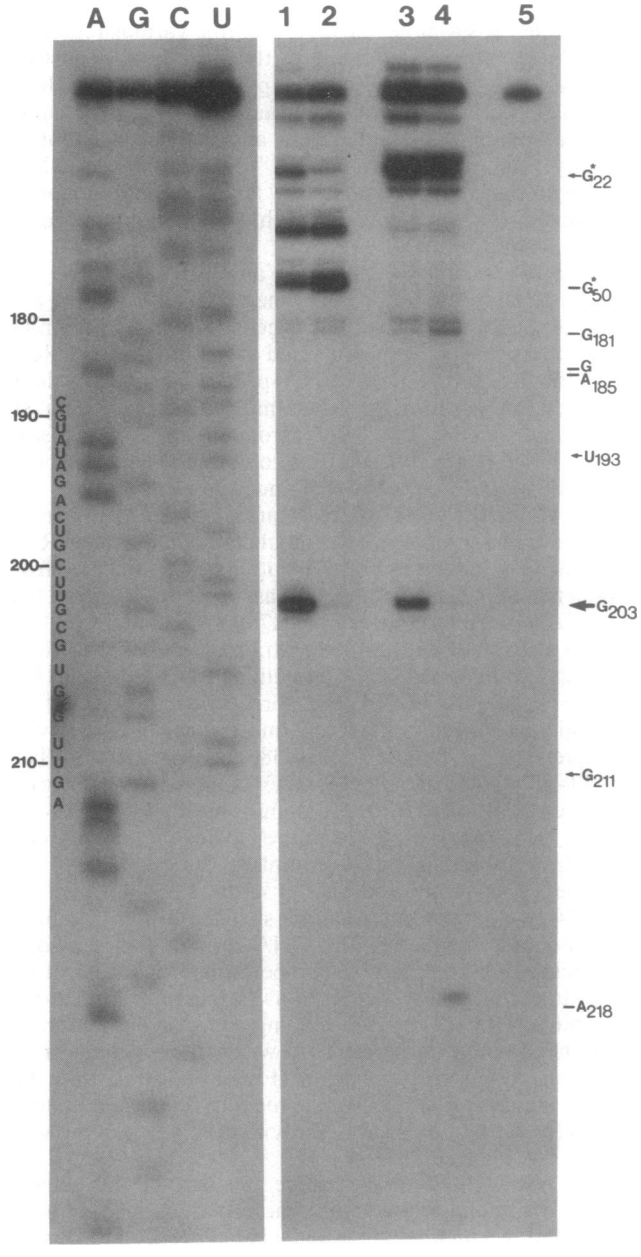

FIG. 3. RNA footprinting analysis using nuclease T1 and α-sarcin. RNA molecules (nt 178–224), labeled at their 3′ ends with ^{32}P (lane 5), were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a partially purified HeLa cell extract and were cleaved with T1 (lanes 1 and 2) or α-sarcin (lanes 3 and 4). Sequencing reactions (A, G, C, U) of the labeled RNA were performed (17). Reaction products were analyzed by PAGE and subjected to autoradiography. Nucleotides derived from nonviral vector sequences are denoted with an asterisk and are numbered according to Promega Biotec (1990 catalogue). The two panels are from different exposures of the same gel.

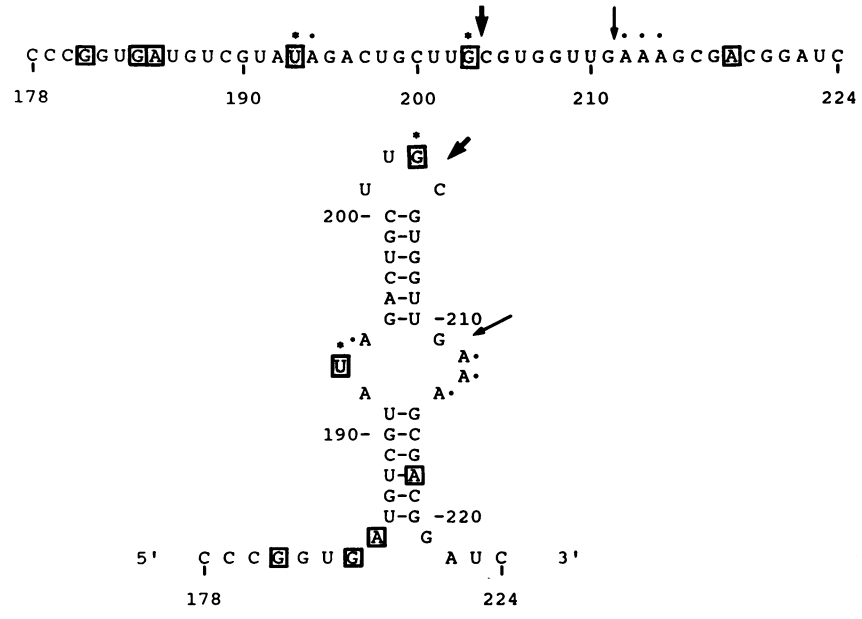

FIG. 4. (*Upper*) Summary of chemical and enzymatic probing of nt 178–224 sequences in the 5′ noncoding region of poliovirus type 1. •, Modification by dimethyl sulfate; ↓, cleavage by T1; □, enhanced reactivity; □̇, diminished reactivity to α-sarcin in the presence of protein. (*Lower*) Computer-predicted RNA hairpin between nt 178 and 224. Only viral sequences are shown.

the presence of extract created several enhanced nuclease cleavage sites at the bottom of the stem in the hairpin structure.

The 50-kDa Protein Contains Cysteine Residues That Are Essential for RNA Binding. Next, we examined the importance of sulfhydryl groups in the formation of the RNA–protein complex, using the diazene carbonyl derivative diamide (19) to oxidize the free sulfhydryl groups. We chose diamide because the induced oxidization is fully reversible by reducing agents such as 2-mercaptoethanol or dithiothreitol. Fig. 5 shows the effect of diamide on RNA–protein complex formation in the gel-retention assay. Neither 25 mM diamide (lane 3) nor 95 mM 2-mercaptoethanol (lane 2) affected the migration of the radiolabeled free nt 178–224 RNA template (lane 1). Addition of 95 mM 2-mercaptoethanol to the partially purified cellular extract did not inhibit RNA–protein complex formation (compare lane 4 with lane 5). In contrast, treatment of the extract with 25 mM diamide for 10 min completely inhibited the RNA–protein interaction (lane 6). This inhibition could be reversed by incubation of the diamide-treated extract with 95 mM 2-mercaptoethanol for an additional 10 min prior to addition of the RNA (lane 7). Treatment of preformed RNA–protein complexes with 25 mM diamide did not abolish gel retention (data not shown). These findings suggest that the RNA binding protein or proteins contain sulfhydryl groups that must be in a reduced state for successful RNA–protein complex formation.

DISCUSSION

We have identified a cellular 50-kDa protein that interacts with a 46-nt sequence element (nt 178–224) in the 5′ noncoding region of the poliovirus RNA. The 50-kDa protein is unlikely to be one of the known translational initiation and elongation factors, because antibodies directed against initiation factors eIF-4A, eIF-2, and eIF-3, and elongation factor EF-1α failed to recognize the protein (data not shown). Protein binding protected a single nt in the loop and 2 nt in the bulge of a predicted RNA hairpin from recognition by the nucleases α-sarcin and T1. The RNA binding protein probably interacts directly with the loop structure, because a single nt change in the loop of the hairpin (U-202 to A-202) severely inhibited RNA–protein complex formation (unpublished observation). In addition, the binding of the 50-kDa protein to the RNA was dependent on the oxidation–reduction status of the protein. Similar regulation by a "sulfhydryl switch" has been observed recently in the interaction of the iron-responsive elements in ferritin and transferrin mRNAs with their binding protein (20).

The RNA–protein complex was remarkably stable upon treatment with 1 M KCl, 1 M urea, and 1 M EDTA (data not shown) and was partially stable during electrophoresis in denaturing SDS/PAGE (Fig. 2). The formation of highly stable nucleic acid–protein complexes that require free sulf-

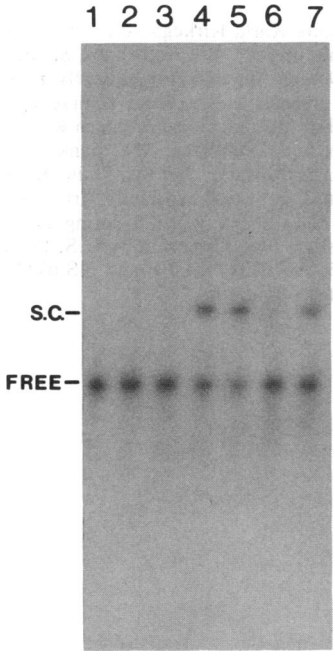

FIG. 5. Oxidation–reduction sensitivity of RNA–protein complex formation. Details are described in the text. An autoradiograph of a polyacrylamide gel is shown. Electrophoretic migrations of unbound RNA (free) and of specific RNA–protein complex (S.C.) are indicated.

hydryl groups in the nucleic acid binding protein have been observed (21–25). It has been suggested that these high-affinity nucleic acid–protein complexes are stabilized by the formation of a transient covalent bond, referred to as a Michael adduct (26), between the nucleic acid and the protein. It has been postulated that a cysteine of the protein performs a nucleophilic attack on the C-6 carbon of either a purine or a pyrimidine nucleotide residue. The proposed single-stranded loop in the RNA sequence studied here contains one cytidine and two uridine residues, any of which could potentially form a transient covalent bond with the RNA binding protein. As mentioned previously, we have observed that replacement of U-202 by an A residue severely inhibited RNA–protein complex formation.

What is the physiological function of the 50-kDa poliovirus RNA complex? The nucleotide sequences comprising the stem–loop structure have been shown to be involved in both translation and replication of poliovirus RNA (reviewed in refs. 2 and 27). Interestingly, Dildine and Semler (28) reported the isolation of a poliovirus mutant, R2, that contained a 45-nt deletion between nt 184 and 228, thereby deleting the predicted RNA hairpin between nt 185 and 221. Preliminary evidence has indicated that no further binding site for the cellular 50-kDa protein was created in R2 (L.N., B. L. Semler, and P.S., unpublished observation). Therefore, the RNA hairpin (nt 185–221) seems to be dispensable for viral growth in human tissue culture cells. However, it should be pointed out that revertant R2 shows a dramatic delay in viral RNA replication compared with wild-type poliovirus (28). We noted that the extraction of the 50-kDa protein from a crude cytoplasmic extract is strictly dependent on the presence of the nonionic detergent Nonidet P-40, indicating that the 50-kDa protein may be membrane associated. Since both replication and translation of poliovirus RNA occur on cellular membranes (1), it is possible that the 50-kDa protein provides an anchor for the membrane association of the viral RNA. To test this hypothesis, we are examining the intracellular fate of both hybrid and polioviral RNA molecules containing the RNA sequences conferring specific binding to the cellular 50-kDa protein.

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