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IDENTIFICATION OF FUNCTIONAL REGIONS IN THE C-TERMINAL DOMAIN OF *ESCHERICHIA COLI* RIBOSOMAL PROTEIN S1 USING MONOCLONAL ANTIBODIES

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Summary. Monoclonal antibodies specific for defined regions of *E. coli* ribosomal protein S1 were used in a R17 mRNA-directed protein synthesis assay to reveal functionally important sites of the protein. Two distinct sites for mRNA binding were identified in the regions 349-437 and 438-547 located in the C-terminal domain of protein S1.

The *Escherichia coli* ribosomal protein S1 has an essential function in the protein synthesis (1,2). S1 binds to synthetic and natural mRNAs (3-5) and is involved in the formation of the initiation complex (6,7). These properties have been assumed to be responsible for the role of S1 on mRNA binding to the ribosome during the initiation of protein synthesis (1,7). The primary structure of S1 has been elucidated by both gene and protein sequencing (8,9). The protein (557 amino acids) is described as a highly elongated protein organized into two distinct domains with a freely rotatable region in between (10-12). Studies using protein fragments and analysis of deletion mutants have indicated that regions from the C-terminal domain are involved in the functional role of S1 (13,14). The N-terminal domain contains the binding site of the 30S ribosomal subunit (13).

Recently, we have described a panel of antibodies directed against defined regions of protein S1 (15). The location of the epitopes along the polypeptide chain of S1 has been mapped. Most of the antibodies bound to the C-terminal half of the molecule, while only one antibody recognized an epitope located in the N-terminal domain. In this paper, we report on the use of these antibodies of defined specificities to show which domains contribute to the function of S1 in the initiation of protein synthesis.

MATERIALS AND METHODS

Monoclonal antibodies. Cell hybridization and monoclonal antibody production have been described in detail elsewhere (15). MAbs[1] were purified from ascitic fluids using protein A-Sepharose CL-4B (Pharmacia) affinity chromatography essentially as described in (16).

Abbreviations. mAb : monoclonal antibody; IgG : immunoglobulin G; ELISA : enzyme-linked immunoassay; SDS : sodium dodecyl sulphate.

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Antibodies were concentrated by ultrafiltration on a hollow fiber system and by vacuum dialysis, and stored at -20°C in TBS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7). All the mAbs used in this study were of the IgG class. Mapping of the epitope location along the polypeptide chain of S1 was investigated by the use of various fragments and mutant forms of the protein (15).

Protein S1 and 30S ribosomal subunits. They were prepared as described by Hahn *et al.* (15).
Enzyme-linked immunoassay (ELISA). An immunoenzymatic procedure was used to examine the possible release of S1 from the 30S subunit upon mAb binding. Microtiter plate wells (Nunc) were coated for 2 h at 37°C with rabbit anti-30S IgGs prepared as described in (15) and diluted at 2 µg/ml in TC buffer (20 mM Tris-HCl pH 7.5, 6 mM magnesium acetate, 100 mM NH_4Cl, 6 mM 2-mercaptoethanol).Binding sites on the plastic were saturated for 30 min with 1% bovine serum albumin in TC buffer containing 0.05% Tween 20. Equimolar amounts of either 30S subunits (5 µg/ml) or S1 protein (400 ng/ml) in TC buffer were incubated for 2 h at 37°C. Appropriate dilutions of ascite fluids were allowed to react with the antigen and the bound mAbs were detected by anti-mouse IgG sheep immunoglobulins coupled to alkaline phosphatase at a 1/10,000 dilution. Plates were then processed as described in (15).

Analysis of the binding of mAb A9 to the 30S subunit. The effect of mAb A9 was also investigated by a centrifugation technique. 30S subunits (2 A_{260}) were incubated in TC buffer for 1 h at 37°C with 0.01 A_{280} of purified antibody A9. 30S-IgG immunocomplexes were separated from unbound antibodies by centrifugation on a 15-30% sucrose gradient in TC buffer (16 h at 24,000 rpm, 4°C, SW41 rotor). Aliquotes of each gradient fraction were then assayed by classical ELISA procedures essentially as described in (15), using : (i) a rabbit anti-30S antiserum (diluted 1/10,000) for the detection of the 30S peak; (ii) a rabbit anti-S1 serum (1/10,000) to reveal the presence of S1; (iii) anti-mouse IgG sheep immunoglobulins conjugated to alkaline phosphatase (1/10,000) to detect mAb A9. Rabbit antibodies were detected by anti-rabbit IgG goat immunoglobulins coupled to alkaline phosphatase, diluted at 1/1000.

Assay for protein synthesis. Phage R17 mRNA was prepared essentially as described in (17). An MRE 600 *E. coli* cell extract prepared according to Goldmann and Gibel (18) was used for the *in vitro* translational assay. R17 mRNA-dependent protein synthesis was carried out in 100 µl of 50 mM Tris-HCl, pH 7, 10 mM magnesium acetate, 50 mM NH_4Cl, 1 mM dithioerythritol, 1.6 mM ATP, 0.3 mM GTP, 8 mM phosphoenolpyruvate, 50 µg/ml pyruvate kinase, 30 µM each of 19 unlabeled amino acids, 20 µM [^{14}C]-valine (125 Ci/mol, CEA), 13 A_{260}/ml cell extract, and 150 µg/ml R17 mRNA, as modified from (19). Before the addition of labeled valine and phage mRNA, the mixture was preincubated for 30 min at 37°C to decrease the background. R17 mRNA-directed amino acid incorporation was then allowed for 30 mn at 37°C. Hot trichloracetic acid insoluble radioactivity recovered on Whatman 3MM paper from 60 µl of reaction mixture was then determined by scintillation counting in Ready Solv-NA (Beckman).

Electrophoretic analysis of in vitro translational products. Increasing amounts from a 25 µl translational assay mixture containing 50 µCi of [^{35}S]-methionine (810 Ci/mmol, Amersham) were heated for 3 min at 100°C in SDS-containing sample buffer and directly analyzed by electrophoresis in a 16% acrylamide/SDS gel (20). The gel was then stained with Amidoblack, dried and autoradiographed for 9 h.

Inhibition of translation by mAbs. Increasing amounts of purified antibodies were added to the translational reaction mixture before the addition of the labeled amino acid and the mRNA, and incubated for 20 min at 20°C. Translation was then achieved as described above.

RESULTS

The properties of a series of monoclonal antibodies reacting with different epitopes in ribosomal protein S1 have been described previously (15). We have shown that all the antibodies recognized their epitope on S1 bound to the 30S subunit in solution. In order to ensure that the antibodies do not cause the release of S1 from the ribosome, their binding to the subunit has been further investigated by an ELISA procedure. Each mAb was incubated with

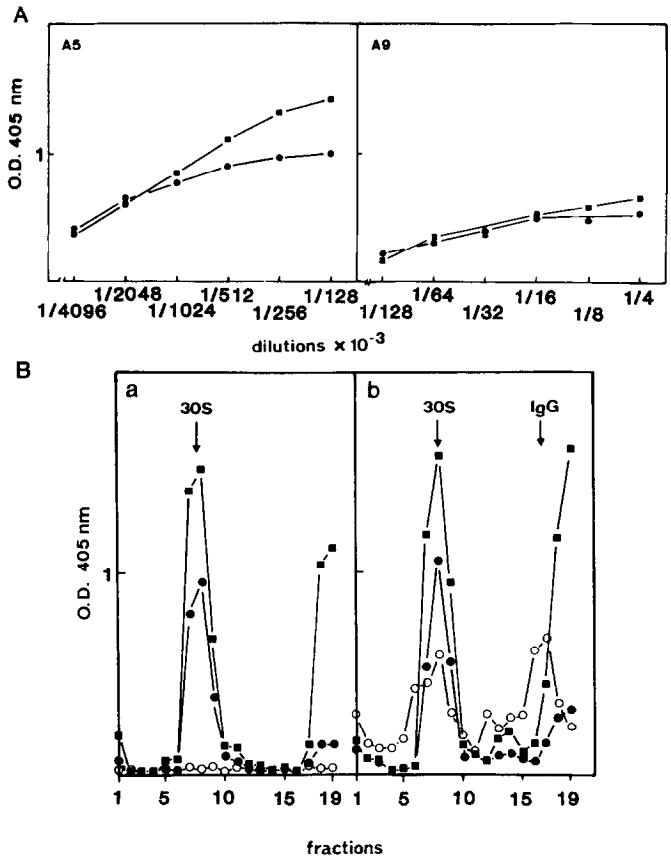

Figure 1. Analysis of the possible release of S1 from the 30S subunit upon mAb binding.
A : effect of the binding of mAbs A5 and A9 as tested by an ELISA procedure (see the Results section); ●—●: protein S1 as antigen; ■—■: 30S subunits as antigen.
B : analysis of the binding of mAb A9 by sucrose gradient centrifugation; aliquotes of each fraction were analyzed by ELISA assays :■—■localization of the 30S subunit peak by a rabbit anti-30S antiserum; o—o detection of mouse mAb A9 by enzyme-conjugated anti-mouse IgG sheep immunoglobulins; ●—● detection of S1 by a rabbit anti-S1 antiserum.
(a) 2 A_{260} of 30S subunits not incubated with mAb A9; (b) 2 A_{260} of 30S subunits incubated in the presence of mAb A9.

30S particles bound to a first layer of anti-30S rabbit IgGs. If the tested anti-S1 mAb causes the loss of S1, immunocomplexes will be washed off during microtiter plate processing. Conversely, if S1 release does not occur, the bound mAb can easily and specifically be detected by an anti-mouse secondary antibody. Nevertheless, a significant fraction of the polyclonal anti-30S antibodies used for coating the plate wells may consist of anti-S1 IgGs that can react with S1 associated to the subunit. This may lead to mAb binding and give appreciable background. The fraction of mAbs bound to S1 directly immobilized by the polyclonal IgGs was evaluated by the same ELISA assay, but using S1 as the antigen. S1 release due to mAb binding to the subunit should give absorbance values close to this background. Higher values will indicate that the binding of the mAb has no effect on the binding of S1 to the 30S subunit.

This study demonstrated that mAbs 7, A5, A1, A6, 20, 22, 14, 4x8 and 3x11 did not cause the release of S1. Typical experimental curves for mAbs A5 and A9 are shown in Fig.1A.

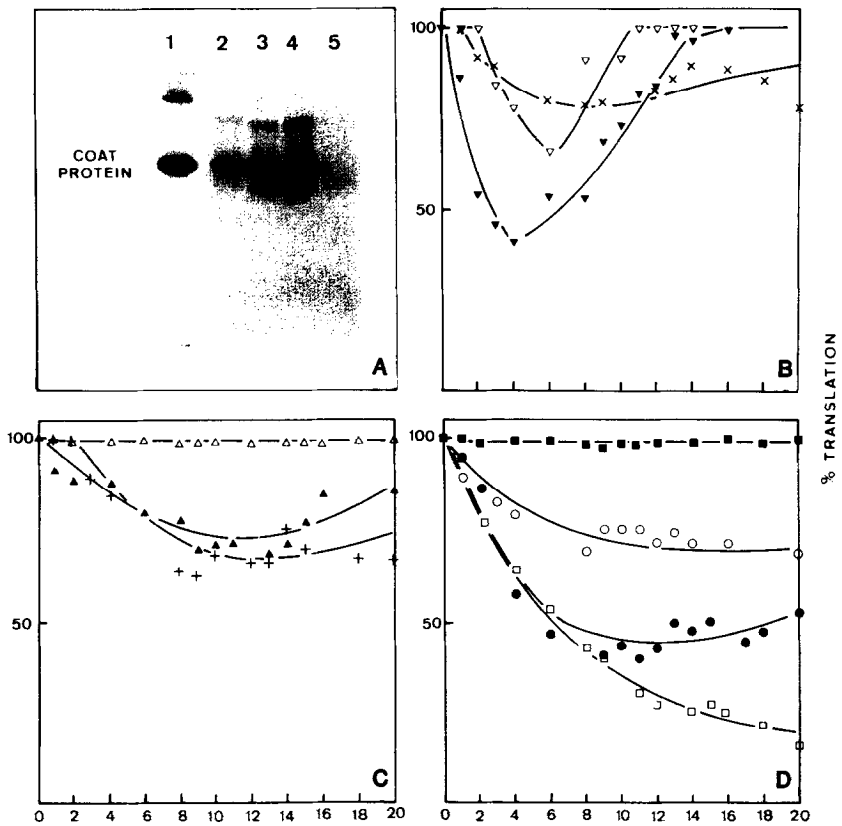

Figure 2. Effect of the anti-S1 mAbs on *in vitro* protein synthesis directed by R17 RNA. A : SDS-polyacrylamide gel electrophoresis of *in vitro* translational products; lane 1 : R17 coat protein obtained by dissociation of 100 µg of R17 phage; lanes 2-4 : autoradiogram of [^{35}S]-labeled translation products (5, 7.5 and 10 µl of reaction mixture); lane 5 : control experiment performed without R17 RNA. **B, C and D** : inhibition of translation by mAbs : ×—×A9; ▽—▽ 20; ▼—▼ 14; △—△ 22; ▲—▲ A6; +—+ 3x11; ■—■ 7; ○—○ A1; ●—● 4x8; □—□ A5. In the absence of mAb, about 6000 cpm were incorporated in precipitable material, the blank value of the assay being 300 cpm.

Results for mAb A9 suggest that the binding of this antibody induces the release of S1. An alternate explanation would be that the epitope for mAb A9 is less accessible for antibody binding when the subunit is immobilized by anti-30S IgGs. The effect of mAb A9 was further investigated by a centrifugation technique. Purified antibody A9 was incubated with 30S subunits and the immunocomplexes were analyzed on a sucrose gradient. The fractions were tested for the presence of mAb A9, 30S particles and S1 by ELISA as described in the Methods section. Fig. 1B shows that anti-mouse IgG sheep immunoglobulins reveal the presence of mAb A9 bound to the 30S subunits. The use of polyclonal anti-S1 antibodies also demonstrated that S1 remains associated to the 30S subunits.

To identify the regions which are involved in the function of S1, 10 purified mAbs of defined specificities were tested for their effect on *in vitro* R17 mRNA-directed protein synthesis. The results in Fig. 2A clearly demonstrate that the major product of translation was the coat protein of phage R17, thus indicating that our *in vitro* translational assay was operating properly (17). As shown in Fig. 2, protein synthesis was inhibited at different extent when the

antibodies were preincubated with the reaction mixture before completing the assay. MAbs 7 and 22 had no effect on protein synthesis (Fig. 2C and D) and therefore demonstrate the specificity of the inhibition observed with the other antibodies. Five mAbs (A9, 20, A6, 3x11 and A1) caused only a 20 to 30% decrease of the translational activity (Fig. 2B, C and D). Antibody 20 had a peculiar effect, since it inhibited protein synthesis at low concentrations, but was inefficient at higher concentrations (Fig. 2B). The same result was observed with mAb 14 which had a strong inhibitory effect only at low concentrations (Fig. 2B) . A tentative explanation would be the increasing precipitation of these 2 IgGs during their preincubation in the translational reaction mixture. Strong inhibition (up to 80%) was observed for mAbs A5 and 4x8 (Fig. 2D).

DISCUSSION

Ten monoclonal antibodies were used to define functionally important domains of protein S1 in the ribosome. They have been shown previously to have epitopes in different regions of the protein (15). MAb A9 binds to the N-terminal region (residues 1-193). The epitope for mAb 7 lies within a narrow region of 17 amino acids (332-348). The epitopes recognized by mAbs A1, A5, A6 and 20 are located between residues 349 to 437. MAbs 14 and 22 bind to the C-terminal domain of S1, most likely in the region 438-547. The epitopes for mAbs 4x8 and 3x11 both map in a short region of 13 amino acids at the C-terminal end of S1 (535-547).

When added to an *in vitro* assay for protein synthesis directed by a natural mRNA, 7 out of the 10 mAbs did not show significant inhibitory effect. Thus, regions which bind mAbs 7, 22, A9, A1, A6, 20 and 3x11 do not participate intimately to the S1 function. The low inhibition observed with some mAbs can be explained by steric hindrance due to the presence of a bound IgG molecule. MAb 14 had a significant effect on protein synthesis (up to 50 %), indicating that the region which forms its epitope in the C-terminal domain (438-547) is involved in the function of S1. MAb 22 which binds close to mAb 14 (15) showed no inhibitory effect. Significant inhibition (60%) was also observed with mAb 4x8 but not with mAb 3x11, albeit the 2 IgGs bind to overlapping sites at the C-terminal end of S1 (15). Since we have shown that the binding of mAb 4x8 induces conformational changes at non-adjacent sites in S1 (15), it is most likely that this inhibition was due to changes in the protein structure which may have affected functional sites. MAb A5 strongly inhibited the translation of R17 RNA. Epitope for mAb A5 which lies between residues 349 to 437 therefore corresponds to a major functional site of S1. It is noteworthy that a distinct antigenic domain defined by mAbs A1, A6 and 20 and located in the same region as the epitope for mAb A5 (15) is not directly involved in the function.

S1 plays a key role in the binding of mRNA during the initiation of protein synthesis. Earlier studies using protein fragments have shown that the region 224-309 provides a binding site for poly(U) (13). Our study identifies two additional and distinct sites for mRNA binding that are located in the regions 349-437 and, most probably, 438-547. Both sites belong to the C-terminal domain which is thought to be the functional domain of S1. Our results correlate well with the following observations : (i) directed mutagenesis has shown that the region

320-430 is essential for the *in vivo* functioning of S1 (14); (ii) cysteine residues at positions 292 and 349 may be part of or in close proximity to the poly(U) binding site (21); (iii) ribosomes reconstituted with an S1 protein fragment (1-348) are inefficient in translating natural mRNA (19); (iv) the mutant protein m1S1 (1-437) is less active in protein synthesis than S1 (13).

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