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Clo DF13 Plasmid Deoxyribonucleic Acid-Directed In Vitro Synthesis of Biologically Active Cloacin DF13 and Clo DF13 Immunity Protein

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Clo DF13 plasmid deoxyribonucleic acid (DNA) was used as a template to direct transcription and translation in a DNA-dependent cell-free system prepared from *Escherichia coli*. Analysis of the in vitro products on sodium dodecyl sulfate-polyacrylamide gels revealed that Clo DF13 DNA directs the synthesis of at least 10 polypeptides ranging in molecular weight from approximately 7,000 to 70,000. Two of these polypeptides could be identified, with respect to their physicochemical and biological characteristics, as the products of the Clo DF13 genes coding for cloacin DF13 and Clo DF13 immunity protein. These results confirm previous findings, obtained with Clo DF13-harboring minicells of *E. coli*, that the structural genes for the latter proteins reside on the Clo DF13 genome.

The bacteriocinogenic plasmid Clo DF13 is an extrachromosomal circular deoxyribonucleic acid (DNA) molecule that originates from *Enterobacter cloacae* (17). Most studies on this plasmid have been carried out in *Escherichia coli*, which can serve as a host for this plasmid as well (20). The nonconjugative Clo DF13 plasmid (molecular weight, 6×10^6) is present in about 10 copies per *E. coli* cell (7). As revealed from studies with cloacinogenic minicells of *E. coli*, the Clo DF13 plasmid directs in vivo the synthesis of at least four messenger ribonucleic acid (RNA) species and eight polypeptides (9). Hitherto the biological functions of only two of these polypeptides, cloacin DF13 (molecular weight, 70,000) and Clo DF13 immunity protein (molecular weight, 10,000), are known. Cloacin DF13 inhibits protein synthesis of sensitive bacterial cells by cleaving 16S ribosomal RNA at a unique site near its 3′ end (2).

Because in cloacinogenic cells an immunity protein is present in addition to cloacin, cloacinogenic cells are immune to the action of their homologous cloacin (6). It has been demonstrated that this immunity protein inhibits the action of cloacin by the formation of a complex with it (6, 8). With the exception of these two proteins, nothing is known about the biological characteristics of the other plasmid-specific proteins.

The aim of our current investigation is to isolate and characterize the gene products of the bacteriocinogenic plasmid Clo DF13 and to study their role in the process of DNA replication and gene expression.

In a first approach we studied these processes in vivo by using chromosomeless minicells of *E. coli* (6-9, 18, 19). However, for the elucidation of the possible role of plasmid-specific gene products in gene expression, an in vitro system might be very useful. For this reason the bacteriocinogenic plasmid Clo DF13 was used to direct coupled transcription and translation in a DNA-dependent cell-free system prepared from *E. coli*. The latter system has already been proven to be very useful for the characterization and identification of the gene products encoded by bacteriophage M13 replicative form DNA (10-13).

In this paper it is demonstrated that Clo DF13 plasmid DNA directs in vitro the synthesis of at least 10 polypeptides. Two of these polypeptides could unequivocally be identified, with respect to their biological and physicochemical characteristics, as the products of the genes coding for cloacin DF13 and the Clo DF13 immunity protein.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strain K-12 AB301 (*F*$^+$, λ^+, *met*$^-$, *his*$^-$, *RNase I*$^-$) was used for the preparation of the DNA-dependent protein-synthesizing system. Cloacinogenic minicells were isolated from *E. coli* K-12 P678-54 (Clo DF13 *rep3*). *Klebsiella edwardsii* var. *edwardsii* N4015 was used as indicator strain for the in vivo cloacin assay.

Isolation of Clo DF13-specific polypeptides syn-

thesized in vivo. In vivo-synthesized Clo DF13-specific polypeptides were isolated from cloacinogenic minicells. Minicells, harboring the mutant plasmid Clo DF13 *rep3*, were isolated, labeled with ^{14}C-amino acids, and lysed as described by Kool et al. (9). It has been demonstrated that these minicells harbor more copies of the (mutated) plasmid genome than minicells harboring the wild-type plasmid (7). The increase in number of plasmid DNA molecules results in the formation of larger amounts of plasmid-specific polypeptides, which in turn facilitates the detection of these products.

DNA-dependent in vitro protein synthesis. The reaction mixture for the in vitro transcription-translation coupled system was the same as described by Konings et al. (11). A typical reaction mixture for the synthesis of Clo DF13 proteins contained, per milliliter: tris(hydroxymethyl)aminomethane(Tris)-chloride, 50 μmol (pH 8.0); ammonium acetate, 110 μmol; potassium acetate, 50 μmol; magnesium acetate, 14 μmol; dithiothreitol, 2.4 μmol; phosphoenolpyruvate, 20 μmol; adenosine 3′,5′-triphosphate, 2 μmol; cytidine, thymidine, and guanosine 3′,5′-triphosphates, 0.5 μmol each; leucovorin, 0.3 μmol; the nonlabeled amino acids, 0.25 μmol each; pyruvate kinase, 25 μg; transfer RNA (tRNA) (stripped), 1 mg; ribosomes, 1.92 mg; and supernatant protein, 2 mg.

If not stated otherwise the proteins were labeled in vitro with L-[^{35}S]methionine (50 μCi/ml; specific activity, 145 Ci/mmol). Circular double-stranded Clo DF13 DNA was usually added at a concentration of 75 to 100 μg/ml; this is close to a saturating amount for this system. After appropriate incubation for transcription and translation, the reaction mixtures were dialyzed extensively against TMN buffer (10 mM Tris-hydrochloride pH 7.8, 10.5 mM magnesium acetate, 60 mM NH_4Cl, and 2 mM dithiothreitol). Part of the dialyzed solution was used for the analysis of the polypeptides on sodium dodecyl sulfate (SDS)/phosphate-polyacrylamide gels (13), while the remaining part was used for the characterization of the biological activities of these polypeptides.

SDS-polyacrylamide gel electrophoresis. The polypeptides made in vitro were analyzed on SDS/phosphate-polyacrylamide slab gels in the presence of 8 M urea (11). After electrophoresis (16 h at 30 V/gel), the gels were stained at room temperature in a solution containing 0.2% Coomassie brilliant blue (12). After destaining, the gels were dried under vacuum on filter paper and autoradiographed by contact with Kodak RP/R54 X-ray film (11).

In vivo cloacin DF13 assay. In vivo cloacin DF13 activity was measured by spotting, after appropriate dilution, aliquots of 5 μl on a lawn of indicator cells (strain N4015; 7).

Purification of the Clo DF13-specific polypeptides synthesized in vitro. Polypeptides, synthesized in vitro in the presence or absence of Clo DF13 DNA, were fractionated on 10% SDS-polyacrylamide gels according to the procedure previously described (6, 19). Native cloacin DF13 and Clo DF13 immunity protein, a gift from B. Oudega, were always run in parallel, and the corresponding positions in the unstained gels were cut out and macerated with a glass stirring rod in 5 volumes of TMN buffer. Gel particles were removed by centrifugation in an SW50.1 rotor of a Beckman L5-65 ultracentrifuge. The supernatant fraction was extensively dialyzed against TMN buffer and finally concentrated with the aid of Carbowax 4000 (6, 19).

In vitro assay for cloacin DF13 and Clo DF13 immunity protein. The biological activity of in vitro-synthesized cloacin DF13 was measured by determining its ability to inactivate ribosomes in vitro, resulting in an inhibition of the phenylalanine incorporation in a poly(U)-directed protein-synthesizing system (14). The ability of the in vitro-synthesized polypeptides to abolish this inhibitory effect was used as an "in vitro" Clo DF13 immunity protein assay (6). Generally, aliquots of the in vivo- or in vitro-synthesized polypeptides were incubated, in the presence or absence of either native cloacin DF13 or Clo DF13 immunity protein, together with the components required for in vitro polyphenylalanine synthesis. A typical incubation mixture for the synthesis of polyphenylalanine contained, per milliliter: Tris-chloride, 20 μmol (pH 7.8); ammonium acetate, 60 μmol; magnesium acetate, 10.5 μmol; dithiothreitol, 2 μmol; $tRNA^{phe}$, 22 μg; pyruvate kinase, 66 μg; phosphoenolpyruvate, 6 μmol; adenosine 3′,5′-triphosphate, 11 μmol; guanosine 3′,5′-triphosphate, 2.2 μmol; S-30 extract, 15 units of absorbancy at 260 nm (A_{260} units); supernatant protein, 60 A_{260} units; and aliquots of 35 to 300 μl of the in vivo- or in vitro-synthesized Clo DF13-specific polypeptides.

After a preincubation period of 20 min at 37 C, the incubation mixtures were cooled in ice and 550 μg of poly(U) and 3.8 μCi of [^{14}C]phenylalanine (specific activity, 60 mCi/mmol) were added. Subsequently incubation was continued for another 20 min at 37 C. The incorporation of [^{14}C]phenylalanine was followed by measuring the radioactivity present in hot trichloroacetic acid-insoluble material (6). To inactivate immunity protein, the in vitro- or in vivo-synthesized Clo DF13-specific polypeptides were, where indicated, heated for 20 min at 100 C before addition to the incubation mixture.

Isolation and purification of Clo DF13 plasmid DNA. The method for the isolation and purification of Clo DF13 plasmid DNA from *E. coli* P678-54 (Clo DF13 *rep3*) has been described (19).

DNA-binding assay. Binding of the in vitro-synthesized polypeptides to Clo DF13 DNA was measured with the aid of the nitrocellulose membrane filter technique described by Veltkamp et al. (19). Before addition of the in vitro-synthesized polypeptides to the incubation mixture, they were first heated at 100 C for 20 min. It has been shown that by this treatment the biological activity of the immunity protein is completely destroyed whereas cloacin DF13 is still biologically active (16). After incubation of the in vitro-synthesized polypeptides and Clo DF13 DNA (30 min at 25 C), the reaction mixtures were filtered through nitrocellulose membrane filters (type B6; Schleicher and Schuell, Inc.). The filters were subsequently washed, dried, and counted as described by Veltkamp et al. (19, 21).

RESULTS AND DISCUSSION

In vitro protein synthesis directed by Clo DF13 DNA. Previously we described a DNA-dependent in vitro protein-synthesizing system and showed the synthesis of a number of bacteriophage M13-specific polypeptides (4, 11, 13). This protein-synthesizing system has been used to examine the in vitro synthesis of polypeptides encoded by Clo DF13 plasmid DNA. Specifically we investigated whether the known plasmid-specific proteins, cloacin DF13 and Clo DF13 immunity protein, are made in this in vitro system.

The general requirements for the Clo DF13 DNA-dependent in vitro system are similar to those for other DNAs and for other DNA-dependent systems (1, 15). The magnesium optimum is rather broad, centering on 13 to 16 mM, whereas the NH_4Cl concentration is optimal in the range from 70 to 140 mM. After a short lag period, the incorporation rises linearly for about 20 min and reaches a plateau after about 40 min of incubation. The synthetic activity of the coupled system is destroyed by pancreatic deoxyribonuclease DNase (2.5 μg/ml). When compared with phage M13 replicative form DNA, Clo DF13 DNA is a less efficient template for coupled protein synthesis. Under optimal ionic conditions and with saturating amounts of DNA, the total protein synthesis with Clo DF13 DNA is only about 15% of that with phage M13 replicative form DNA.

Pattern of polypeptides encoded by Clo DF13 DNA. To determine the size and number of the polypeptides synthesized in vitro under the direction of Clo DF13 DNA, the in vitro-synthesized polypeptides were fractionated on SDS/phosphate-polyacrylamide gels (Fig. 1b). For comparison, native cloacin DF13 and Clo DF13 immunity protein (Fig. 1d) as well as Clo DF13-specific polypeptides synthesized in Clo DF13 *rep3*-harboring minicells (Fig. 1c) were run in parallel. From these results it can be concluded that addition of Clo DF13 plasmid DNA to the coupled system results in the formation of about 11 polypeptides (numbered I through XI) with molecular weights ranging from 7,000 to 70,000. Infrequently the (weak) synthesis of a polypeptide migrating between bands VIII and IX and co-migrating with one of the major in vivo-synthesized Clo DF13-specific polypeptides (Fig. 1c) could also be demonstrated.

At least four of the in vitro-synthesized polypeptides (polypeptides I, II, IX, and X) co-migrated with Clo DF13-specific polypeptides isolated from Clo DF13 DNA-harboring (mini)-cells (Fig. 1c, and d). The electrophoretic mobilities of polypeptides IX and X were almost identical to the electrophoretic mobilities of two of the polypeptides synthesized in the endogenous system (Fig. 1a). The polypeptide band X in the endogenous system, however, was much less intensive than the corresponding band in the Clo DF13 DNA-directed system. These results therefore strongly suggest that the polypeptide band X in Fig. 1b includes, besides a product of the endogenous system, a Clo DF13 DNA-specific polypeptide. In this connection it is worthwhile to mention that no polypeptides co-migrating with polypeptide X were detectable in the endogenous system when the synthesis of Clo DF13-specific proteins was studied in an RNA-dependent protein-synthesizing system under the direction of in vitro-synthesized Clo DF13 mRNA (Konings, unpublished data).

No answer can be given yet to the question of why most of the in vitro-synthesized Clo DF13-specific polypeptides differ from the plasmid-specific polypeptides synthesized in Clo DF13 DNA-harboring minicells (Fig. 1c). One explanation might be that some of the in vitro-synthesized polypeptides (Fig. 1b) are either the result of premature termination of polypeptide synthesis or they are precursor molecules of native Clo DF13-specific polypeptides (cf. reference 11). However, one should take into account that, under the conditions used, presumably not all plasmid-specific polypeptides are synthesized in the Clo DF13 DNA-harboring minicells.

Storage of native cloacin DF13 (Fig. 1d, polypeptide I) resulted, for reasons still unknown, in the appearance of a polypeptide with an electrophoretic mobility slightly different from that of the native product (Fig. 1d, polypeptide II; F. K. de Graaf, personal communication). Since in the in vitro system biologically active cloacin DF13 was made (see below) and since the in vitro-synthesized polypeptide II (Fig. 1b, polypeptide II) had the same electrophoretic mobility as the in vivo-synthesized polypeptide II, it might well be that the in vitro-synthesized polypeptide II was also the result of "aging" of biologically active cloacin DF13.

So far the function and biological characteristics of only two Clo DF13-specific polypeptides, cloacin DF13 and Clo DF13 immunity protein, are known (2, 6, 8). Since the in vitro-synthesized polypeptides I and X do have the same electrophoretic mobility as these Clo DF13-specific proteins (Fig. 1b, and d), we investigated whether these polypeptides also share the same biological characteristics. The results of these studies are described below.

Characterization of the polypeptides synthesized in vitro under the direction of Clo

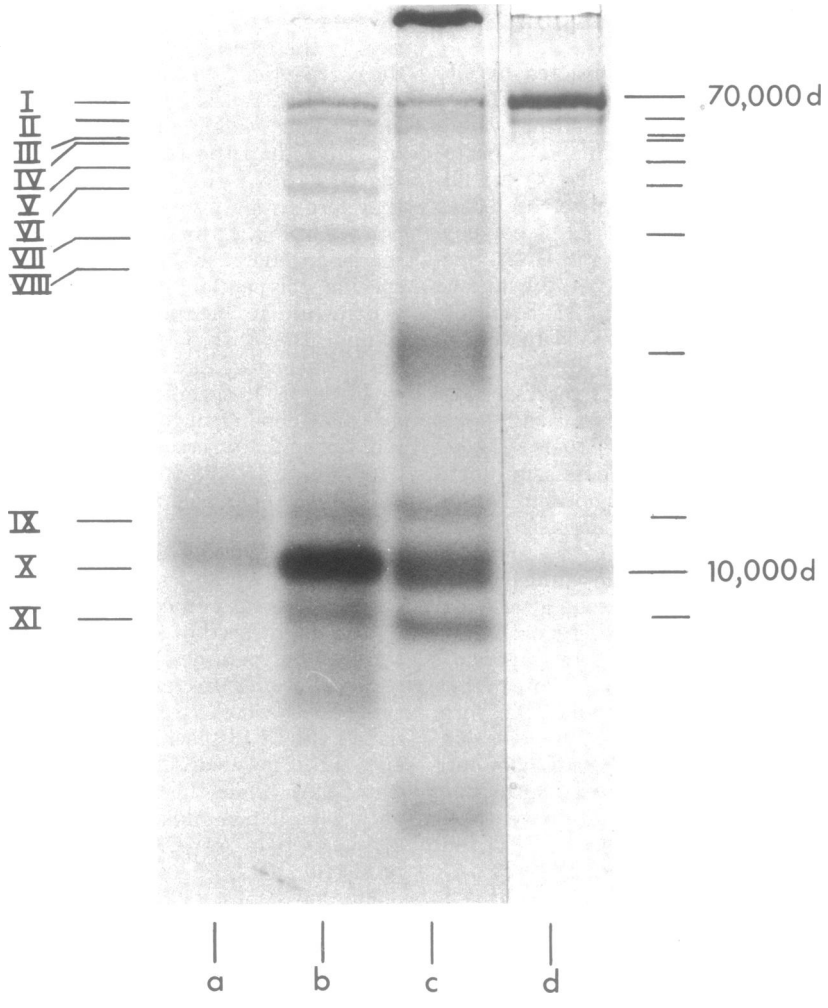

FIG. 1. *Autoradiogram of the polypeptides synthesized in vitro under the direction of Clo DF13 DNA and separated by polyacrylamide gel electrophoresis. (a) Products of the endogenous system; (b) products synthesized in the presence of Clo DF13 DNA; (c) ^{14}C-labeled Clo DF13 marker proteins isolated from Clo DF13 rep3-harboring minicells of E. coli; (d) Clo DF13 marker proteins, cloacin DF13 (molecular weight 70,000) and Clo DF13 immunity protein (molecular weight 10,000), stained with Coomassie brilliant blue.*

DF13 DNA. Biologically active cloacin DF13 can be characterized by at least four different functions: (i) it binds selectively to the receptor sites of sensitive cells (3); (ii) it inhibits protein synthesis, both in vivo as well as in vitro, by cleaving 16S ribosomal RNA at a unique site near its 3′ end (2); (iii) it forms a tight complex with its homologous immunity protein (6, 8); and (iv) it binds tightly and selectively to a unique region of Clo DF13 plasmid DNA (19; Veltkamp and Nijkamp, unpublished results).

To obtain a preliminary indication of whether biologically active cloacin DF13 is made in the cell-free system, we tested by spot-test analysis whether the mixture of the polypeptides synthesized in vitro in the presence of Clo DF13 DNA was able to inhibit the growth of cloacin DF13-sensitive cells. For comparison, we also tested the activity of native cloacin DF13 and of the mixture of polypeptides synthesized in vitro in the absence of Clo DF13 DNA. Only the mixture of polypeptides synthesized in the presence of Clo DF13 DNA and native cloacin DF13 was able to inhibit the growth of cloacin DF13-sensitive cells (Fig. 2a–e).

These results strongly suggest that biologically active cloacin DF13 is made in the coupled system. The latter conclusion is furthermore supported by the observation that no killing

activity could be demonstrated when both reaction mixtures were spotted on a lawn of cloacin DF13-insensitive cells (data not shown).

To examine whether the mixture of polypeptides synthesized in vitro in the presence of Clo DF13 DNA also possessed "in vitro" cloacinogenic activity, we tested whether aliquots of the dialyzed reaction mixtures were able to inhibit the phenylalanine incorporation in a poly(U)-dependent protein-synthesizing system. As shown in Table 1 (line 1), the mixture of polypeptides synthesized in the presence of Clo DF13 DNA (reaction mixture A) inhibited only slightly, in contrast to native cloacin DF13 (Table 1, line 7), the phenylalanine incorporation. These results suggest either that only very small amounts of biological active cloacin DF13 are made in the coupled system or that the biological activity of cloacin DF13 is masked by the presence of biologically active in vitro-synthesized immunity protein (cf. references 6,8). The latter explanation suggests that biological active immunity protein is also made in the Clo DF13-dependent system. To test which of these possibilities is valid, we heated the reaction mixtures for 20 min at 100 C. Previously it was shown that by this treatment the biological activity of immunity protein, in contrast to that of cloacin, is completely destroyed (16).

TABLE 1. *Effect of the in vitro-synthesized polypeptides on the phenylalanine incorporation in a poly(U)-dependent cell-free system*[a]

Addition to the incubation mixture	Incorporation (%)
1. None	100
2. Reaction mixture A (10 μl)	83.0
3. Reaction mixture A (10 μl; heated)	31.6
4. Reaction mixture A (10 μl; heated) plus native immunity protein (20 μl)	95.3
5. Reaction mixture B (10 μl)	94.8
6. Reaction mixture B (10 μl; heated)	94.6
7. Native cloacin DF13 (20 μl)	18.1
8. Native cloacin DF13 (20 μl) plus native immunity protein (20 μl)	96.5
9. Native cloacin DF13-Clo DF13 immunity protein complex (20 μl)	92.9
10. Native cloacin DF13-Clo DF13 immunity protein complex (20 μl; heated)	21.3

[a] The mixtures of polypeptides synthesized in vitro, in the presence (reaction mixture A) or absence (reaction mixture B) of Clo DF13 DNA, were prepared as described in the text. With the exception of poly(U) and [^{14}C]phenylalanine, all components required for in vitro polyphenylalanine synthesis were mixed with the indicated amounts of the in vitro- or in vivo-synthesized polypeptides. After a preincubation period of 20 min at 37 C, the incubation mixtures were cooled in ice, poly(U) and [^{14}C]phenylalanine were added, and incubation at 37 C was continued for another 20 min. The incorporation of phenylalanine was followed by measuring the radioactivity present in hot trichloroacetic acid-insoluble material. To inactivate immunity protein, the in vivo- or in vitro-synthesized Clo DF13 specific polypeptides were, where indicated, heated for 20 min at 100 C. One hundred percent net incorporation was 93,000 counts/min per 200 μl of incubation mixture.

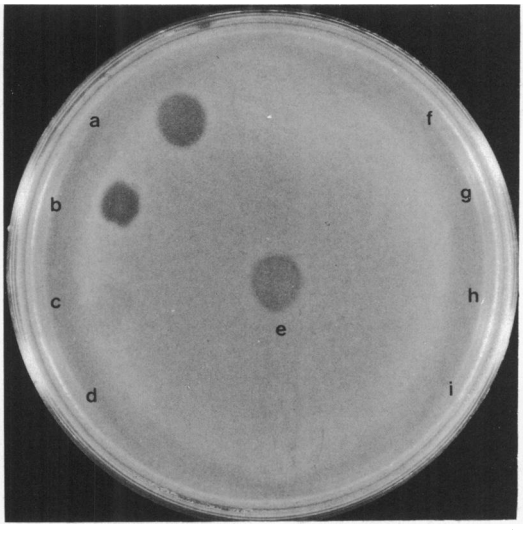

FIG. 2. *In vivo cloacin DF13 assay: effect of the in vitro-synthesized polypeptides on the growth of cloacin DF13-sensitive cells. The following 5-μl aliquots were spotted on the lawn of indicator cells: (a) undiluted mixture of polypeptides synthesized in vitro in the presence of Clo DF13 DNA; (b) 1:1 dilution of (a); (c) 1:4 dilution of (a); (d) 1:9 dilution of (a); (e) 0.1 μg of purified in vivo-synthesized cloacin DF13; (f–i) undiluted and diluted samples of the mixture of polypeptides synthesized in vitro in the absence of Clo DF13 DNA (dilutions are identical to a–d).*

As shown in Table 1 (line 3), the heat-treated reaction mixture A now inhibited very strongly the phenylalanine incorporation. This result was independent of whether native immunity protein was added to reaction mixture A before heat treatment. However, as one would expect on the basis of the results published previously, simultaneous addition of native immunity protein and heat-treated reaction mixture A to the poly(U)-dependent system abolished this inhibitory effect (Table 1, line 4). Similar results were obtained when, as a control, native cloacin DF13 instead of heat-treated reaction mixture A was added to the phenylalanine incorporating system (Table 1, lines 7 to 10). On the contrary, no cloacinogenic activity could be demonstrated in the mixture of polypeptides synthesized in the absence of Clo DF13 DNA (Table 1, lines 5 and 6).

From these data it can be concluded that both "in vitro" cloacinogenic and "in vitro" immunity protein activity are present in the mixture of polypeptides synthesized in vitro under the direction of Clo DF13 DNA. Apparently cloacin

DF13 and Clo DF13 immunity protein are predominantly present in the in vitro reaction mixture as a cloacin DF13-immunity protein complex (cf. references 6, 8). The fact that such a complex is unable to inhibit the phenylalanine incorporation in a poly(U)-dependent system but is able to kill cloacin-sensitive cells has already been demonstrated and discussed by Kool et al. (6, 8).

To obtain definite answer to the question of whether the in vitro-synthesized polypeptides I and X are, respectively, responsible for the "in vitro" cloacinogenic and immunity protein activity, the in vitro-synthesized polypeptides were purified by means of SDS-polyacrylamide gel electrophoresis (6, 19). Previously it was shown that by this method cloacin DF13 can be separated from its homologous immunity protein. Furthermore it has been demonstrated that both polypeptides are in vitro still biologically active after this purification step (5, 6).

Addition of the purified polypeptide I to the poly(U)-dependent cell-free system resulted in an inhibition of the [^{14}C]phenylalanine incorporation (Fig. 3A). This inhibition was strongly dependent on the amount of polypeptide I added. As one would expect on the basis of the results described above, simultaneous addition of (increasing amounts) of native Clo DF13 immunity protein abolished this inhibitory effect (Fig. 3A). Similar results were obtained when, instead of polypeptide I, native cloacin DF13, which also had passed the purification step on the SDS-polyacrylamide gel, was added to the polyphenylalanine-synthesizing system (Fig. 3A). On the contrary, none of the other polypeptides synthesized in vitro in the presence or absence of Clo DF13 DNA were able to inhibit the phenylalanine incorporation (Fig. 3A).

Polypeptide X was the sole in vitro-synthesized polypeptide that could abolish the inhibitory effect of native cloacin DF13 on the phenylalanine incorporation in the poly(U)-dependent system (Fig. 4A). This inhibition of cloacin activity was strongly dependent on the amount of polypeptide X added. These results are very similar to the results described above for native Clo DF13 immunity protein.

From these results, the conclusion seems to be justified that the in vitro-synthesized polypeptides I and X are, respectively, products of the Clo DF13 genes coding for cloacin DF13 and Clo DF13 immunity protein.

It should be mentioned that purification of native cloacin DF13 and Clo DF13 immunity protein on SDS-polyacrylamide gels results in a

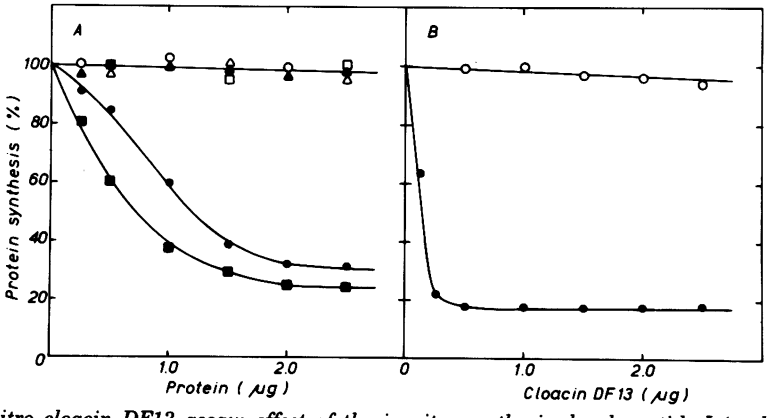

FIG. 3. *In vitro cloacin DF13 assay: effect of the in vitro-synthesized polypeptide I (molecular weight, 70,000) and native cloacin DF13 on the phenylalanine incorporation in a poly(U)-dependent cell-free system. The amounts of polypeptide I and native cloacin DF13 added to the incubation mixture were as indicated. (A) Effect of polypeptides that had been purified by means of SDS-polyacrylamide gel electrophoresis. Symbols: (●) Effect of increasing amounts of polypeptide I; (○) effect of simultaneous addition of increasing amounts of polypeptide I and native (2.0 μg) Clo DF13 immunity protein; (■) effect of increasing amounts of native cloacin DF13; (□) effect of simultaneous addition of increasing amounts of native cloacin DF13 and native (2.0 μg) Clo DF13 immunity protein; (▲) effect of polypeptides synthesized in the endogenous system; (△) effect of simultaneous addition of polypeptides synthesized in the endogenous system and native (2.0 μg) Clo DF13 immunity protein. (B) Effect of native cloacin DF13 that had been purified by means of column chromatography. Symbols: (●) Effect of increasing amounts of native cloacin DF13; (○) effect of simultaneous addition of increasing amounts of native cloacin DF13 and native (2.0 μg) Clo DF13 immunity protein. The results are expressed as the percentage of a control incubation without cloacin DF13 and Clo DF13 immunity protein. The values are corrected for the radioactivity contributed by the in vitro-synthesized ^{35}S-labeled polypeptides. Other details are given in the legend in Table 1. One hundred percent net incorporation was 67,295 counts/min.*

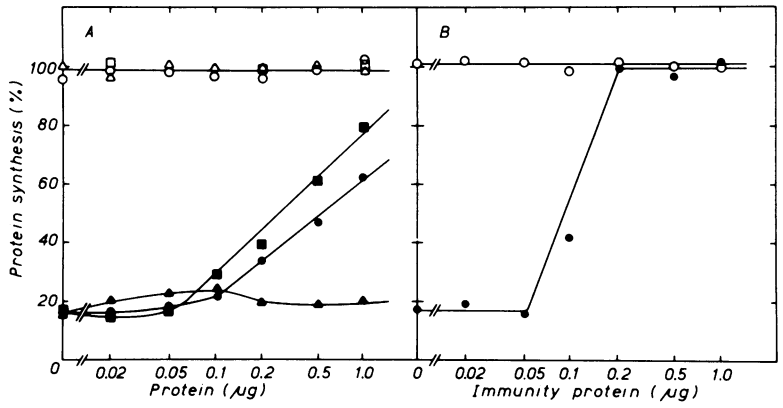

FIG. 4. *In vitro Clo DF13 immunity protein assay: effect of the in vitro-synthesized polypeptide X and native Clo DF13 immunity protein on the phenylalanine incorporation in a poly(U)-dependent cell-free system. The amounts of polypeptide X and native Clo DF13 immunity protein added to the cell-free system are as indicated. (A) Effect of polypeptides that had been purified by means of SDS-polyacrylamide gel electrophoresis. Symbols: (○) Effect of increasing amounts of polypeptide X; (●) effect of simultaneous addition of increasing amounts of polypeptide X and native (0.5 μg) cloacin DF13; (□) effect of increasing amounts of native Clo DF13 immunity protein; (■) effect of simultaneous addition of increasing amounts of native Clo DF13 immunity protein and native (0.5 μg) cloacin DF13; (△) effect of polypeptides synthesized in the endogenous system; (▲) effect of simultaneous addition of polypeptides synthesized in the endogenous system and native (0.5 μg) cloacin DF13. (B) Effect of native Clo DF13 immunity protein that had been purified by means of column chromatography. Symbols: (○) Effect of increasing amounts of native Clo DF13 immunity protein; (●) effect of simultaneous addition of increasing amounts of native Clo DF13 immunity protein and native (0.5 μg) cloacin DF13. Results are expressed as the percentage of a control incubation in the absence of cloacin DF13 and Clo DF13 immunity protein. The values are corrected for the radioactivity contributed by the in vitro-synthesized ^{35}S-labeled polypeptides. Other details are given in the legend to Table 1. One hundred percent net incorporation was 59,115 counts/min.*

partial loss of their biological activity. The latter conclusion is deduced from the observations that the purified polypeptides have a weaker effect on the phenylalanine incorporation in the poly(U)-dependent system that equal amounts of their native counterparts.

Previously it was demonstrated that only one of the Clo DF13-specific polypeptides, i.e., cloacin DF13, is able to form a tight complex with Clo DF13 plasmid DNA (19). To determine whether the in vitro-synthesized polypeptides also are able to bind to Clo DF13 DNA, we incubated ^{3}H-labeled Clo DF13 DNA with increasing amounts of the heat-treated (20 min at 100 C) mixture of polypeptides synthesized in vitro in the presence of Clo DF13 DNA.

Addition of increasing amounts of the in vitro-synthesized polypeptides results in an increased retention of Clo DF13 DNA by the nitrocellulose membrane filters (Fig. 5). A plateau was reached when about 80% of the DNA (total input, 69,300 ^{3}H dpm) and 15% of the in vitro-synthesized polypeptides (total input, 1,530,000 ^{35}S dpm) was bound to the filters. No specific binding of DNA could be demonstrated when Clo DF13 DNA was incubated with the polypeptide synthesized in vitro in the absence

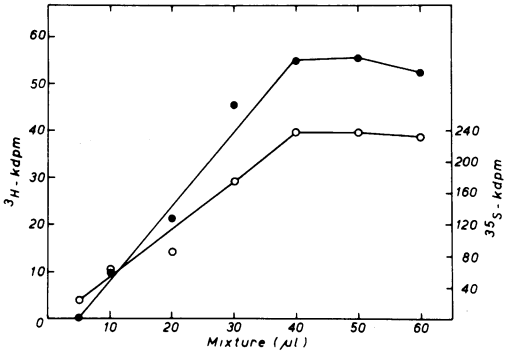

FIG. 5. *Binding of the in vitro-synthesized polypeptides to ^{3}H-labeled supercoiled Clo DF13 DNA. To inactivate immunity protein, the in vitro-synthesized polypeptides were heated for 20 min at 100 C before being added to the incubation mixture. After incubation for 30 min at 25 C, duplicate samples of 1 ml were taken and filtered through the nitrocellulose membrane filters. The filters were subsequently washed, dried, and counted as described in the text. The values indicated are corrected for the nonspecific binding of DNA and protein to the filters. Symbols: (●) Amount of ^{3}H-labeled DNA retained by the filters; (○) amount of ^{35}S-labeled in vitro-synthesized polypeptides retained by the filters.*

of Clo DF13 DNA. These data indicate that at least one of the polypeptides synthesized in vitro under the direction of Clo DF13 DNA is able to bind to this DNA. Since only one of the Clo DF13-specific polypeptides, cloacin DF13, is able to form a tight complex with the plasmid DNA, these results further support the conclusion that biologically active cloacin DF13 is made in the DNA-dependent cell-free system.

Although the coupled system showed some evidence of regulation of gene expression, more cloacin DF13 and Clo DF13 immunity protein were made than any of the other plasmid-specific polypeptides (Fig. 1), it probably does not express the full regulatory potential of the plasmid-harboring cell, as can be shown by comparison of the autoradiograms of the Clo DF13-specific polypeptides synthesized in vivo and in vitro. This may be in part due to the failure of some regulatory system to function in vitro. Now that it is possible to study the in vitro synthesis of plasmid-specific proteins, studies of the regulatory phenomena are more feasible and are under way.

ACKNOWLEDGMENT

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