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Evidence for Involvement of *trans*-Acting Factors in Selection of the AUG Start Codon during Eukaryotic Translational Initiation

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The molecular mechanism with which an appropriate AUG codon is selected as the start site for translational initiation by eukaryotic ribosomes is not known. By using a cell-free translation system, small RNA molecules containing single AUG codons, surrounded by various nucleotide sequences, were tested for their abilities to interfere with the translation of a reporter mRNA. RNAs containing the AUG in an ACCAUGG context (Kozak consensus sequence) were able to inhibit translation of the reporter mRNA. In contrast, RNAs containing the AUG in a less favorable context for start site selection (for example, CAGAUGC) had no effect on the translation of the reporter mRNA. The effect mediated by the ACCAUGG-containing RNAs was not due to sequestration of ribosomal subunits or to particular structural features in these RNAs. To identify potential *trans*-acting factors that might be preferentially bound by ACCAUGG-containing RNAs, ACCAUGG- and CAGAUGC-containing RNAs with a single 4-thiouridine residue at the AUG were incubated with partially fractionated extracts, and AUG-binding proteins were identified after irradiation of the complexes with UV light and subsequent analysis by gel electrophoresis. The analysis of such complexes in competition experiments revealed that proteins, approximately 50 and 100 kDa in size, were found to bind directly at the AUG codon embedded in the ACCAUGG motif. One of these proteins has been identified as the La autoantigen. These findings indicate that *trans*-acting factors may play a role in AUG start site selection during translational initiation.

In eukaryotic cells, protein synthesis is initiated by binding of 40S ribosomal subunits to mRNA molecules at or near their capped 5′ ends (28, 33). The 40S subunits, carrying the initiator Met-tRNA$_i$ and certain translation initiation factors such as eukaryotic initiation factors eIF2 and eIF3 (33, 34), subsequently scan the mRNA in a 5′-to-3′ direction until an appropriate AUG is encountered; this AUG is used as the start site for protein biosynthesis (24, 28). The AUG triplet that is used as the start site is influenced by the base composition of its adjacent nucleotides. In vertebrates, efficiently used AUG start codons are embedded in the sequence motif 5′-CC(A/G)C CAUGG-3′ (26), in which the purines at positions −3 and +4 (the A in the AUG is designated +1) seem to be most critical. In addition, more recent experiments have shown that the presence of an adenosine residue at the +5 position helps in the selection of the start AUG codon (16). Interestingly, the presence of purines at position −3 but not at +4 seems to characterize efficient AUG codons in *Saccharomyces cerevisiae* (5′-AAAAUGU-3′) (5) and in *Drosophila melanogaster* (5′-AAAAUGN) (3).

Experiments with the yeast *S. cerevisiae* have identified several players in translational start site selection. First, it was shown that an AGG codon could be used efficiently as a start codon if the anticodon sequence in the initiator tRNA was mutated to CCU and the methionyl-tRNA synthetase *MES1* gene contained a particular point mutation (6, 10). Thus, codon-anticodon interactions are crucial in start site selection, although components of the translation apparatus also have a direct influence. This was demonstrated with the isolation of yeast suppressor strains in which methionine was inserted at non-AUG initiator codons. These suppressor strains contained mutations in the genes encoding the α (*SUI2*) (7) or β (*SUI3*) (11) subunits of eukaryotic translation initiation factor eIF2. This provided the first evidence that 40S subunit-associated factors were involved in start site selection. In addition, a single point mutation in *SUI1* (44) mediated selection of a UUG triplet as a start codon in the absence of an AUG start codon in *HIS4* mRNAs. The *SUI1* gene is distinct from the genes encoding eIF2 subunits and has been suggested to cooperate with eIF2 in AUG start site selection. Although these experiments point to a role of the initiator tRNA and certain translation initiation factors in start site selection, the mechanism of start site selection remains unknown.

In multicellular eukaryotes, the sequences surrounding the start site AUG codon determine the efficiency with which the AUG is used as a start codon. The presence of a purine in position −3 is most important for efficient AUG usage; for example, replacing the −3 purine with a pyrimidine diminishes the AUG usage by approximately fivefold (25). In the absence of a −3 purine, the presence of a guanosine at position +4 is essential for efficient AUG usage (25). Although interactions between the 5′-CC(A/G)CCAUGG-3′ sequences in the mRNA and specific regions located in 18S rRNA have been postulated (25, 37), experimental evidence for this interaction has not been provided. Thus, the mechanism of recognition of the start site AUG codon, surrounded by particular bases, is not clear. It is likely that start site AUG recognition is accomplished by 40S subunit-associated factors such as the initiator tRNA and eIF2. In addition, it is also possible that RNA-binding proteins interact specifically with the 5′-CC(A/G)C

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TABLE 1. RNAs containing single AUG codons embedded in various sequence contexts

Type	Competitor RNA[a] Sequence (−3 +4)	Assay[b]
C	GGGAGACCGCGGAACACGCACAGCAG**ACCAUGG**GAGACGCGAACCCC	UV
	GGGAGACCGCGGAACACGCACAGCAG**ACCAUGG**GAGACGCGAACCCCGGGUACCGAGCUCGAAUU	TRLN
	GGGAGACCGCGGAACACGCACAGCAG**ACCAUGG**GAGACGCGAACCCCGGGUAAUCACUAGUGCGGCC	TRLN
GUG	GGGAGACCGCGGAACACGCACAGCAG**ACCGUGG**GAGACGCGAACCCCGUACCGAGCUCGAAUU	TRLN
NC1	GGGAGACCGCGGAACACGCACAGCAG**C**AA**AUGA**CACGCGAACCCC	UV
	GGGAGACCGCGGAACACGCACAGCAG**C**AA**AUGA**CACGCGAACCCCGGGUACCGAGCUCGAAUU	TRLN
NC2	GGGAGACCGCGGAACACGCACAGCAG**C**AG**AUGC**ACGACGCGAACCCC	UV
	GGGAGACCGCGGAACACGCACAGCAG**C**AG**AUGC**ACGACGCGAACCCCGGGUACCGAGCUCGAAUU	TRLN
	GGGAGACCGCGGAACACGCACAGCAG**C**AG**AUGC**ACGACGCGAACCCCGGGUAAUCACUAGUGCGGCC	TRLN
NC3	GGGAGACCGCGGAACACGCACAGCAG**C**GA**AUGG**ACCGACGCGAACCCC	UV
	GGGAGACCGCGGAACACGCACAGCAG**C**GA**AUGG**ACCGACGCGAACCCCGGGUACCGAGCUCGAAUU	TRLN
NC4	GGGAGACCGCGGAACACGCACAGCA**C**AG**AUGG**ACGACGCGAACCCCGGGUACCGAGCUCGAAUU	TRLN
pG3	GGGAGACCGGAAUUCGAGCUCGGUACCCGGGAUCCUCUAGAGUCCA	TRLN

[a] The AUG start codon and the nucleotides at positions −3 and +4 are in boldface. The AUG and the stop codons are underlined. pG3 (pGEM3) is an RNA molecule lacking AUG codons.

[b] The assays in which the competitor RNAs were employed are indicated. UV, UV-cross-linking assay; TRLN, translation competition assay.

CAUGG-3′ sequence element, marking the embedded AUG as a start codon.

We explored this latter possibility, i.e., that additional protein factors are involved in designation of the 5′CC(A/G)CC AUGG3′ sequence element as the start site for protein synthesis. We present data that suggest that *trans*-acting factors are involved in recognizing the 5′-CC(A/G)CCAUGG-3′ motif as a translational initiation site in a cell-free translational extract prepared from human HeLa cells. Furthermore, using site-specific UV cross-linking, we have identified HeLa cell proteins, approximately 50 and 100 kDa in size, which interact specifically with the AUG codon in a context-dependent manner. The 50-kDa La autoantigen was identified as one of the proteins that interact with the start site AUG codon. This finding is discussed with respect to the recent findings that La is involved in the modulation of the translational efficiencies of two different viral mRNAs.

MATERIALS AND METHODS

Plasmid constructions. Plasmids that could direct the synthesis by T7 RNA polymerase of RNA molecules that contained only one uridine residue at the AUG were constructed. Plasmid pUC18C (consensus AUG) and plasmid series pUC18NC (nonconsensus AUG) directed RNAs containing an AUG codon embedded in the Kozak consensus sequence, ACCAUGG, and in a variety of nonconsensus sequences, respectively. Briefly, pUC18C was constructed by first performing a PCR with template DNA strand 5′-**CCCGGG**GTTCGCGTCTC CCATGGTCTGCTGTGCGTGTTCCGCGGTCTC-3′ (a *Sma*I restriction site is shown in boldface, and sequences complementary to the single AUG are underlined), T7 promoter-primer 5′-AGAGCTCTAATACGACTCACTATAG GGAGACCGCGGAAC-3′ (the T7 promoter is underlined), and a second primer, 5′-ACCCGGGGTTCGCG-3′. The plasmid series pUC18NC was constructed by PCR with the primers listed above and DNA template 5′-CCCG GGGTTCGCGTCGT(**C/G**)CAT(**T/C**)(**T/C**)GCTGCTGTGCGTGTTCCGCG GTCTC-3′. This template was designed to contain various nucleotides at positions −1, −2, and +4, as shown in boldface. Following PCR, the double-stranded DNA products were purified according to standard techniques (1) and directly ligated into the pGEM-T vector (Promega Biotec). The inserts in pGEM-T were sequenced by the dideoxy sequencing method (1), excised after digestion with *Sma*I and *Sph*I, isolated, and subcloned into the pUC18 vector (United States Biochemicals) to yield plasmids pUC18C, pUC18NC1, pUC18NC2, pUC18NC3, and pUC18NC4, which were used to synthesize RNAs shown in Table 1.

In addition, plasmid pUC18GUG, which can be transcribed to yield RNAs containing a guanine residue in place of the adenine residue at the +1 position (i.e., ACCGUGG), was constructed. Briefly, DNA oligonucleotide 5′-**CCCGGG** GTTCGCGTCTCCCACGG(G/T)CTGCTGTGCGTGTTCCGCGGTCTC-3′ (sequences complementary to the GUG codon are underlined) was used as a template in a PCR with the T7 promoter-primer and the second primer as explained above. The double-stranded products were ligated into the pGEM-T vector. After excision with *Sal*I and *Sma*I, the insert was isolated and cloned into the pUC18 vector. The final plasmids were sequenced to confirm the presence of the predicted sequences. As shown in Table 1, the NC1 and NC4 RNAs are missing two nucleotides and one nucleotide, respectively, and the NC3 RNA has one extra nucleotide; these changes presumably were acquired during the PCR.

The C-ORF and NC-ORF series of plasmids were constructed as follows. PCR was used to generate a 130-bp DNA fragment encompassing the T7 promoter, the 5′ noncoding region, and the various AUG codons (C and NC1 to NC4) with the pUC series of plasmids (see above) as templates. Therefore, five different primers (AUG primers) were designed that were complementary to each AUG and its context in the five different pUC plasmids (see above) and contained an *Eco*RI site at their 5′ ends: for pUC18C, 5′-CTGAATTCGCCATGGTCTGCTGT GCG-3′; for pUC18NC1, 5′-CTGAATTCGTCATTTGCTGCTGTGC-3′; for pUC18NC2, 5′-CTGAATTCTGCATCTGCTGCTGTGC-3′; for pUC18NC3, 5′-CTGAATTCGCCATTCGCTGCTGTGCG-3′; and for pUC18NC4, 5′-CTGAAT TCGCCATCTGTGCTGTGCG-3′. An additional primer (the T7 primer) was designed that was complementary to sequences located upstream of the T7 promoter in the pUC18 plasmid; this primer contained a *Pvu*II site at its 5′ end (5′-GACTCAGCTGGGTTTTCCCAGTCAC-3′). After PCR amplification of the various pUC plasmids with the AUG primers and the T7 primer, amplified DNA fragments were ligated into plasmid T7Luc$_A$(H) (18) that had been digested with *Pvu*II and *Eco*RI. In this way, the various AUG codons were placed in frame with a shortened luciferase-coding region. The presence of the desired inserts was verified by DNA sequencing. After digestion with *Hpa*I and addition of T7 RNA polymerase, RNAs that could be translated to yield truncated luciferase proteins (37 kDa in size) were synthesized.

In vitro RNA synthesis. Prior to RNA synthesis, the pUC18 plasmids were digested with *Eco*RI (for translation competition assays) or *Sma*I (for UV-cross-linking assays). The pGEM-T plasmid series was used to synthesize RNAs bearing translational stop codons (for the translation competition assays). Briefly, pGEM-T plasmids were digested with *Sph*I and *Not*I. The small, 111-bp fragment was purified by gel electrophoresis and used as a template in in vitro transcription reactions. RNAs were transcribed from linearized templates with T7 RNA polymerase (kindly provided by Bruce Burnett and Charles McHenry, University of Colorado Health Sciences Center). All competitor RNAs used for the translation competition assay were synthesized according to the RIBOMAX protocol (Promega Biotec), using 100 μg of template DNA per ml and 10 μg of RNA polymerase per ml in a final volume of 300 μl, for 4 h at 37°C and then treated with 1 U of DNase RQ1 per μg of template (Promega Biotec). The RNAs were extracted once with phenol-chloroform-isoamyl alcohol, and the

aqueous phase was adjusted to 3.75 M NH_4 acetate (NH_4OAc) and precipitated by addition of 2.5 volumes of ethanol. RNAs were further purified after electrophoresis in a 5% polyacrylamide–7 M urea gel as described previously (43). The isolated RNAs were quantitated by measuring the A_{260} (40 μg/ml/A_{260} unit).

RNA substrates for UV-cross-linking assays were synthesized with T7 RNA polymerase in the presence of [α-^{32}P]CTP (800 Ci/mmol) and 4-thio-UTP, synthesized as described by Stade et al. (40). 4-Thiouridines were selectively incorporated at the unique AUG codon. Briefly, *Sma*I-linearized plasmids (80 μg/ml) were incubated in transcription buffer (40 mM Tris-HCl [pH 7.4], 10 mM dithiothreitol [DTT], 2 mM spermidine, 6 mM $MgCl_2$) in the presence of 30 μCi of [α-^{32}P]CTP, 100 μM CTP, 500 μM GTP, 500 μM 4-thio-UTP, and 20 U of RNasin (Promega Biotec) (40). Transcripts were made after treatment with 4 μg of T7 RNA polymerase per ml for 2 h at 37°C. After treatment with DNase RQ1 (see above), radiolabeled RNAs were purified from the transcription reactions as described above, except that the wet gel was exposed to film to visualize the RNA. Unlabeled competitor RNAs were synthesized in large-scale RIBOMAX reactions as described above.

The reporter mRNAs, encoding full-length or truncated luciferase, were synthesized with T7 RNA polymerase from plasmid T7Luc$_A$(H) digested with *Bam*HI (18) or from the T7-ORF series (see above) digested with *Hpa*I.

Preparation of translation extracts from human HeLa cells. The preparation of extracts from human HeLa cell lysates and conditions for in vitro translation were as described previously (21). Briefly, approximately 5 liters of suspension-grown HeLa cells (5×10^5 cells per ml) was collected by centrifugation at 1,000 × *g*. The cell pellet was washed three times with isotonic buffer (35 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.4], 146 mM NaCl, and 11 mM glucose). After the final centrifugation, the cell volume was measured and the cells were resuspended in 1.5 times volume of hypotonic buffer [10 mM HEPES-KOH (pH 7.4), 15 mM KCl, 1.5 mM $Mg(OAc)_2$, and 6 mM 2-mercaptoethanol] and kept on ice for 2 min. The cells were then lysed by 50 strokes with a Dounce homogenizer and transferred to a 50-ml tube. After the addition of 5/18 volume (of the original cell pellet) of 10× incubation buffer [200 mM HEPES-KOH (pH 7.4), 120 mm KCl, 50 mM $Mg(OAc)_2$, 60 mM 2-mercaptoethanol], the suspension was sedimented at 1,000 × *g* for 5 min. The supernatant was sedimented again at 5,000 × *g* for 15 min, and ATP, GTP, creatine phosphate, and creatine phosphokinase were added to the resulting supernatant to yield final concentrations of 1, 0.2, and 8 mM and 0.2 mg/ml, respectively. The lysate was then aliquoted and stored at −80°C. Usually 1 ml of lysate was thawed and preincubated at 37°C for 30 min in order to "run off" the ribosomes from endogenous mRNAs. The lysate was then fractionated on a G-25 Superfine spin-column (Bio-Rad) in dialysis buffer [10 mM HEPES-KOH (pH 7.4), 90 mM KOAc, 1.5 mM $Mg(OAc)_2$, 1 mM DTT], and the excluded portion was saved for nuclease treatment. Micrococcal nuclease treatment was initiated by addition of 1 mM $CaCl_2$, 50 μg of creatine phosphokinase per ml, and 0.15 kU of nuclease S7 (Boehringer Mannheim) per ml to the lysate. After incubation at room temperature for 5 min, EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] was added to 2 mM, and the lysate was tested for translational activity. Lysates were stored at this point at −80°C.

In vitro translation and competition assays. The final reaction volumes were either 25 μl (reactions for which results are displayed in Fig. 1 and 2), 15 μl (reactions for which results are displayed in Fig. 3), or 17 μl (reactions for which results are displayed in Fig. 4). In each experiment, the reaction mixtures contained 30% (vol/vol) HeLa cell lysate in 20 mM HEPES-KOH (pH 7.4)–135 mM KOAc–1.1 mM $Mg(OAc)_2$–1.7 mM DTT–0.16 mM spermidine–9 μM amino acids (lacking methionine)–0.8 mM ATP–0.04 mM GTP–7.5 mM creatine phosphate–15 μCi of [^{35}S]methionine (100 Ci/mmol) per ml–23 μg of luciferase mRNA per ml (17 μg/ml for Fig. 2) plus various amounts of competitor RNAs (for Fig. 1, 2, and 4). First, the competitor RNAs were preincubated with the reaction mixtures lacking the luciferase reporter RNA for 5 min at 30°C. Next, luciferase mRNA was added, and the reaction mixture was incubated for an additional 30 min. An aliquot of each reaction mixture was used to measure luciferase activity (9), and the remaining reaction volume was analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE).

UV-cross-linking assay. HeLa cell monolayers were washed in phosphate-buffered saline, and a Nonidet P-40 lysate was prepared as described previously (38). Next, the lysate was clarified by centrifugation at 10,000 × *g* for 20 min at 4°C, and the supernatant was fractionated by successive ammonium sulfate precipitations (0 to 20%, 20 to 40%, 40 to 60%, and 20 to 60%) as described by Englard and Seifert (13). Each ammonium sulfate precipitate was resuspended in dialysis buffer (10 mM HEPES-NaOH [pH 7.9], 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) and dialyzed overnight against two changes of buffer.

For UV cross-linking, 24 μg of a partially purified fraction obtained from the 20 to 60% ammonium sulfate precipitation was incubated in a 20-μl reaction volume (13 mM HEPES-NaOH [pH 7.4], 3 mM $MgCl_2$, 1.3 mM ATP, 5 mM creatine phosphate, 270 mM KCl, 0.7 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 0.25 mM EDTA, and 14% glycerol) lacking or containing unlabeled competitor RNA for 10 min at 30°C in 1.5-ml Eppendorf tubes. Following the addition of the 4-thio-UTP-containing radiolabeled RNA (10^6 cpm; 6 nM final concentration), the entire reaction mixture was incubated for an additional 10 min at 30°C. Alternatively, 200 μg (20 μl) of the HeLa translation lysate [in 10 mM HEPES-KOH (pH 7.4), 90 mM KOAc, 1.5 mM $Mg(OAc)_2$, 1 mM DTT] was incubated with 3×10^6 cpm of 4-thio-UTP-containing radiolabeled C RNA in a 30-μl reaction volume containing 3 μl of 10× binding buffer (85 mM HEPES-NaOH [pH 7.4], 30 mM $MgCl_2$, 13 mM ATP, 50 mM creatine phosphate, 10 mM DTT, and 40% glycerol) and 2 μl of 3 M KCl for 10 min at 30°C. Next, the tubes were laid flat in a rack (Stratagene) approximately 3 cm from the UV light source, covered with a glass plate, and exposed to 312-nm light in a Stratalinker model 1800 (Stratagene) at 3,000 μW/cm^2 for 20 min at 4°C. After the cross-linking, the sample was digested with 0.25 mg of pancreatic RNase A (United States Biochemicals) per ml for 30 min at 37°C. The cross-linked RNA-protein complexes were then visualized after separation on SDS-containing 10% polyacrylamide gels by autoradiography. Similarly, 1 nM 4-thio-UTP-containing radiolabeled RNA was incubated with 40 nM purified recombinant La protein (a generous gift of D. Kenan and J. Keene, Duke University Medical Center), dialyzed against 20 mM Tris-HCl (pH 7.5)–100 mM KCl–0.2 mM EDTA–1 mM DTT–10% glycerol, under the same conditions. After UV cross-linking and digestion with RNase A, the cross-linked products were analyzed by SDS-PAGE. It is noteworthy that La-C RNA complexes could be formed over a wide range of KCl concentrations (15 to 500 mM) (30).

Immunoprecipitations. The immunoprecipitations were performed as described previously (39) with modifications. Briefly, UV-cross-linked and RNase A-digested samples were added to immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA [pH 8.0]) to 95 μl, and 5 μl of human serum directed against the La autoantigen, kindly provided by D. Kenan and J. Keene (Duke University Medical Center), was added. Following incubation on ice, 120 μl of a 50% slurry of protein A-Sepharose (Sigma) was added. The resin was washed extensively with 50 mM Tris-HCl (pH 7.5)–0.5 M NaCl–5 mM EDTA–1% Nonidet P-40–5% sucrose–0.2% SDS, and the retained material was analyzed by SDS-PAGE.

Biochemical probing of RNA structures. Structural probing of C and NC2 RNAs was performed with 5′-end-labeled RNA as described previously (22), except that tRNA (1 μg/μl) was added to the samples prior to performance of the sequencing reactions. RNase T_1 was obtained from GIBCO-BRL.

RESULTS

Synthesis of RNA molecules containing single AUG codons surrounded by various nucleotide sequences. Plasmids were constructed (see Materials and Methods) that could direct the synthesis of short RNA molecules whose nucleotide sequences were very similar to each other except in the sequences surrounding the single AUG start codon present in the RNAs (Table 1). The RNAs contain 5′ noncoding regions which are 29 nucleotides in length, the average size for most mammalian mRNAs (26), followed by short coding regions containing or lacking UAA stop codons (Table 1). Kozak demonstrated that purines at positions −3 and +4 (the A in the AUG start codon is designated +1) promote optimal usage of an AUG as a start codon (27, 28). Thus, the RNAs designated C in Table 1, containing a single AUG codon that is surrounded by sequences **A**CC<u>AUG</u>**G**, should designate the AUG triplet as optimal for the start of protein biosynthesis. In contrast, RNAs containing the AUG triplet flanked by pyrimidines at both −3 and +4 or by a pyrimidine at either −3 or +4 should function poorly as AUG start codons (27, 28). Therefore, RNAs containing the AUG in various nucleotide sequence contexts, i.e., C (**A**CC<u>AUG</u>**G**), NC1 (**C**AA<u>AUG</u>**A**), NC2 (**C**AG<u>AUG</u>**C**), NC3 (**C**GA<u>AUG</u>**G**), and NC4 (**C**AG<u>AUG</u>**G**), were tested for their effects on translation of a consensus AUG-containing reporter mRNA.

Short RNA molecules containing a consensus AUG, but not a nonconsensus AUG, inhibit translation of reporter mRNAs. We tested the effect of C and NC RNAs on the translational efficiency of a reporter mRNA in an in vitro translation system prepared from human HeLa cells. Luciferase-encoding mRNAs (9), approximately 1,700 nucleotides in length with a 22-nucleotide 5′ noncoding region and an AUG start codon embedded in an ACC<u>AUG</u>G sequence motif, were used as reporter mRNAs. The effects of C and NC RNAs on luciferase mRNA translation are shown in Fig. 1A. The translational efficiency of the reporter mRNA in the absence of any exogenously added RNA was set at 100%. A slight enhancement in

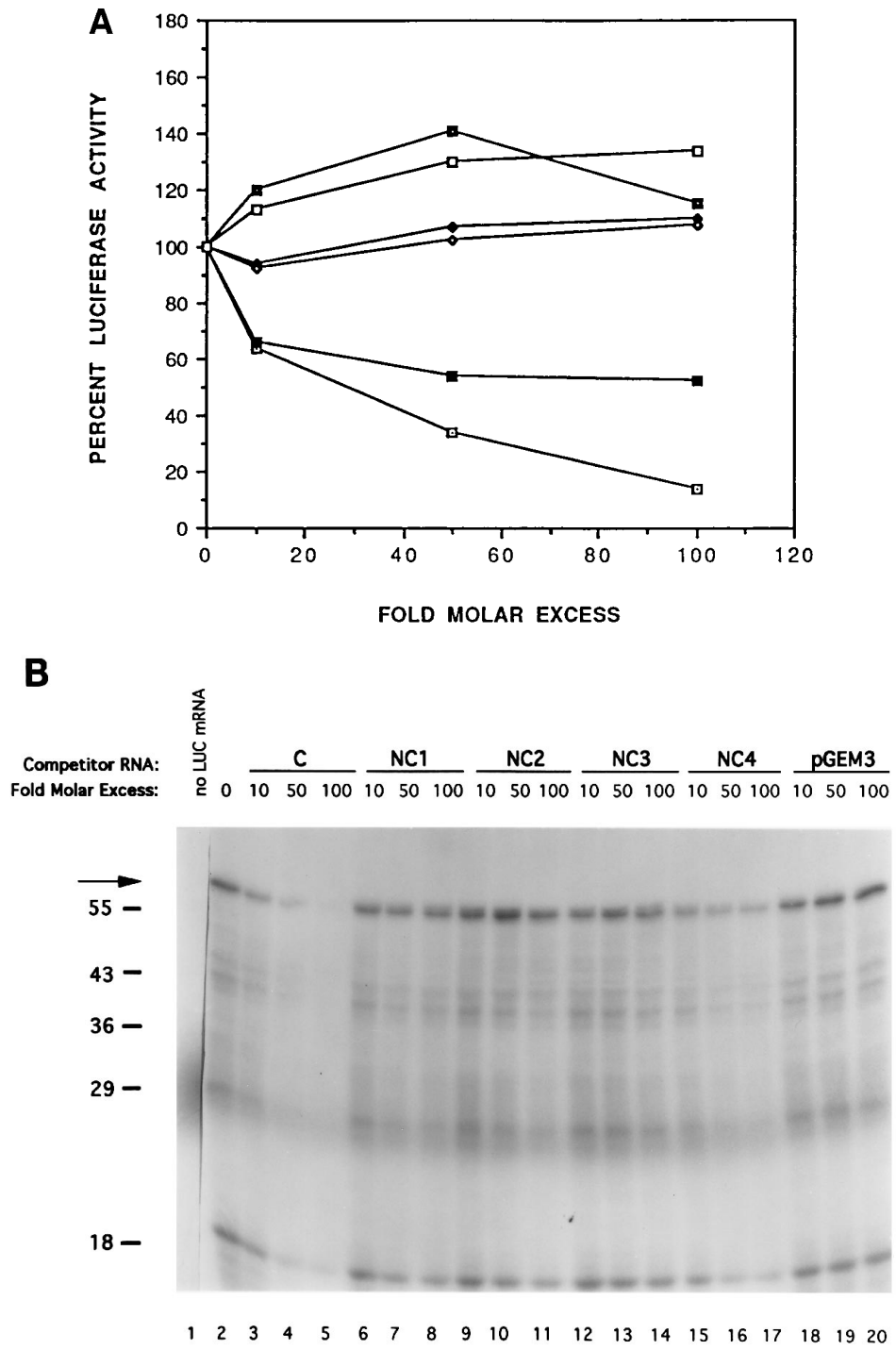

FIG. 1. Effect of RNAs containing AUGs in various sequence contexts on translation of luciferase mRNA (0.04 μM) in vitro. See Materials and Methods for details. The effect of 10 (0.4 μM)-, 50 (2 μM)-, and 100 (4 μM)-fold molar excesses of C, NC1, NC2, NC3, NC4, or pGEM3 RNA is shown. (A) Percent luciferase activity relative to the reaction in the absence of RNAs. Symbols: □, pGEM3 (no AUG); ◆, NC1 (CAAAUGA); ◘, NC2 (CAGAUGC); ◇, NC3 (CGAAUGG); ■, NC4 (CAGAUGG); ⊡, C (ACCAUGG). (B) An aliquot of the same reaction mixture was analyzed by SDS–10% PAGE, and the gel was fixed, treated with Fluoro-Hance (RPI), and dried. An autoradiograph is shown. The arrow indicates the migration of luciferase protein. The positions of the molecular mass markers (in kilodaltons) are indicated on the left. The translation reaction mixtures were digested with RNase A (0.25 mg/ml) before being loaded on the gel.

reporter mRNA translation was observed in the presence of RNAs containing AUG codons embedded in nonconsensus (NC2) sequences or partially consensus (NC1 and NC3) sequences or in the presence of a control RNA of approximately the same length lacking an AUG codon (pGEM3). In contrast, the addition of RNAs with a consensus AUG codon (C RNA) decreased the translational efficiency of the reporter mRNA; the presence of a 30-fold molar excess of C RNA reduced the synthesis of luciferase by approximately 50% (Fig. 1A). NC4 RNAs displayed an intermediate inhibitory effect. This was

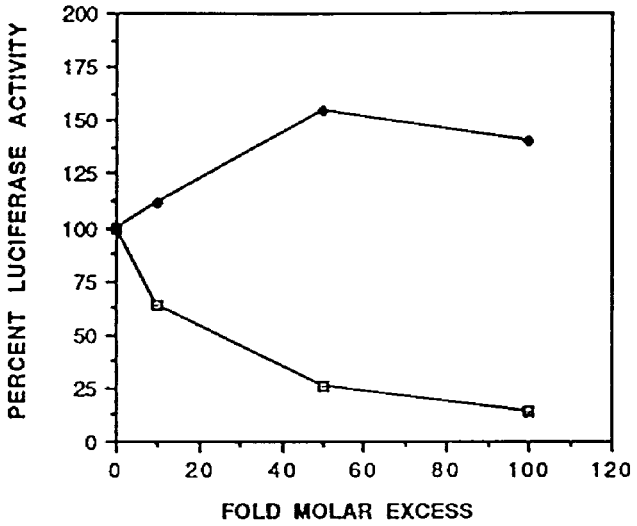

FIG. 2. Dependence of translational inhibition on the presence of an AUG codon. The effect of 10 (0.3 μM)-, 50 (1.5 μM)-, and 100 (3 μM)-fold molar excesses of C or GUG RNA on the translation of 0.03 μM luciferase mRNA in a 25-μl reaction mixture is shown. The translation conditions were as described in Materials and Methods, except that [^{35}S]methionine was omitted and replaced with unlabeled methionine (0.03 mM final concentration). The percent luciferase activity relative to the reaction in the absence of competitor RNA is shown. Symbols: ◆, GUG (5′ACCGUGG3′); ⊡, C (5′ACCAUGG3′).

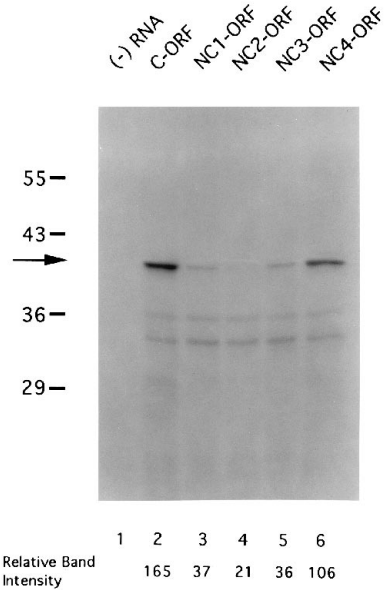

FIG. 3. Translation of mRNAs containing C or NC 5′ noncoding regions. The mRNAs were translated in the presence of [^{35}S]methionine in the HeLa translation lysate as described in the legend to Fig. 1B, but without addition of competitor RNA. Lane 1, translation products from a reaction mixture lacking exogenously added RNA; lanes 2 to 6, translation products from reaction mixtures containing C-ORF, NC1-ORF, NC2-ORF, NC3-ORF, and NC4-ORF mRNAs, respectively. An autoradiograph of the SDS-polyacrylamide gel is shown. The arrow marks the position of the predicted 37-kDa, truncated luciferase polypeptide, which migrates at 41 kDa in this gel system. The identities of the smaller bands migrating at approximately 36 and 34 kDa are not known. The relative band intensities of truncated luciferase proteins are shown at the bottom. The positions of the molecular mass markers (in kilodaltons) are indicated on the left. This experiment was repeated three times with identical results.

surprising, because both NC3 and NC4 RNAs contain cytosines at −3 and guanosines at +4 (Table 1). However, because of a single nucleotide deletion in the 5′ noncoding region of NC4, the sequences upstream of NC3-AUG and NC4-AUG are different (Table 1). Whether this accounts for the observed intermediate inhibition of NC4 RNAs remains to be determined. These experiments were performed with uncapped mRNA and uncapped competitor RNAs; however, the same results were obtained with capped mRNA (30). Furthermore, C RNAs in which the AUG codon was changed to a GUG codon did not show any inhibitory effect on reporter mRNA translation, as shown in Fig. 2. These experiments indicated that an AUG codon, embedded in an optimal sequence context for a start site codon, inhibited in *trans* the synthesis of active luciferase protein by luciferase mRNAs.

To test whether C RNAs inhibited translation of the reporter mRNA at the initiation step, radiolabeled translation products synthesized in the presence of C, NC, and pGEM3 RNAs were analyzed. Figure 1B shows that no radiolabeled products were synthesized in the absence of luciferase mRNA (lane 1). Addition of luciferase mRNA resulted in the synthesis of several radiolabeled products, including the full-length luciferase protein, 60 kDa in size (lane 2). Addition of C RNAs abolished the synthesis of all radiolabeled products in a dose-dependent manner (lanes 3 to 5). In contrast, addition of NC RNAs and pGEM3 RNAs did not significantly affect translation of the reporter mRNA (lanes 6 to 20). Quantitation of the 60-kDa protein bands by densitometry indicated that the luciferase activity displayed in Fig. 1A directly correlated with the amount of labeled product observed in Fig. 1B (30). Also, Fig. 1B shows that C RNAs did not cause aberrant initiation events or premature termination of elongation, because no new initiation products are observed (lanes 4 and 5).

The effect of AUG context in the competitor RNAs on their competitive efficiencies was striking. To determine if the behaviors of the short RNAs in the translational competition assays correlated with their abilities to be translated with different efficiencies, we assayed the translation efficiencies of reporter mRNAs bearing C or NC noncoding regions. As outlined in Materials and Methods section, C or NC noncoding regions were linked to open reading frames (ORFs), and the translational efficiencies of these mRNAs were determined. As predicted from the translation competition results (Fig. 1), C-ORF mRNAs were translated more efficiently than NC1-ORF, NC2-ORF, and NC3-ORF mRNAs (Fig. 3). Furthermore, the translational efficiencies of C-ORF and NC-ORF mRNAs correlated well with the translational competition of the C and NC RNAs (Fig. 1). This correlation was particularly striking for NC4-ORF mRNA and NC4; NC4 both acted as a weak inhibitor of translation and could be translated, although less efficiently than the C-ORF mRNA. Translation of C-ORF and NC-ORF mRNAs was also tested in rabbit reticulocyte lysates. All ORF mRNAs were translated well in this system, and, as expected from earlier studies on the effects of AUG context in the rabbit reticulocyte lysate system (28), the context of the AUG codons influenced the efficiencies with which the mRNAs were translated, but the differences between the C-ORF and NC-ORF mRNAs were minimal (30). The data in Fig. 1, 2, and 3 suggest that the ability of the short RNAs to inhibit translation is directly related to the presence and the context of the AUG codon and the efficiency with which it is used as a start codon.

The inhibition of luciferase mRNA translation by C RNAs could have resulted from sequestration of 43S ternary complexes or 80S ribosomes because of the absence of a translational stop codon in the C RNAs. Alternatively, *trans*-acting factors that recognize specifically the **ACC**AUG**G** motif in C RNAs may have become limiting for efficient mRNA transla-

tion. To distinguish between these possibilities, mRNA translation was monitored in the presence of C and NC RNAs containing UAA translational stop codons.

Translation competition by the C RNA is not due to sequestration of ribosomal subunits. C and NC2 RNAs either containing or lacking UAA stop codons (Table 1) were tested for their abilities to affect the translation of the reporter mRNA. Figure 4A shows that C RNAs containing (C/UAA) or lacking (C) stop codons inhibited reporter mRNA translation with approximately equal efficiency. In contrast, various amounts of NC2 RNAs containing (NC2/UAA) or lacking (NC2) stop codons did not influence the translational efficiency of the reporter mRNA. To monitor whether the introduced stop codons were operational, the translation of C and NC RNAs was monitored. In the absence of stop codons, the last aminoacyl-tRNA is predicted to remain covalently attached to a [^{35}S]methionine-labeled peptide synthesized from the competitor RNAs. Inspection of Fig. 4B shows that in the presence of both C and NC2 RNAs lacking stop codons, a novel translation product, migrating between the 18,000- and 29,000-molecular-weight markers, is seen (lanes 1 to 6 and 14 to 19). This product was not seen if the samples were treated with RNase A before electrophoresis (Fig. 1B). Addition of stop codons to C and NC2 RNAs (Table 1) abrogated the synthesis of this protein species, which presumably represents tRNA-peptide complexes (Fig. 4B, lanes 7 to 12 and 20 to 25). It is likely that in the presence of the stop codons, tRNA and ribosomal subunits were released at the UAA stop codons; synthesis of the predicted small peptide, approximately 7 amino acids in length, could not be monitored because it was too small to be detected. Of course, it cannot be completely ruled out that C RNAs with stop codons temporarily sequester ribosomes longer than NC RNAs with stop codons. However, this scenario was less likely, because the observed effects of C and NC RNAs, with or without stop codons, on reporter mRNA translation were very similar at various RNA concentrations (Fig. 4) (30). More likely, these data show that the introduced stop codons were functional and that C RNAs inhibited reporter mRNA translation by a mechanism that did not involve a simple sequestration of ribosomal subunits.

It could be argued that despite their nearly identical sequences, the few nucleotide changes surrounding the AUG codons in C and NC2 RNAs caused large differences in the overall RNA structures. Structural features in the competitor RNAs could have accounted for the observed effects on reporter mRNA translation. To test this possibility, the structures of C and NC2 RNAs were examined by enzymatic probing methods.

Structural analysis of C and NC2 RNA molecules. Figure 5A and B shows the results of the enzymatic probing of 5′-radiolabeled C and NC2 RNAs. Radiolabeled RNA C (Fig. 5A) and RNA NC2 (Fig. 5B) were treated with increasing amounts of RNase A (lanes 2 to 4), which cleaves after single-stranded pyrimidine residues, or RNase T_1 (lanes 5 to 8), which cleaves after single-stranded guanosine residues. Figure 5C summarizes the primary data shown in Fig. 5A and B. Most guanosine and cytidine residues in both C and NC2 RNAs were accessible to nuclease cleavage, with the exception of the very-terminal nucleotides. The nucleases cleaved most efficiently the nucleotides located 5′ proximal of the AUG and less efficiently the nucleotides 3′ of the AUG triplet. Overall, the structures in both C and NC RNAs are mostly in single-stranded conformations. Therefore, the different effects of C and NC2 RNAs on reporter mRNA translation are unlikely to be due to helical conformations in the C RNA resulting in translational inhibition of the reporter mRNA.

HeLa cell cytoplasmic proteins can bind selectively to AUG codons surrounded by optimal start site sequences. Translational competition experiments suggested that C RNAs sequester some factor that limits the translational efficiency of the reporter mRNA. We searched for proteins that could bind with higher affinity to C RNAs than to NC2 RNAs. Because the uridine in the AUG is the only uridine in the RNA (Table 1, UV assay RNAs), 4-thio-UTP could be selectively incorporated at this position by T7 RNA polymerase (40). This photoreactive nucleotide could then be activated by irradiation at 312 nm, resulting in a covalent bond between the 4-thio-UTP and any protein that was in close contact with this nucleotide (40). Importantly, neither radiolabeled RNAs nor proteins suffer detectable damage after irradiation under these conditions (30, 40).

C RNAs were synthesized in the presence of 4-thio-UTP and [α-^{32}P]CTP, incubated with partially purified extracts from HeLa cells, irradiated at 312 nm, and treated with RNases. The cross-linked products were analyzed by SDS-PAGE. Specifically, extracts were fractionated by successive ammonium sulfate precipitations, and the individual fractions were tested in the cross-linking assay. As shown in Fig. 6, several proteins from the 20 to 60% ammonium sulfate precipitate could be cross-linked to the AUG codon present in C RNA (lane 1). Treatment of the radiolabeled RNA, in the absence of the ammonium sulfate precipitate, with RNase A digested the RNA (30). Similarly, treatment of the cross-linked reaction mixture, in the presence of the ammonium sulfate precipitate, with proteinase K eliminated the appearance of the bands seen in Fig. 6, lane 1 (30), indicating that the observed radiolabeled bands were cross-linked protein-RNA complexes.

To test whether the cross-linked products represented specific RNA-protein complexes, competition experiments were performed. Increasing amounts of unlabeled C or NC2 competitor RNAs were preincubated with the 20 to 60% ammonium sulfate precipitate and radiolabeled, 4-thiouridine-containing C RNA was added, UV irradiation was performed, and cross-linked products were analyzed by SDS-PAGE. Figure 6 shows that increasing amounts of both C and NC2 RNAs competed for binding of several proteins to the radiolabeled C RNA (lanes 2 to 7). In contrast, increasing amounts of C RNA, but not of NC2 RNA, competed for binding of a 50-kDa protein to radiolabeled C RNA. Quantitation of the data showed that an approximately 150-fold molar excess of C RNA was needed to eliminate 50% of the binding of the 50-kDa protein to radiolabeled C RNA, whereas a 360-fold molar excess of NC2 did not affect the binding of the 50-kDa protein to C RNA. The binding of a 100-kDa protein to radiolabeled C RNA was also specifically inhibited in the presence of unlabeled C RNA; however, the difference between C and NC2 competitor RNAs was not as striking. These findings indicated that there was at least one HeLa cell protein that interacts specifically with the AUG codons embedded in optimal start site sequences.

A 50-kDa start site AUG-binding protein can be immunoprecipitated with serum directed against the La autoantigen. A potential candidate for the 50-kDa AUG-binding protein in C RNA (start site AUG-binding protein) was the human La autoantigen (reviewed in reference 42). The La autoantigen had been reported to be involved in AUG start site selection of poliovirus RNA (32, 41). It has been known for some time that translation of poliovirus RNA in rabbit reticulocyte lysates initiates at several internally located AUG codons in addition to the predicted start AUG codon at position 743 (12). The addition of La to rabbit reticulocyte lysates greatly abolished the incorrect translational start sites in poliovirus RNAs, while

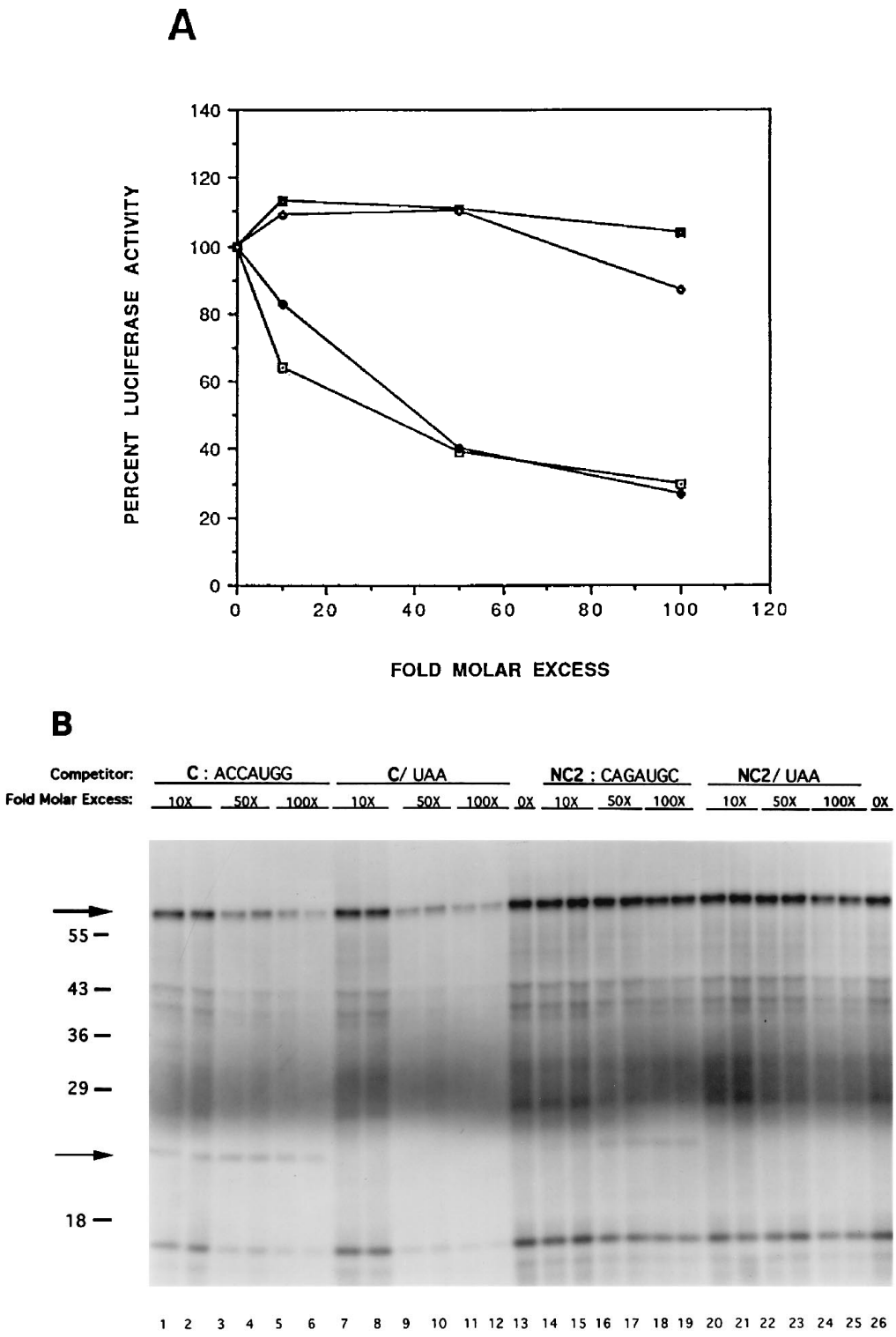

FIG. 4. The inhibition caused by addition of the consensus AUG-containing RNA is independent of an in-frame stop codon. The experiment was performed as described for Fig. 1, except as noted in Materials and Methods. (A) Percent luciferase activity relative to the reaction in the absence of RNAs. Symbols: ■, NC2; ◇, NC2/UAA; ⊡, C; ◆, C/UAA. (B) An aliquot of the same reaction mixture was analyzed by SDS–10% PAGE, and the gel was fixed, treated with Fluoro-Hance (RPI), and dried. An autoradiograph is shown. The arrows indicate the migrations of luciferase protein (60 kDa) and of tRNA-protein complexes. The positions of the molecular mass markers (in kilodaltons) are indicated on the left. The translation reaction mixtures were not digested with RNase A before being loaded on the gel.

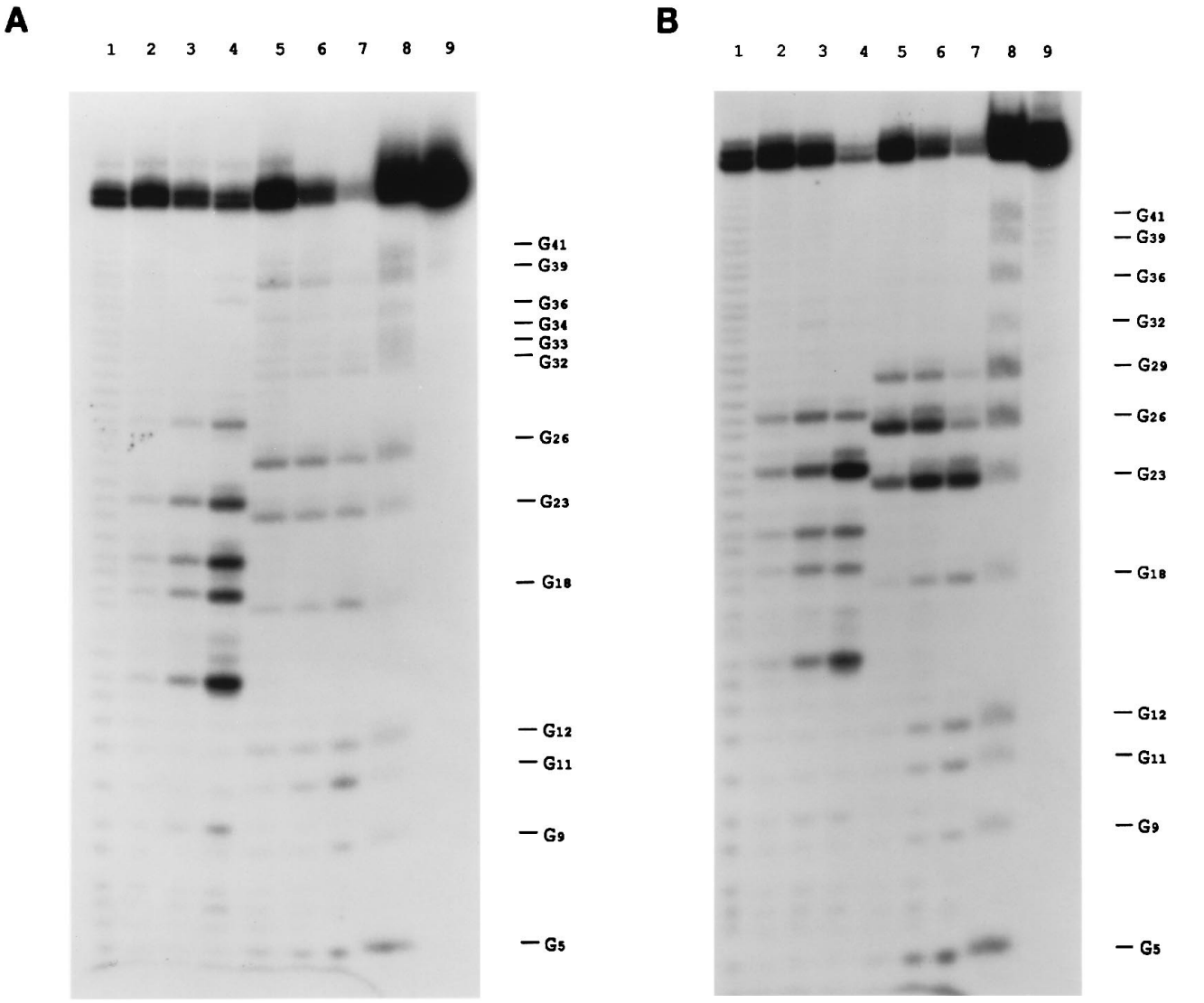

FIG. 5. Enzymatic probing of C and NC2 RNA molecules. The results of RNase A and RNase T_1 enzymatic probing of C (A) and NC2 (B) RNAs are shown. RNA structural analysis was performed in TMK buffer (30 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 270 mM KCl). In both panels, lanes 1 and 9 contain untreated RNA; lanes 2 to 4 show the effect of 20, 60, and 100 ng of RNase A per ml, respectively; lanes 5 to 7 show the effect of 0.5, 2, and 8 U of RNase T_1 (final volume, 10 μl), respectively; and lane 8 shows the sequence Gs generated by treatment with 2 U of T_1 under denaturing conditions (7 M urea, 50°C). (C) Summary of RNase cleavages. Squares, nucleotides susceptible to RNase A cleavage; circles, nucleotides susceptible to RNase T_1 cleavage; solid symbols, strong cleavage; open circles, weak cleavage.

translation from the correct start site was slightly stimulated (32, 41). Although La has been shown to bind to an AUG-containing hairpin structure located in the viral 5′ noncoding region (31), whether La can interact with the start site AUG codon in poliovirus mRNA as well has not been tested.

To test whether the 50-kDa start site AUG-binding protein and the La autoantigen were the same protein, C RNAs were UV cross-linked to proteins obtained from a 20 to 60% ammonium sulfate-precipitated HeLa cell extract and immunoprecipitated with human serum containing antibodies directed against the La autoantigen (Fig. 6, lanes 8 to 14). The immune serum was able to immunoprecipitate a 50-kDa protein cross-linked to radiolabeled C RNA (Fig. 6, lane 8). No radiolabeled proteins could be detected when comparable amounts of an unrelated rabbit immune serum was used (30). The slight shift of the immunoprecipitated complex was likely due to the presence of large amounts of antibody heavy chains, which migrate at approximately 50,000 molecular weight in the gel matrix. In support of this statement, addition of serum to the sample shown in lane 1 of Fig. 6 distorted the migration of the 50-kDa protein in a similar manner (30).

To test whether binding of La was specific to the AUG embedded in C RNA, competition assays were performed by

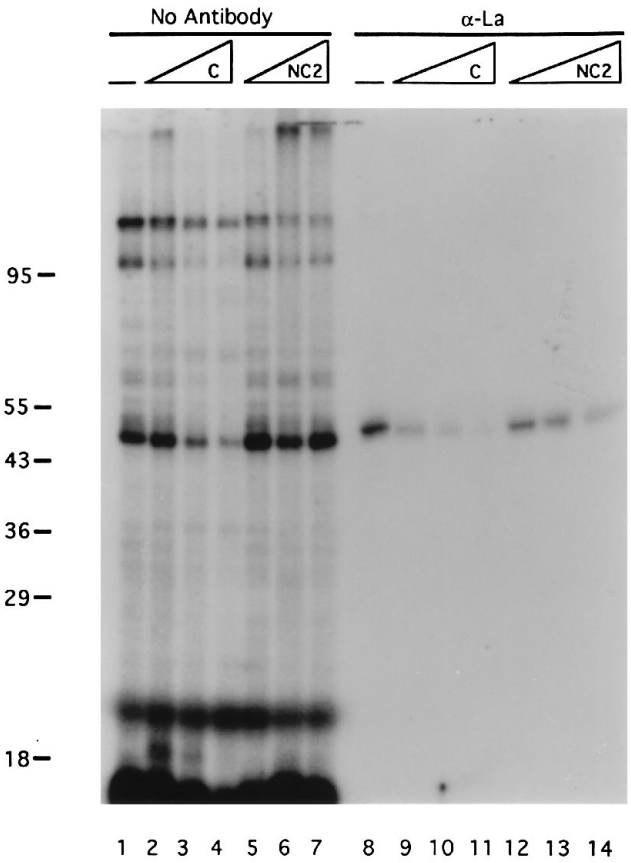

FIG. 6. Site-directed cross-linking and immunoprecipitation of cytoplasmic HeLa cell proteins that interact with the uridine residue in the ACCAUGG motif in the C RNA. A fraction obtained after the 20 to 60% ammonium sulfate precipitation was incubated without C or NC2 RNA (lanes 1 and 8) or with increasing amounts of C RNA (lanes 2 to 4 and 9 to 11) or NC2 RNA (lanes 5 to 7 and 12 to 14); radiolabeled C RNA was added, and the sample was irradiated at 312 nm. After RNase A digestion, samples were analyzed by SDS–10% PAGE. An autoradiograph is shown. Lane 1, no unlabeled competitor RNA; lanes 2 and 5, 90-fold molar excess of unlabeled competitor RNA; lanes 3 and 6, 180-fold molar excess of unlabeled competitor RNA; lanes 4 and 7, 360-fold molar excess of unlabeled competitor RNA; lanes 8 to 14, RNA-protein complexes from reactions in lanes 1 to 7, respectively, immunoprecipitated with human serum directed against the La autoantigen, as described in Materials and Methods. The migrations of known molecular weight markers (in thousands) are indicated on the left.

using the immunoprecipitation assay. Unlabeled C RNA (Fig. 6, lanes 9 to 11) competed for La binding to C RNA more efficiently than did unlabeled NC2 RNA (lanes 12 to 14). Quantitation of the autoradiograph showed that approximately twice as much NC2 RNA as C RNA was required to compete for a comparable amount of immunoprecipitable La-C RNA complex. Thus, La was present in the 50-kDa species that cross-linked specifically to C RNAs. That the La-C RNA complex was somewhat less specifically inhibited than the 50-kDa protein–C RNA complex could be the result of the different experimental protocols employed or of La being only one protein migrating at 50 kDa.

Because the translation competition studies were performed with cytoplasmic lysates which had not been fractionated by ammonium sulfate precipitation, it was important to test whether La-C RNA complexes were formed in translation-competent extracts. Therefore, lysates obtained after ammonium sulfate precipitation and HeLa translation lysates were incubated with radiolabeled 4-thio-UTP-containing C RNA,

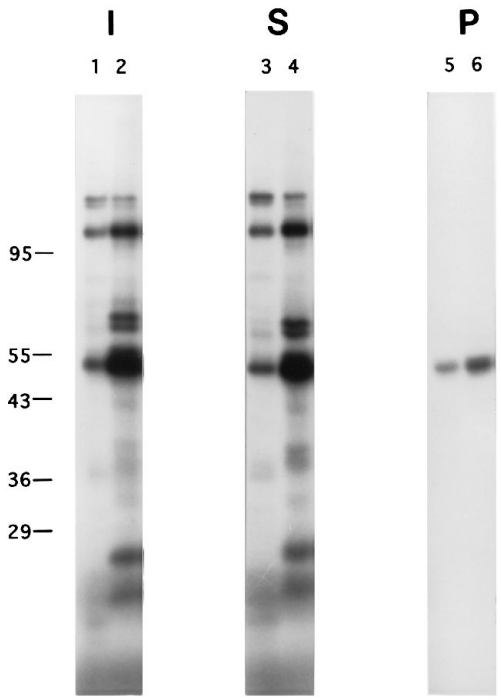

FIG. 7. Immunoprecipitation of a 50-kDa protein from the HeLa translation lysate cross-linked to C RNA with antibodies directed against the La autoantigen. Lanes 1, 3, and 5, 2×10^6 cpm of 4-thio-UTP-radiolabeled C RNA incubated with 24 μg of proteins precipitated with 20 to 60% ammonium sulfate in a 20-μl reaction mixture; lanes 2, 4, and 6, 3×10^6 cpm of radiolabeled C RNA incubated with 200 μg of proteins from the micrococcal nuclease-treated HeLa translation lysate in a 30-μl reaction mixture. The samples were UV irradiated and treated with RNase A. Lanes 1 and 2, 1 and 1.5 μl, respectively, of the samples; lanes 5 and 6, material after immunoprecipitation with La antibodies from 18 and 27 μl, respectively. I, input; S, supernatant; P, pellet. Sizes of molecular weight markers (in thousands) are shown on the left.

UV cross-linked as described above, and then immunoprecipitated with La antibodies. Figure 7 shows that human serum directed against the La autoantigen could immunoprecipitate cross-linked C RNA–50-kDa protein complexes from both ammonium sulfate-treated (lane 5) and translation-competent (lane 6) lysates. Additional C RNA-protein complexes were detected in the extracts which were not immunoprecipitated by La antibodies (lanes 1 and 2). Analysis of the supernatants of the immunoprecipitation reactions revealed the presence of those non-50-kDa protein–C RNA protein complexes and of slightly diminished amounts of C RNA–50-kDa protein complexes which likely represented nonimmunoprecipitated C RNA-La complexes, non-La-containing C RNA-protein complexes, or both. These experiments show that La binds and can be cross-linked to C RNA both in partially purified extracts and in translation-competent lysates from HeLa cells. Curiously, 50-kDa protein–C RNA complexes were only poorly detected in lysates prepared from cells treated with the nonionic detergent Nonidet P-40 (30).

La-C RNA complexes that could be UV cross-linked were also formed by using purified recombinant La (Fig. 8A, lane 1). Increasing amounts of C RNAs competed with binding of La to radiolabeled C RNA; approximately a 35- to 40-fold molar excess of C RNA was needed to obtain a 50% reduction in the binding of La to the C RNA (Fig. 8B). In contrast, more than a 150-fold molar excess of NC2 RNA was required for a 50% reduction in the binding of La to the C RNA (Fig. 8B). We conclude from these studies that La binds preferentially to AUG codons embedded in optimal start site sequences.

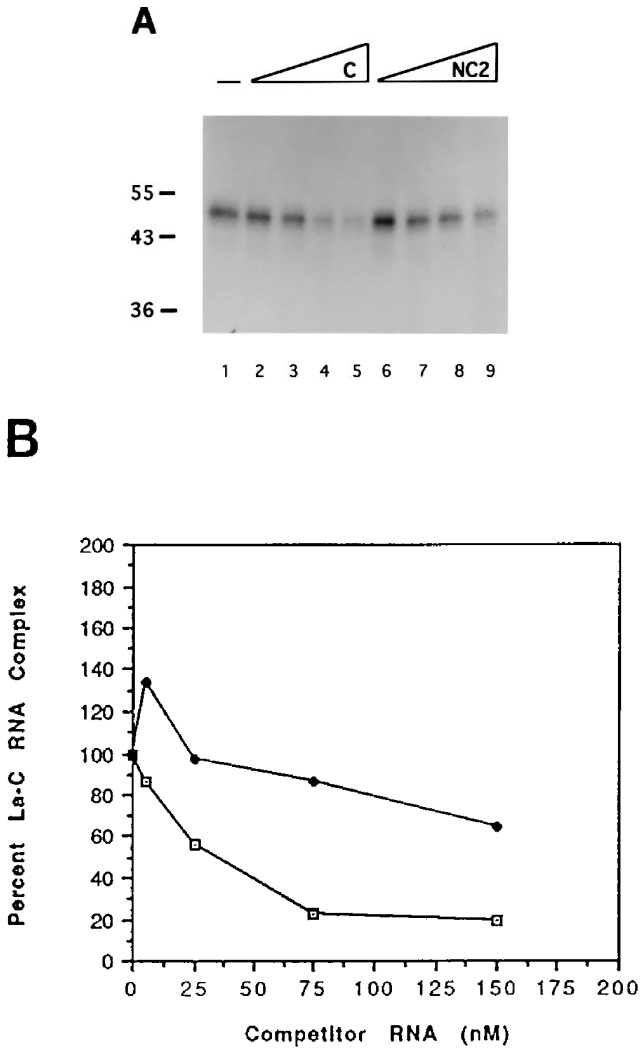

FIG. 8. Site-directed cross-linking of purified recombinant La to the uridine residue in the ACCAUGG motif in C RNA. (A) Autoradiograph of an SDS gel. Lane 1, La cross-linked to the radiolabeled 4-thio-UTP-substituted C RNA in the absence of competitor RNAs; lanes 2 to 5, 5-, 25-, 75-, and 150-fold molar excesses of C RNA, respectively; lanes 6 to 9, the same concentrations of NC2 RNA, respectively. The migrations of known molecular weight markers (in thousands) are indicated on the left. (B) The bands in panel A were quantitated by densitometry. Percent La-C RNA complex formation was determined by setting the band intensity in lane 1 at 100%. The slight stimulation of binding with a fivefold molar excess of NC2 was not observed in subsequent experiments. Symbols: ◆, NC2 RNA; ⊡, C RNA.

DISCUSSION

It is clear that 43S RNA-associated factors, such as the initiator tRNA and certain translational initiation factors such as eIF2 (6, 11, 44), are involved in the recognition of an AUG codon as the start site for protein synthesis by the scanning 43S ribosomal ternary complex (28, 33). We explored the possibility that additional factors interact specifically with sequences at the AUG start site codon and thereby direct the 40S subunit to the appropriate AUG start codon. To do this, we designed a competition assay in which the translational efficiency of a reporter mRNA was monitored in the presence of small RNA molecules containing single AUG codons surrounded by sequences that should favor or disfavor usage of that AUG as a start codon.

Factors modulate the start site selection in vitro. The competition experiments indicated that the ACCAUGG-containing C RNAs inhibited reporter mRNA translation, while the CAGAUGC-containing NC2 RNAs did not display inhibitory effects. This effect was seen in the presence or absence of translational stop codons in the C and NC2 RNAs, indicating that the observed effects were not simply due to ribosomal subunit sequestration by the C RNAs. Explanations for the actions of C and NC RNAs include the possibility that C RNAs may activate the DAI kinase, the double-stranded RNA-activated inhibitor of protein synthesis (19, 20, 29). We consider this less likely because the overall structural features of C and NC2 RNAs are predominantly single stranded. C RNAs could sequester *trans*-acting factors involved in elongation of the reporter mRNA. However, inspection of radiolabeled products synthesized in the presence of C or various NC RNAs did not reveal the accumulation of prematurely terminated translation products. The most likely explanation, therefore, is that C RNAs sequester factors that are involved in translational initiation. Consistent with this hypothesis is that the same AUG codons which conferred translation inhibition in C and NC4 RNAs exerted greater translational efficiency than, for example, NC2 when linked in frame to a reporter mRNA.

Cellular proteins bind specifically to AUG codons in a context-dependent manner. Eukaryotic translation initiation factors, which are not necessarily associated with ribosomes, have been found to interact with AUG-containing RNAs. Eukaryotic initiation factor eIF4B has been observed to bind to sequences in satellite tobacco necrosis virus RNA containing the AUG initiation codon (2) and was found to bind preferentially to AUG triplets (14). Initiation factor eIF2 can interact with various RNA molecules that contain AUG codons (23, 35); however, the exact binding sites for eIF2 on these RNAs have not been reported. More recently, Dasso et al. provided evidence that selection of the 5′-proximal start site in influenza virus N2 RNA can be influenced by eIF2 levels (8). It was found that when the mRNA concentration in the reticulocyte lysate-primed translation reaction mixture was high, the 5′-proximal AUG was bypassed and the second AUG was used for the translational start. This was observed regardless of the sequence context of the two AUG codons. It was hypothesized that high mRNA concentrations sequestered factors involved in selection of the 5′-proximal AUG (8). Interestingly, exogenously added eIF2 could restore first-AUG usage under the condition of high mRNA concentration (8). When purified eIF2 (generously provided by W. Merrick, Case Western University) was tested for its ability to cross-link to C and NC2 RNAs, the binding affinities for the two RNAs were found to be very similar (30), and specific restoration of C RNA-inhibited translation of reporter mRNAs was not observed (30).

To determine whether proteins can bind specifically to AUG codons surrounded by optimal start site sequences, site-directed cross-linking experiments were performed. It was found that at least two proteins, 50 and 100 kDa in size, could be specifically cross-linked to a 4-thiouridine-substituted AUG in C RNAs. Subsequently, the La autoantigen, 50 kDa in size, was found to be at least one component of this 50-kDa species.

A precedent for the involvement of La in translational initiation comes from two previous studies. Several AUG triplets in poliovirus mRNA that are not used as start codons during infection are used as translational initiation codons in rabbit reticulocyte lysates (12). Meerovitch and colleagues reported that purified La antigen can restore the preferential use of the correct AUG initiation codon in poliovirus mRNA during translation in rabbit reticulocyte lysates (32). Thus, La can function as a translation start site correction factor. La has also

been shown to alleviate the translational repression mediated by TAR, which is located in the 5′ noncoding region of human immunodeficiency virus type 1 mRNA (41). These findings provide examples in which a protein known to be involved in the formation of polymerase III transcripts in the nucleus (15, 36) also participates in translation of viral mRNAs. Eight to 12% of the total amount of La is found in the cytoplasm of HeLa cells (17).

The translational competition assays and RNA binding studies have indicated a role of La in AUG start site selection. However, several other proteins could be cross-linked with high affinity to C RNAs as well; any of those proteins could have a putative role in AUG start site selection. If La has a role in start site selection, it is likely of modest impact, because rabbit reticulocyte lysates which contain only a small amount of La (32) mediate the efficient translation of mRNAs. Interestingly, we observed a more striking difference in translational efficiencies between C-ORF and NC2-ORF in the HeLa lysate than in the rabbit reticulocyte lysate (30). It is possible that the small amount of La in the rabbit reticulocyte lysate is sufficient to mediate a moderate level of AUG start site selection in this system. To address this possibility, purified recombinant La was added to the rabbit reticulocyte lysate as described previously (32), and translation of C-ORF and NC2-ORF was monitored. However, the addition of La did not change the pattern of translation of C-ORF or NC2-ORF RNAs (30). This finding suggests that La-C RNA interactions are not involved in start site selection in the rabbit reticulocyte lysate system, possibly because of the fact that translation of C-ORF and NC2-ORF RNAs is already quite efficient in this system. Indeed, mRNAs are translated very much more efficiently in the rabbit reticulocyte lysate than in most other translation systems, such as lysates prepared from cultured cells. Thus, a stimulating or enhancing role of proteins in translational initiation may not be discovered in the rabbit reticulocyte system. Alternatively, a La-associated factor(s), which was absent in the preparation of purified recombinant La from *Escherichia coli*, may mediate the effect of La on start site selection.

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