

Com, the phage Mu *mom* translational activator, is a zinc-binding protein that binds specifically to its cognate mRNA

(zinc finger/mRNA-binding protein/gel retardation/footprinting)

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ABSTRACT Bacteriophage Mu controls an unusual DNA-modification function encoded by the *mom* gene, which is located in an operon that consists of two overlapping genes. The *com* gene, located proximal to the 5' end of the common mRNA transcript, encodes a polypeptide of 62 amino acids that is required for translation of *mom*. Analysis of the derived amino acid sequence reveals that Com contains zinc-binding finger motifs, suggesting that Com may be a zinc-activated regulatory protein. Atomic absorption analysis showed that there is about one zinc bound per molecule of Com. We have subcloned the *com* gene into an expression vector and thus have overproduced and purified the Com protein. By gel retardation analysis with various ³²P-labeled RNAs (made by *in vitro* transcription with T7 RNA polymerase), we show that Com binds specifically to *com-mom* mRNA. A single C → U substitution mutation, located 26 nucleotides upstream from the *mom* translation start codon, abolishes Com binding. The nature of the Com target sequence was deduced from *in vitro* footprinting analyses. The results are consistent with the existence of a complex stem-loop structure within the overlap of the *com-mom* open-reading-frames. Com binding to its target site results in the destabilization of a proposed translation-inhibitor stem-loop (TIS) to expose the Shine-Dalgarno sequence and *mom* translation initiation codon. This suggests that Com interaction with a specific site on its cognate mRNA alters the mRNA secondary structure to activate translation of *mom*.

Bacteriophage Mu controls an unusual DNA-modification function (1, 2) that protects viral DNA against cleavage by a variety of site-specific DNA endonucleases (3, 4). The *mom* gene is located at the right end of the Mu genetic map in an operon comprising two overlapping genes. The *com* gene, located proximal to the 5' end of the common mRNA transcript, overlaps *mom* (see ref. 5 for review). Com is a trans-acting positive regulator (6) that appears to act at the level of translation of the *mom* open reading frame (ORF) (7, 8). Wulczyn *et al.* (9) used *mom-lacZ* translational fusion plasmids to study the sequence requirements for Com regulation. They proposed a model in which Com binding to a specific region in the *mom* operon mRNA acted to relieve translational repression caused by the presence of a translation-inhibitor stem-loop structure (denoted here as TIS) within the overlap of the *com-mom* ORFs. From an analysis of various deletion mutants, they concluded that the Com target site is located in a short sequence located 5' to the TIS. While our manuscript was in revision, these workers provided additional evidence for their model (10). We have independently approached the study of Com function through an *in vitro* analysis of its interaction with *com-mom* mRNA. We describe a procedure for purifying Com to near homogeneity and show that it is, in fact, a *mom*-specific mRNA-

binding protein. Footprinting studies demonstrate that (i) the secondary structure of the target and TIS is more complex than previously proposed, (ii) Com specifically binds to *com-mom* mRNA at a site 5' to the putative TIS, and (iii) Com binding destabilizes the mRNA secondary structure to expose the Shine-Dalgarno (SD) ribosome-binding sequence and *mom* start codon. Finally, consistent with the presence of zinc-binding finger (11) motifs, physical analyses have revealed that Com does indeed contain a tightly bound zinc ion.[¶]

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* DH5 α [$\Delta(lac-arg)U169\ endA1\ recA1\ hsdR17\ supE\ thi\ gyrA\ relA\ \lambda^R/F' \phi80dlacZ\ \Delta M15$] was from BRL. *E. coli* RZ1032 {= HfrKL PO/45 [*lysA*(61–62)] *dut1 ungl thi1 relA1 Zbd-279::Tn10 supE44*} (12) was from G. Pruss. *E. coli* C600-(pC1857) and plasmid pCP40 (13) were from W. Fiers. Phage M13rv1 (14) and phagemid pGC2 (15) were from R. Myers. Plasmid pT7T3-18 was from Pharmacia.

Enzymes and Fine Chemicals. T4 DNA ligase, T4 polynucleotide kinase, and avian myeloblastosis virus reverse transcriptase (RTase; United States Biochemical); RNase-free DNase, placental RNase inhibitor, and T7 RNA polymerase (BRL); *E. coli* DNA polymerase I Klenow fragment, Sephadex G-100, and cobra venom RNase V1 (CV1) (Pharmacia); [α -³²P]UTP (Amersham); GeneClean (Bio 101, La Jolla, CA); 1-cyclohexyl-3-(2-morpholino)carbodiimide metho-*p*-toluenesulfonate (CMCT) and Sephadex G-50 (Sigma); and dimethyl sulfate (DMS; Aldrich) were purchased from the firms indicated in parentheses. Oligodeoxyribonucleotides used in site-directed mutagenesis were synthesized at the University of Rochester sequencing facility.

Plasmid Construction. Plasmid pLW3 contains an *EcoRI*–*BamHI* fragment, excised from a derivative of the *mom-lacZ* fusion plasmid pMLF-2 (16), subcloned into the corresponding sites of phagemid pGC2. A new *EcoRI* site was introduced into pLW3 by site-directed mutagenesis (Fig. 1) between the *com-mom* promoter and the *com* SD sequence to create pLW3RI. An intact *com* gene along with part of *mom* was excised from pLW3RI with *EcoRI* and *BamHI* and cloned into pCP40 and pT7T3-18 to generate pLW40 and pLW9, respectively (Fig. 1). Transcription of the *com-mom* segment is controlled at the λP_L promoter in pLW40 and at

Abbreviations: CMCT, 1-cyclohexyl-3-(2-morpholino)carbodiimide metho-*p*-toluenesulfonate; CV1, cobra venom RNase V1; DMS, dimethyl sulfate; ORF, open reading frame; nt, nucleotide(s); SD, Shine-Dalgarno; TIS, translation-inhibitor stem-loop; wt, wild type.
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[¶]A preliminary report of these findings was presented at the Cold Spring Harbor meeting on the Molecular Genetics of Bacteria and Phages, Aug. 21–26, 1990, Cold Spring Harbor, NY.

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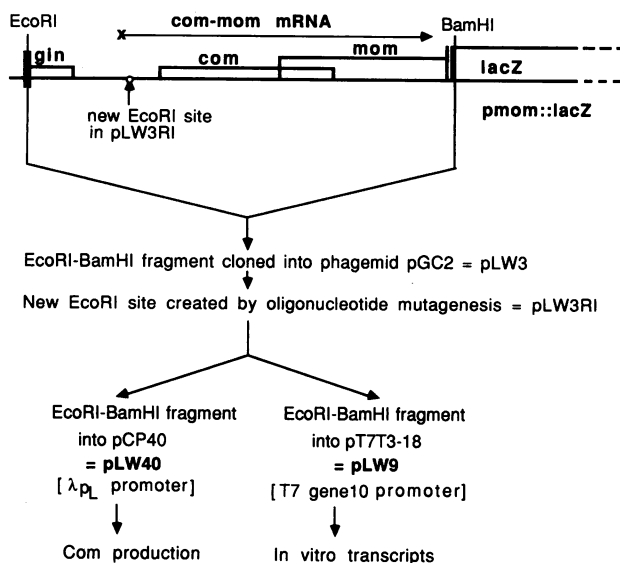


FIG. 1. Strategy for construction of recombinant plasmids for overproduction of Com and for *in vitro* synthesis of labeled Mu *com-mom* mRNA transcripts. See *Materials and Methods* for details.

the phage T7 gene 10 promoter in pLW9. In pLW40, expression from λP_L is regulated by the thermolabile cI857 repressor encoded by plasmid pcI857.

Plasmid pLW9 Δ Bsm was derived as follows. pLW9 was digested with *Bsm* I, which cleaves this plasmid at only two sites, both located within the *mom* gene and separated by about 50 nucleotides (nt). The resulting "overhangs" were removed by S1 nuclease digestion and the plasmid was recircularized by ligation. The C \rightarrow U substitution at position -26 was produced by oligonucleotide site-directed mutagenesis (12).

Labeling of *In Vitro* mRNA Transcripts. Plasmid DNA was cleaved with *Sal* I and the unit-length fragment was purified. *In vitro* production of labeled transcripts with T7 RNA polymerase was according to the supplier's instructions. The wild-type (wt) and -26 U transcripts are 369 nt long, whereas the Δ Bsm transcript is 319 nt. The 5' end of each transcript contains an additional 9 nt (contributed by the vector) compared with the native *com-mom* mRNA. The entire *com* ORF and approximately one-third of the *mom* ORF (5' end) are present on each transcript.

Gel Retardation Analysis. Com binding to labeled RNA was monitored by gel retardation analysis. The binding reaction mixture (10 μ l) contained 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 0.6 ng of ³²P-labeled RNA (\approx 1670 cpm/ng; 5 nM final concentration), 12 ng of yeast tRNA, and various concentrations of Com (0.054–0.27 μ M). After addition of Com to start the reaction, incubation was at 22°C for 10 min, at which time 2 μ l of loading buffer (0.25% bromophenol blue/0.25% xylene cyanol/15% Ficoll) was added. Samples were subjected to 5% PAGE with 89 mM Tris/89 mM boric acid at 22°C for 20 hr at 30 V.

Zinc Analysis. Atomic absorption spectroscopy using an Instrumentation Laboratory (Lexington, MA) IL 57 spectrometer was performed by T. Pan in the laboratory of J. E. Coleman.

Footprinting and Secondary Structure Analyses. Footprinting and secondary structure analyses were carried out using CMCT, DMS, and CV1 as probes. Reaction mixtures (50 μ l; 50 mM Tris-HCl, pH 7.6/50 mM NaCl) contained 150 ng of wt RNA (25 nM); see Fig. 5 for concentrations of Com and agents. After 10 min of incubation with Com at 22°C, DMS, CMCT, or CV1 was added (10 mM MgCl₂ was included for

CV1 digestion). The reactions were quenched in stop buffer after 60 min (final concentrations, 250 mM Tris-HCl, pH 7.6/500 mM NaCl/500 mM 2-mercaptoethanol/0.25 mM Na₂EDTA) and extracted successively with phenol and chloroform/isoamyl alcohol, 24:1. The RNA was recovered after ethanol precipitation and centrifugation. The pellets were dried *in vacuo* and suspended in 2.1 μ l of primer extension mixture (12 mM Tris acetate, pH 7.4/70 mM NH₄Cl/7 mM 2-mercaptoethanol with 2 ng of end-labeled sequencing primer). After heating for 3 min at 65°C, the primer was annealed during rapid cooling at -80°C over 30 min. Following the addition of dNTPs (0.37 mM final for each) and Mg(OAc)₂ (1 mM final), primer extension was started by the addition of 6 units of reverse transcriptase. After incubation for 15 min at 37°C, the reaction was terminated in standard formamide/dye loading buffer with 30 mM Na₂EDTA. The samples were heated for 3 min at 95°C and subjected to 5% PAGE with 8 M urea. Control sequencing reactions were carried out in parallel using pLW9 RNA (200 ng) and standard dideoxy-NTP/dNTP mixtures.

RESULTS AND DISCUSSION

Overproduction and Purification of Com. *E. coli* DH5 α containing plasmids pLW40 and pcI857 was grown in drug-selection medium at 28°C to an OD₅₅₀ of 0.5. The temperature was raised to 42°C and incubation continued for 3 hr. All further operations were at 4°C. After low-speed centrifugation the cells were washed and resuspended in 20 mM Tris-HCl, pH 8.0/5 mM MgCl₂/7 mM 2-mercaptoethanol/1 mM Na₂EDTA/1 mM phenylmethylsulfonyl fluoride. Following sonication and low-speed centrifugation, the pellet (which contained Com, presumably in inclusion bodies) was suspended in S buffer [20 mM Tris-HCl, pH 8.0/1 mM Na₂EDTA/25 mM NaCl/7 mM 2-mercaptoethanol/5% (vol/vol) glycerol]. After washing the pellet, Com was solubilized in HS buffer (S buffer with 1 M NaCl). The suspension was clarified by centrifugation; Com was precipitated in 70% saturated (NH₄)₂SO₄ and harvested by centrifugation. The resulting pellet was resuspended in HS buffer. Samples (1–1.5 ml) were subjected to gel filtration through a Sephacryl S-100 column (2.5 \times 60 cm) equilibrated in MS buffer (S buffer with 0.5 M NaCl). Selected Com-containing fractions (at least 95% pure by SDS/PAGE and Coomassie blue staining; data not shown) were pooled, concentrated by precipitation in 80% saturated (NH₄)₂SO₄, and stored in HS buffer at -80°C. Com retained full RNA-binding activity for at least 6 months. Amino-terminal sequence analysis was consistent with the derived sequence predicted for Com. The yield was about 7 mg of Com per liter of cells based on the assay of Bradford (17). However, amino acid composition analysis showed that the Bradford assay overestimated Com concentration by a factor of 2.5; hence, the protein concentrations given in the text and figure legends represent corrected values.

Com Contains Equimolar Bound Zinc. Potential zinc-binding finger motifs have been noted in a large number of proteins, including *E. coli* aminoacyl-tRNA synthetases as well as the gene 32 and UvrA proteins (11, 18). Examination of the derived amino acid sequence revealed that there are two such motifs present in Com. The first is CX₂CX₁₁HX₄C (or H), where C and H are the single-letter designations for cysteine and histidine, respectively; X denotes any amino acid. As seen in Fig. 2A, there are two such sequences in Mu Com, arranged in tandem. One is CX₂CX₁₁HX₄C (residues 6–26) and the other is CX₂CX₁₁HX₄H (residues 26–46). In the latter, CX₂C is Cys-Pro-Arg-Cys, the same motif found in the *E. coli* isoleucyl-tRNA synthetase, which is known to bind its cognate tRNA.

The second motif is CX₂CX₁₆CX₂CX₉CX₇C (Fig. 2B; residues 6–47) belonging to the C_x family (19). The sequence

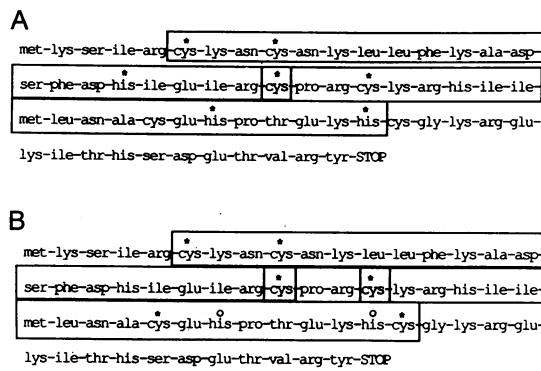


FIG. 2. Zinc-binding finger motifs in Mu Com. The entire derived 62-amino acid sequence is shown. (A) Motifs in which both the cysteine and histidine side chains are used to coordinate a single zinc ion. Two mutually exclusive, overlapping (at Cys-26) motifs are boxed; stars denote the cysteine and histidine residues capable of serving as zinc ligands. The cysteine residue common to both motifs is highlighted by stippling. (B) Motif in which cysteine residues only (stars) are used to coordinate two zinc ions. The cysteine residues that would serve as shared ligands are highlighted by stippling. A variation of this motif is one in which His-41 and His-46 (denoted by circles) are utilized in place of the flanking cysteine residues.

between the first and fourth cysteine residues is identical in loop length to the motif found in *E. coli* isoleucyl-tRNA synthetase, which binds zinc ion (20). However, two zinc ions could be bound if Cys-26 and Cys-29 were used as shared ligands to both ions. A variation of the second motif is CX₂CX₁₆CX₂CX₁₁HX₄H (residues 6–46); in Fig. 2B the carboxyl-terminal histidine residues, 41 and 46, are denoted with circles.

In view of these considerations an atomic absorption analysis of purified Com was carried out. The results indi-

cated 0.9 mol of zinc per mol of Com (data not shown). The presence of tightly bound zinc was unambiguously demonstrated by x-ray absorption fine spectroscopy, which showed a strong zinc edge absorption at 9.659 keV as well as detailed fine structure (J. Penner Hahn, R. Witkowski, G. McClendon, and S.H., unpublished work).

Com Binds Specifically to *com-mom* mRNA. To analyze whether Com interacts specifically with *com-mom* mRNA, we carried out gel retardation assays with defined labeled mRNAs made by *in vitro* transcription with T7 RNA polymerase. Binding to wt mRNA was compared with binding to an internal deletion derivative, ΔBsm (see structure a in Fig. 3); the latter serves as a negative control because it lacks part of the sequence required for Com recognition (9). As predicted, Com formed a specific complex with wt RNA, but not with ΔBsm RNA (Fig. 4A). This suggests that Com binds specifically to *com-mom* mRNA and that some internal sequence is required for target recognition and binding. This was confirmed by the inability of Com to bind mRNA containing the single-base substitution -26 C to -26 U, located 25 nt 5' to the *mom* start codon (Fig. 4B).

It should be noted that the transcripts used in these assays were not full-length *com-mom* RNAs; however, the results obtained with them indicate that they serve as a good model system for studying Com interaction with the native transcript.

Footprinting and Secondary Structure Analyses. Wulczyn *et al.* (9) proposed a model in which Com binding to a specific target site on the *com-mom* mRNA destabilizes an adjacent stem-loop structure (Fig. 3, structure a) to expose the SD sequence and *mom* start codon; more recently (10), the presence of an additional stem-loop was proposed (Fig. 3, structure b). Our analysis suggests the existence of a more complex structure (Fig. 3, structure c), which has a calculated ΔG about 1.4 kcal/mol lower than that of structure b. To

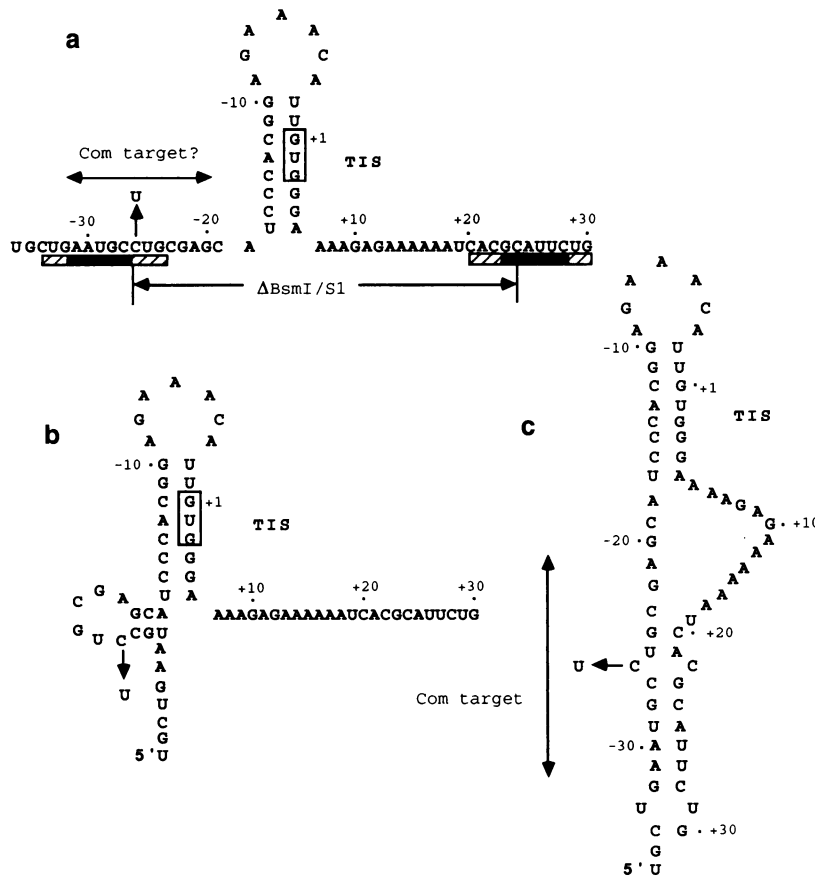


FIG. 3. Nucleotide sequence and possible secondary structures in the mRNA within the *com-mom* ORF overlap. Structure a is the structure originally proposed (9) to contain the Com target site and the adjacent stem-loop, denoted TIS, which inhibits *mom* translation initiation. The likely *mom* ORF start codon, GUG, is boxed. It is about 161 nt downstream from the normal *com-mom* mRNA transcription initiation site. The numbering of bases is with respect to the 5' G (+1) in the start codon. The hatched rectangles underscore a pair of 11-mer inverted repeats (9 of 11 nt match) not noted in earlier analyses (9, 10); the solid portion of the rectangle underscores a perfect hexameric repeat. The extent of the deletion produced by Bsm I and S1 nuclease treatments and the -26 C to -26 U substitution are indicated. Structure b is a recently proposed (10) modification of structure a. Structure c is a third possible secondary structure. The 11-mer inverted repeats create an additional stem region (containing the putative Com target) adjacent to the TIS.

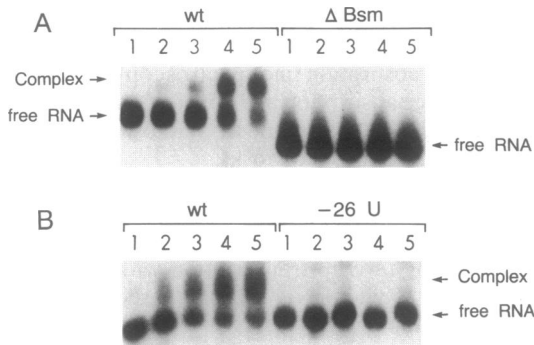


FIG. 4. Gel retardation analysis of Com binding to *com-mom* mRNAs. Various amounts of purified Com were incubated with ^{32}P -labeled wt, -26 U (369 nt), or ΔBsm (319 nt) RNAs. Reaction mixtures were subjected to PAGE and the dried gels were autoradiographed. Positions of free RNA and Com-RNA complexes are indicated. Lane 1, no Com; lane 2, $0.054\ \mu\text{M}$ Com; lane 3, $0.108\ \mu\text{M}$ Com; lane 4, $0.162\ \mu\text{M}$ Com; lane 5, $0.27\ \mu\text{M}$ Com.

better understand the sequence and/or structure elements required for Com interaction with *com-mom* mRNA, we carried out *in vitro* footprinting analyses using chemical probes (CMCT and DMS) and a secondary structure-specific RNase (CV1). In unpaired regions, CMCT modifies N1 of guanine and N3 of uracil (with a preference for U), whereas DMS modifies N3 of cytosine and N1 of adenine (with a preference for A); CV1 cleaves without base specificity within paired regions, generating 3'-hydroxyl and 5'-phosphoryl termini. Sites modified with CMCT or DMS can be identified by (end-labeled) primer extension with reverse transcriptase, which terminates 1 nt prior to the modified residue. Following PAGE analysis, these bands are displaced by 1 nt (shorter) relative to corresponding fragments terminated in the control dideoxy sequencing lanes (21, 22).

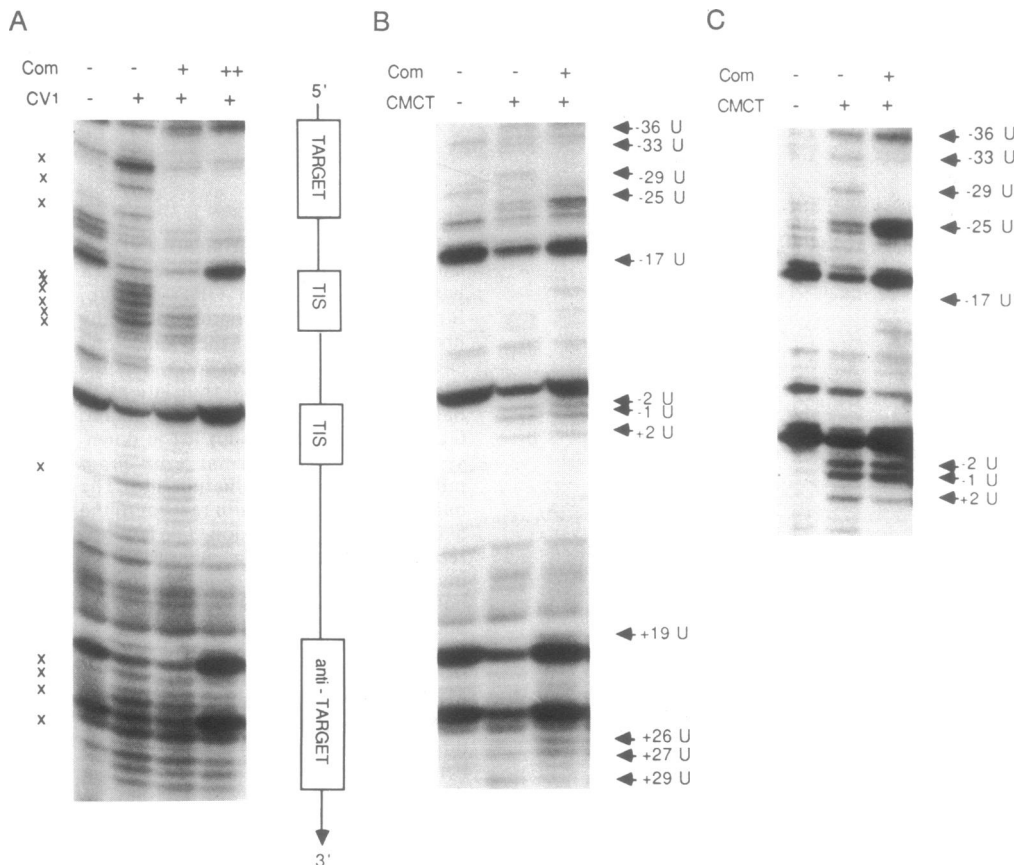


FIG. 5. CMCT and CV1 probing of *com-mom* mRNA in the presence and absence of Com. Various amounts of purified Com were incubated with wt *com-mom* mRNA (25 nM) in the presence of CV1 (4.5×10^{-4} units/ml (A) or CMCT (1 mg/ml and 2 mg/ml in B and C, respectively) (for CV1 cleavage, 10 mM $\text{Mg}(\text{OAc})_2$ was included in the reaction). Com was at 250 nM (+) or 500 nM (++)). CV1 sites cleaved only in the absence of Com are indicated (x). The results of two independent CMCT-modification analyses are shown in B and C; for convenience, the sequencing gel lanes are omitted, but the U positions are indicated. Anti-target denotes the sequence complementary to the one protected by Com binding.

To begin analyzing possible secondary structure(s) within the *com-mom* overlap, we first used CV1 as a probe of paired regions. In the absence of Com, CV1 cleaved sites in regions corresponding to both the Com target and the TIS (Fig. 5A). This indicates there must be base pairing in both of these regions, providing strong evidence for structure c. In contrast, when Com was present, CV1 cleavage at these sites was inhibited. These results do not distinguish between the possibility that Com binding destabilizes the paired regions or that Com binds to and shields them from CV1 cleavage. Moreover, the results do not indicate where Com actually binds.

Therefore, we carried out a footprinting analysis using CMCT as the probe. This agent modified primarily U residues at a variety of sites in both the target and TIS regions (Fig. 5B). Because CMCT modifies unpaired bases, the results suggest that structure c must be in equilibrium with unpaired structures. [In this regard, Wulczyn and Kahmann (10) observed that G residues located in the TIS and target sites were sensitive to cleavage (to varying degrees) by T1 RNase; since this enzyme cleaves only at unpaired G residues, their results also support such an equilibrium.] However, when Com was present, there were CMCT-reactivity enhancements at -25 U, +26 U, and +27 U. In contrast, -33 U, -29 U, and -17 U remained unreactive, despite destabilization of the target stem and TIS; we suggest that Com makes direct contact with these residues. This should be contrasted with -36 U, which was always sensitive to CMCT. Although U residues at -2, -1, and +2 were weakly reactive in the absence of Com and showed minimal enhancement after addition of Com, the results are consistent with structure c being the major species in the equilibrium mixture. The protection afforded by Com at -33 U and -17 U is not clearly visible in Fig. 5B; however, an excerpt of results from a separate experiment shows this protection quite nicely (Fig. 5C). We conclude that the 5' boundary of the Com

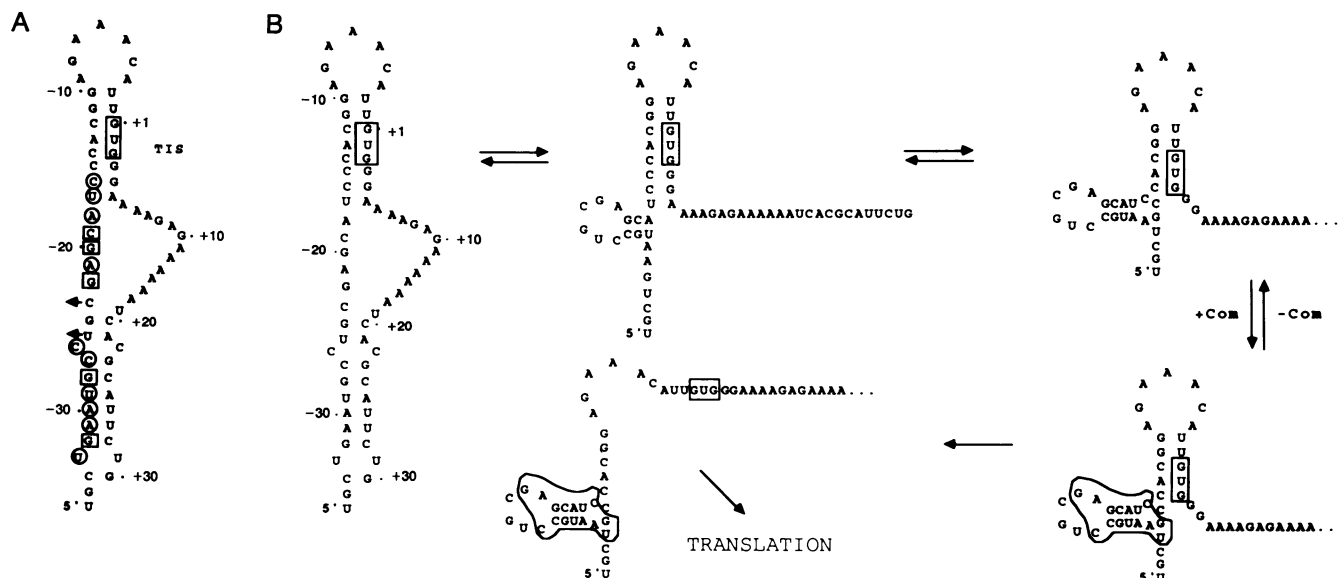


FIG. 6. (A) Com contact sites deduced from chemical footprinting analyses. Small arrows are at sites whose reactivity to CMCT or DMS was enhanced by Com; therefore, they are excluded as contact sites. Circled letters denote bases protected against the above agents by addition of Com. Letters in squares denote Com-protected residues (10) for which we had no data. (B) Model describing Com interaction with *com-mom* mRNA. Structure c is in equilibrium with a variety of possible stem-loop structures. Com binds to one that is similar to structure b (see Fig. 3), except for a slightly longer stem. This melts the shortened TIS and allows translation. Sites protected by Com binding are enclosed.

target site extends upstream to at least -33 U but stops before -36 U; and the 3' boundary extends downstream to at least -17 U. Analogous footprinting experiments were also carried out with DMS. The results (not shown) are summarized as follows. (i) In the absence of Com, DMS readily modified all A and C residues in the unpaired regions of structure c (except -19 C and -23 C). Residues +28 C, +25 A, +22 C, +21 A, -16 C, -23 C, -26 C, -27 C, -30 A, -31 A, and -34 C, all predicted to be in paired regions of structure c, were weakly (or not at all) reactive with DMS. This was also observed in part by Wulczyn and Kahmann (10), but they did not present data for +20 to +35. (ii) In the presence of Com, there were enhancements of DMS reactivity at +28 C, +25 A, +21 A, -12 C, -14 C, -15 C, and -23 C. These results are consistent with the destabilization of both stems in structure c, making A and C residues accessible to DMS. In the presence of Com the weak reactivity seen at residues -18 A, -21 A, -26 C, -27 C, -30 A, and -31 A was lost, and -16 C remained unreactive, suggesting that Com interacts directly with these bases. Consistent with this inference is the fact that the -26 C to -26 U mutation abolishes Com binding [from the gel retardation assay (Fig. 4), as well from footprinting analysis (data not shown)]. From this we conclude that the 3' boundary of the Com target extends to -16 C.

Based on the protections against chemical modification, a summary of the apparent Com contact sites is illustrated in Fig. 6A. From this it is evident that Com protects only one strand of the target stem in structure c. Thus, we must ask, does Com bind the target stem and destabilize it, or does Com bind some other structure that alters the equilibrium mixture? One model for Com interaction with *com-mom* mRNA is shown in Fig. 6B. Verification of the model awaits analysis of Com binding to transcripts with various mutations in the +20 to +30 region.

To our knowledge, Com is the smallest zinc-binding protein known, and it represents the first example of a translation factor that specifically binds its cognate mRNA and alters the secondary structure without requiring ATP. In this regard, it has been proposed that RNase III stimulates translation of phage λ cIII mRNA by binding to one of two alternative

structures, affecting the equilibrium in favor of the translatable form (23). Although the precise mechanism of Com activation is not known, it is evident that the *mom* story continues to grow in complexity and interest.

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