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Identification of Messenger Ribonucleic Acids and Proteins Synthesized by the Bacteriocinogenic Factor Clo DF13 in Purified Minicells of *Escherichia coli*

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It has previously been shown that the cloacinogenic factor Clo DF13 (Clo DF13) segregates into minicells of strain *Escherichia coli* P678-54 that harbors Clo DF13 and that this Clo DF13 factor is the only deoxyribonucleic acid (DNA) present in these otherwise chromosomeless minicells. The study reported here shows that minicells prepared from P678-54(Clo DF13) are able to incorporate radioactive precursors into ribonucleic acid (RNA) and protein. The RNA synthesized in these purified minicells is Clo DF13 specific, as shown by RNA-DNA hybridization experiments. The results indicate that all the de novo synthesized gene products in Clo DF13 minicells are Clo DF13 specific. Polyacrylamide gel electrophoretic patterns show that in these minicells at least three polypeptides (molecular weight about 70,000, 20,000, and 11,000) and one major species of messenger RNA (mRNA) (*S* value about 21.3) are synthesized. To investigate the factor in its induced state, we isolated a Clo DF13 mutant with an enhanced level of cloacin production. Minicells harboring this Clo DF13 mutant produce five additional polypeptides (molecular weight about 58,000, 44,000, 28,000, 16,000, and 14,000). Three additional mRNA species (*S* value about 19.5, 14, and 12) could be distinguished. The total molecular weight of the eight polypeptides corresponds to 85% of the total coding capacity of the mRNAs (303,000). The total molecular weight of the four mRNAs is 2.55×10^6, which covers 85% of the Clo DF13 DNA (molecular weight 6×10^6).

Bacteriocins are extracellular antibiotic proteins produced by a class of bacterial plasmids called bacteriocinogenic factors. In addition to the structural gene(s) for the production of bacteriocins, other genes might also be present, depending on the complexity of the factor. These genes might govern properties such as regulation, immunity, fertility, susceptibility to male-specific phages, repression of fertility, restriction of phages, and resistance to ultraviolet irradiation (for recent reviews, see 6).

The only gene products that have been studied extensively are the bacteriocins (26). Other gene products have not yet been identified.

The aim of our work is to identify the nature and function of the gene products of the cloacinogenic factor DF13 (Clo DF13), which originates from *Enterobacter cloacae*. Clo DF13 is a relatively small plasmid with a contour length of 3.0 μm and a molecular weight of 6×10^6 (31). It directs the synthesis of an antibiotic protein, the cloacin DF13, which is known to abolish protein synthesis of sensitive strains by inhibiting the binding of formylmethionine-transfer ribonucleic acid (tRNA) to the messenger RNA (mRNA)-30*S* ribosome complex. This inhibition is accompanied by a progressive extrusion of potassium ions (13). de Graaf et al. (12) have shown that this inhibition of protein synthesis is caused by a subtle conformational change of the cloacin-treated 30*S* ribosomal subunits.

Besides the structural gene(s) for the synthesis of cloacin DF13, one can expect at least two other genes to be located on the Clo DF13 plasmid. First, there probably is a gene responsible for immunity against homologous cloacin. This immunity character is specified by the plasmid itself: if a bacteriocinogenic factor is transferred to a sensitive strain, this strain will become immune within 15 min (9). Attempts to isolate a plasmid mutant with an altered immunity have been unsuccessful (19). Secondly, there must be a gene involved in the regulation of the cloacin synthesis: under usual conditions, the Clo DF13 factor is present in the repressed state, and only a small amount of spontaneously

produced cloacin is observed. After induction with mitomycin C, the production of cloacin is greatly increased (14). We began the present work with the isolation of Clo DF13-specific proteins and mRNAs by using purified minicells. Minicells produced by the *E. coli* mutant P678-54 are the result of aberrant cell divisions (1) and contain little, if any, chromosomal deoxyribonucleic acid (DNA). However, they were found to carry DNA of colicinogenic factors, drug resistance transfer factors, and the sex factor, F, when these plasmids were present in the minicell-producing mutant (16, 17, 22). Roozen et al. (27) and Kool et al. (20) have shown that plasmid-containing minicells, but not minicells without plasmids, are able to incorporate radioactive precursors into acid-insoluble RNA or protein. In previous studies we have shown that the Clo DF13 factor segregates into minicells, and that this Clo DF13 factor is the only DNA present in these minicells (20, 31a).

We report here that the Clo DF13 factor directs, under repressed conditions, the synthesis of at least three polypeptides and one species of mRNA. Minicells containing a Clo DF13 plasmid mutant with a higher production of cloacin produce five additional polypeptides and three additional mRNA species.

MATERIALS AND METHODS

Bacterial strains. The minicell-producing strain *E. coli* K-12 P678-54 (1) has the following genetic markers (the nomenclature follows the proposals of Demerec et al. [8]): *thr*, *leu*, *lac Y*, *minA*, T6^s, *gal*, *minB*, *str*^{r}, *thi* (for *min* genotype, see Roozen et al. [26]). A P678-54 strain harboring Clo DF13 was isolated in our laboratory from a conjugation experiment, using P678-54 as acceptor and *E. coli* K-12 Hfr R4(Clo DF13) as donor strain (31). The Clo DF13 factor originates from *E. cloacae* (29).

Media. The media used were composed as follows: (i) brain heart infusion (BHI; Difco), 3.7% BHI (wt/vol); (ii) minimal medium, 0.3% $KH_2PO_4 \cdot 2H_2O$, 0.7% $Na_2HPO_4 \cdot 2H_2O$, 0.1% NH_4Cl, 0.05% NaCl, 0.001% $CaCl_2$, 0.25% $MgSO_4$, and 0.5% glucose (wt/vol). This medium was supplemented with 0.5% Casamino Acids (Difco) (wt/vol) unless otherwise indicated. Demineralized water was used as solvent. When required, other supplements were used at the following final concentrations (μg/ml): thiamine-hydrochloride, 1; threonine, 40; and leucine, 20.

Purification of minicells. The minicell purification procedure of Roozen et al. (27) was slightly modified. A culture of strain P678-54(Clo DF13) or P678-54 was grown in 1.6 liters of BHI medium at 37 C. The culture was centrifuged in the cold for 20 min at 4,000 rpm in an MSE 18 rotor (6 by 250 ml). The supernatant liquid was then centrifuged at 12,000 rpm in the rotor (6 by 250 ml) for 15 min. The pellets were suspended in 6 ml of cold buffered saline with gelatine (BSG) (6). Minicells were isolated from these suspended pellets by three successive sedimentations through 40-ml linear gradients of 5 to 20% (wt/vol) sucrose in BSG. These gradients were centrifuged at 3,700 rpm for 30 min in a Christ Minifuge. After the third sucrose gradient, the minicells were suspended in the minimal medium as described above at an absorbancy of 0.2 at 620 nm. This purified minicell fraction usually contained less than 100 contaminating bacterial cells per 10^8 minicells.

Isolation of labeled proteins. Purified minicells, suspended in minimal medium (10 ml) supplemented with thiamine, threonine, and leucine, were incubated for 20 min at 37 C. Then, a mixture of uniformly labeled [^{14}C]amino acids ([^{14}C]AA; The Radiochemical Centre, Amersham, England) with a specific activity of 52 mCi/matom of carbon was added to the minicells in a final concentration of 2 μCi/ml. After incubation for 90 min at 37 C, the minicells were centrifuged at 15,000 rpm for 7 min in an MSE 18 rotor (8 by 50 ml) and washed with minimal medium. The minicell pellet was suspended in 70 μliters of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 1% 2-mercaptoethanol, 25% sucrose, and 3.7% ethylenediaminetetraacetic acid (EDTA). Then, 70 μliters of buffer containing 0.5% lysozyme in 0.25 M Tris-hydrochloride (pH 8.0) was added. The mixture was incubated for 15 min at 2 C and lysed by treatment with 0.4% sodium dodecyl sulfate (SDS) for 30 min at 37 C. The lysate was centrifuged at 10,000 $\times$ *g* for 15 min, and the supernatant was used in further experiments.

Isolation of labeled RNA. RNA was extracted from 10 ml of purified minicells suspended in minimal medium supplemented with thiamine and Casamino Acids. The culture was incubated for 20 min at 37 C before the addition of the label. Then [^{14}C]adenine and [^{14}C]uridine (The Radiochemical Centre, Amersham; specific activities 287 mCi/mmol and 405 mCi/mmol, respectively) were added to a final concentration of 0.7 μCi/ml. Incubation was continued for 7 min at 37 C. Cell metabolism was then inhibited by pouring the culture onto 15 ml of frozen minimal medium containing 0.01 M sodium cyanide.

The minicells were centrifuged and resuspended in 0.5 ml of buffer containing 0.06 M Tris-hydrochloride (pH 7.5), 0.05 M EDTA, and 3 mg of proteinase K per ml (34). Then, 3 ml of a solution containing 0.5% SDS and 0.25% macaloid (purified Bentonit; binds and inactivates proteins, e.g., ribonuclease [RNase]) in the same Tris-EDTA buffer was added, and minicells were lysed by heating this suspension for 3 min in a 97 C water bath as described by Bremer and Yuan (3). The lysate was cooled and RNA was extracted three times at room temperature (29) with a phenol-cresol mixture (18). Then, 0.2 volume of 30% sodium acetate and 2 volumes of ethanol were added. The RNA was allowed to precipitate overnight at −20 C and was collected by centrifugation.

The precipitate was redissolved in 1.8 ml of 5% sodium acetate and reprecipitated by the addition of 4 ml of ethanol. After 4 h at −20 C, the precipitate was again collected and dissolved in 80 μliters of electro-

phoresis buffer G (0.04 Tris-acetate [pH 7.8], 0.03 M sodium acetate, 0.001 M EDTA, and 0.2% SDS) containing 20% glycerol.

Polyacrylamide-gel electrophoresis of proteins. The proteins of the minicell lysates were separated on 10% SDS-polyacrylamide gels by the method of Weber and Osborn (33). Thirty-five microliters of the lysates of P678-54(Clo DF13) or P678-54 (control) minicells was mixed with 5 μliters of frontmarker (bromophenol blue) and 30 μliters of gel buffer. The samples were applied on gels (7 cm long) and run for 3 h at 8 mA per gel in a Shandon electrophoresis apparatus (Shandon, Frankfurt am Main, Germany). Gels were stained overnight with a 0.25% solution of Coomassie brillant blue (Serva) and destained for 2 days in a mixture of CH_3OH-HAc-H_2O (5:7.5:87.5, vol/vol/vol).

After staining and destaining, the gels were sliced in a longitudinal gel slicer (Canalco Europe). The center slabs were transferred to a sheet of filter paper and dried at 60 C on a gel drying plate (Canalco Europe; Vlaardingen, The Netherlands). Autoradiography was carried out by exposing Ilford Red Seal 100 FW X-ray film to the dried gels for about 3 weeks. After development, the films were scanned with a Kipp DD2 densitometer. To calculate the molecular weights of the labeled proteins from the relative migration values, we used six proteins (serum albumin, catalase, aldolase, chymotrypsinogen, myoglobin, and cytochrome *c*) as references.

Polyacrylamide gel electrophoresis of RNA. The various species of RNA extracted from minicells were separated on 2.1% polyacrylamide gels containing 0.5% agarose by the method of Summers (30). RNA samples (30 μliters in electrophoresis buffer containing 20% glycerol) were layered on top of the gels. Electrophoresis was carried out at room temperature at a current of 3 mA per gel (0.6 cm in diameter and 7.0 cm long) until the frontmaker (bromophenol blue) had left the gel. The gels were scanned at 260 nm in the Joyce-Loebl Chromoscan to determine the migration distance of the nonlabeled marker RNAs (16*S* and 23*S* ribosomal RNA (rRNA) from *E. coli* P678-54, 17*S* and 26*S* yeast rRNA). The gels were then sliced longitudinally as described above, and gel slabs were dried for 1 h at 60 C on filter paper (2 mm thick; Schleicher & Schüll no. 2294). Distortion of the gels does not occur when they are dried in this way. The dried gels were exposed to X-ray film and scanned as described above. The average *S* values and molecular weights of the RNA species in the scanned peaks were determined from the plot of migration distance of the markers against both their molecular weights and their sedimentation coefficients according to Peacock and Dingman (25) and Lewicki and Sinskey (23).

Preparation of RNA for hybridization experiments. For labeling and extracting of RNA, 10 ml of purified minicells was suspended in minimal medium supplemented with thiamine and Casamino Acids. After preincubation for 20 min at 37 C, [^{3}H]uridine (15 Ci/mmol, The Radiochemical Centre, Amersham) was added at a final concentration of 40 μCi/ml, and incubation was continued for 7 min at 37 C. Incorporation was then inhibited by pouring the culture onto crushed ice containing 0.01 M sodium cyanide. The minicells were centrifuged and lysed as described for the isolation of labeled RNA. The lysate was extracted three times at 60 C with water-saturated phenol and adjusted to the salt concentration of 2× standard saline citrate (SSC, 0.15 M NaCl-0.015 M sodium citrate, pH 7).

The radioactivity incorporated into RNA was determined with the trichloroacetic acid albumine method as described by Bøvre and Szybalski (2).

Preparation of DNA for hybridization experiments. Clo DF13 plasmid DNA was prepared from strain *E. coli* P678-54(Clo DF13): cells were grown in 6 liters of BHI medium to an optical density at 660 nm of 1.0. The culture was then centrifuged, washed, and suspended in 37.5 ml of 0.05 M Tris-hydrochloride (pH 8.0) containing 25% sucrose. The cells were converted to protoplasts and lysed with Brij 58 as described by Veltkamp and Nijkamp (32). The lysate was then centrifuged for 40 min at 38,000 × *g* to remove most of the chromosomal DNA (4). To the supernatant, 50 μg of heated pancreatic RNase and 10 U of T_1 RNase were added per ml of solution, and the RNA was digested for 30 min at 37 C. Pronase, self-digested for 1 h at 37 C, was then added at a level of 50 μg/ml and incubated for 30 min at 37 C. The solution was then deproteinized three times at room temperature with water-saturated phenol, dialyzed against 0.05 M Tris-hydrochloride (pH 8.0) containing 0.06 M EDTA, and subjected to dye-buoyant centrifugation as previously described by Kool et al. (20). The lower band in the CsCl gradient, representing the covalently closed Clo DF13 plasmid DNA, was collected, dialyzed against several changes of 1 mM EDTA, and stored at −20 C.

Chromosomal DNA was isolated from strain *E. coli* P678-54 by the method described by Bøvre and Syzbalski (2).

Filter hybridization procedures. Plasmid DNA was denatured by the method of Goebel and Schrempf (11). The DNA solution was adjusted to 0.1 N NaOH and heated for 15 min at 100 C. The samples were then quickly cooled in ice and neutralized with 0.1 N HCl. More than 70% of the supercoiled DNA is denatured in this way. Chromosomal DNA was denatured by heating for 10 min at 97 C. Thereafter, the denatured DNA was chilled and kept on ice by the method of Gillespie and Spiegelman, as modified by Bøvre and Szybalski (2). Denatured DNA samples were mixed with cold 10× SSC to give the salt concentration corresponding to 6× SSC and was passed through a membrane filter type B6 (Schleicher & Schüll BA 85/1). The DNA filters were washed with 100 ml of 6× SSC and dried at room temperature for 20 h. Finally, the DNA filters were "baked" at 80 C in a vacuum oven for 3 h.

Hybrids were formed by placing the DNA filters in scintillation vials containing 1.5 ml of [^{3}H]RNA in 2× SSC (half saturated with phenol). Annealing was carried out for 20 h at 66 C, after which the vials were chilled in ice. The filters were removed from the vials, washed with 50 ml of 2× SSC, and incubated in 2 ml of 2× SSC that contained 40 μg of heated pancreatic RNase and 20 U of T_1 RNase (10). After incubation for 1 h at 25 C, the filters were rinsed with 10 ml of 2×

SSC on each side and then washed by filtration with 50 ml of 2× SCC. Finally, the filters were dried and counted in 10 ml of toluene-2,5-diphenyloxazole (PPO)-1,4-bis-(5-phenyloxazolyl)benzene (POPOP).

Chemicals. Bovine serum albumine, catalase, aldolase, myoglobin, chymotrypsinogen, and cytochrome *c* were purchased from Serva (Heidelberg, Germany). Proteinase K was obtained from Merck (Darmstadt, Germany). Agarose and SDS (specially pure) came from BDH Chemicals Ltd. (Poole, England). Pancreatic RNase and T_1 RNase came from Worthington Biochemical Corp. Casamino Acids were obtained from Difco. Macaloid was purchased from Baroid Division (Houston), and Atlas Chemie (Essen, Germany) supplied the Brij 58.

RESULTS

Synthesis of RNA in purified Clo DF13-containing minicells. Because plasmid-containing minicells are able to incorporate radioactive presursors into RNA (21, 27), we wanted to show that this was also valid for Clo DF13-containing minicells. For this reason, minicells isolated from P678-54(Clo DF13) and P678-54 were incubated in the appropriate medium, and the incorporation of [^{3}H]uridine was examined. Figure 1 shows that minicells containing a Clo DF13 factor are able to incorporate [^{3}H]uridine into acid-insoluble material, whereas the plasmid-free minicells show a considerably lower level of incorporation. Polyacrylamide gel analysis of ^{14}C-labeled material from plasmid-free minicells incubated with [^{14}C]uridine shows only a small amount of 16*S* and 23*S* rRNA and a rather high background of radioactivity (Fig. 8B). These rRNA species are synthesized by the contaminating cells because the amount of these labeled rRNAs varies with the amount of contaminating cells in the purified minicell preparation (Fig. 4B and 8B). However, the incorporation of labeled uridine by plasmid-free minicells and the resulting radioactive background on the gels cannot be explained by the presence of these contaminating cells, because when [^{3}H]uridine is added to cells of P678-54(Clo DF13) at a concentration of 7,000 cells/ml, which is about 10 times higher than the contaminating cell titer in a purified minicell fraction, only insignificant amounts of incorporation are observed (Fig. 1). Also, we found that the total amount of uridine incorporation by plasmid-free minicells is not lowered by a decreasing number of contaminating cells in the purified minicell fraction. These results suggest that the observed incorporation of [^{3}H]uridine by plasmid-free minicells is not simply the result of the contaminating cells, but might be caused by transcription of a small amount of random fragments of chromosomal DNA trapped in these minicells. This is also shown by RNA-DNA hybridization experiments (Table 1), where at least 2% of the RNA isolated from Clo DF13-containing minicells can hybridize with chromosomal *E. coli* DNA. The possibility that a few minicells might contain entire chromosomes has been excluded by Veltkamp and Nijkamp (manuscript in preparation). The incorporation of [^{3}H]uridine by Clo DF13-containing minicells is considerably higher than incorporation in plasmid-free minicells. There-

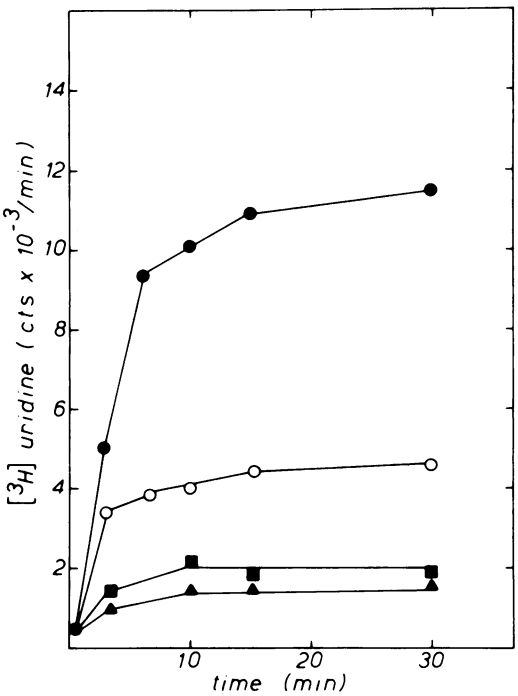

FIG. 1. *Incorporation of [^{3}H]uridine into cold trichloroacetic acid-insoluble material by cells and purified minicells. Cells or minicells were preincubated for 20 min at 37 C. Then [^{3}H]uridine (15 Ci/mmol) was added in a final concentration of 40 μCi/ml. At the intervals indicated, 50-μliter samples were precipitated with cold 10% trichloroacetic acid. After washing twice with 5% trichloroacetic acid, ethanol-ether, and ether, the filters were dried, placed in scintillation vials containing 10 ml of Toluene-PPO-POPOP, and counted in a liquid scintillation counter. Rifampin, when present, was added at the start of the preincubation in a final concentration of 100 μg/ml. Incorporation of [^{3}H]uridine by minicells from P678-54(Clo DF13) in the absence (●) and presence (■) of rifampin. Incorporation of [^{3}H]uridine by plasmid-free minicells isolated from P678-54 (○) and by cells of P678-54(Clo DF13) at a concentration of 7,000 cells/ml (▲). These cells were treated three times with 8% sucrose, diluted with BSG buffer, and subjected to centrifugation to obtain cells comparable with the cells contaminating the purified minicell fraction.*

fore, this incorporation cannot be caused only by transcription of random fragments of chromosomal DNA. When rifampin, which is known to inhibit the initiation of RNA synthesis, is added to the Clo DF13-containing minicells, the incorporation of [^{3}H]uridine is almost completely inhibited (Fig. 1). From these results we conclude that Clo DF13-containing minicells are able to synthesize RNA. To obtain further evidence that this RNA synthesis is a property of the Clo DF13 factor, we carried out hybridization experiments between RNA isolated from highly purified, Clo DF13-containing minicells and Clo DF13 DNA. Table 1 shows that more than 65% of this RNA hybridizes with Clo DF13 DNA, whereas only 2% of the RNA hybridizes with chromosomal DNA. Additional evidence that only a minor amount of the [^{3}H]RNA from Clo DF13-containing minicells represents chromosomally determined RNA is obtained from experiments with plasmid-free minicells. When purified plasmid-free minicells are incubated with [^{3}H]uridine under the same conditions as described for Clo DF13 minicells, the total amount of [^{3}H]RNA that can be extracted from these minicells is only 3%, compared to the amount of [^{3}H]RNA that can be extracted from Clo DF13-containing minicells.

The results described above indicate that the RNA that is extracted from Clo DF13-containing minicells represents Clo DF13-specific RNA.

Although plasmid-free minicells incorporate uridine 39% as well as plasmid-containing ones, only 3% as much labeled RNA can be extracted. At least 44% of this incorporation is the result of nonspecific uptake or binding, for this percentage is rifampin sensitive (Fig. 1). The remaining 22% incorporation by plasmid-free minicells is most probably due to transcription of small fragments of chromosomal DNA, and, therefore, this RNA most likely consists of incomplete, small pieces of RNA. We think that these small, incomplete RNA pieces are easily degraded by RNase and that, therefore, only 3% of this RNA is extractable from these plasmid-free minicells.

Synthesis of protein in purified Clo DF13-containing minicells. We have shown previously that Clo DF13-containing minicells are able to synthesize Clo DF13-specific proteins (20). To get some information about the extent of protein synthesis by chromosomally determined RNA species in these minicells, we carried out the following experiments. Minicells were isolated from strain P678-54(Clo DF13) and P678-54. These minicells were incubated with [^{14}C]AA, and the incorporation of these radioactive precursors into hot acid-insoluble material was measured. Figure 2 shows that Clo DF13-containing minicells are able to incorporate [^{14}C]AA into hot trichloracetic acid-insoluble material, whereas the plasmid-free minicells incorporate considerably less label. The incorporation of [^{14}C]AA by plasmid-free minicells is not influenced by the degree of purity of the isolated minicells. This suggests that this incorporation is not the result of the contaminating cells usually present in our minicell preparations. To prove this, we incubated cells of P678-54(Clo DF13) with [^{14}C]AA under the same conditions as used in the minicell experiments. Figure 2 shows that, in comparison with minicells, the cells show insignificant amounts of incorporation when they are incubated at a concentration of 7,000 cells/ml, which is about 10 times higher than the contaminating cell titer in the purified minicell fractions. When ^{14}C-labeled material isolated from the plasmidless minicells is analyzed on polyacrylamide gels, no distinct band of labeled cellular proteins is present in these gels, and only a rather high background of radioactivity can be observed (Fig. 6B). These results suggest that the observed [^{14}C]AA incorporation by plasmid-free minicells is caused by translation of a small amount of host mRNA present in these minicells.

The incorporation of [^{14}C]AA by Clo DF13-containing minicells is considerably higher than in plasmidless minicells, and therefore this incorporation cannot be explained only by the translation of the small amount of host mRNAs. In the presence of chloramphenicol, which is known to inhibit protein synthesis, this incorpo-

TABLE 1. *Hybridization between [^{3}H]RNA extracted from Clo DF13 minicells and Clo DF13 plasmid DNA or E. coli chromosomal DNA*[a]

Input [^{3}H] RNA from Clo DF13 minicells (counts/min)	Clo DF13 DNA (μg)	*E. coli* DNA (μg)	[^{3}H]RNA retained on filters (counts/min)[b]	[^{3}H]RNA input hybridized to DNA (%)
38,125	10.0		25,685	67.3
38,125	0.1		24,193	64.1
38,125		10.0	748	2.0

[a] The hybridization values obtained from hybridization experiments between mutant Clo DF13 RNA and mutant Clo DF13 DNA did not differ significantly from the wild-type Clo DF13 values. Therefore, the hybridization values given above are also valid for the mutant Clo DF13.

[b] The background count for DNA-free filters has been subtracted.

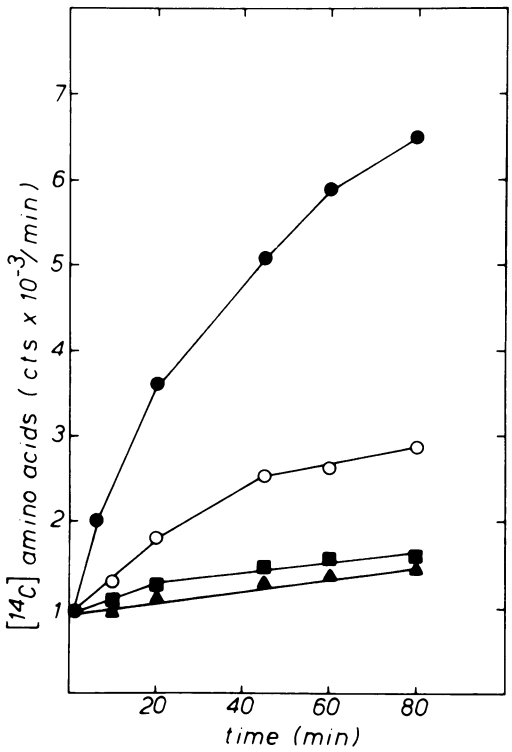

FIG. 2. *Incorporation of [^{14}C]AA into hot trichloroacetic acid-insoluble material. Cells or minicells were preincubated for 20 min at 37 C. Then [^{14}C]AA (52 mCi/matom of carbon) were added in a final concentration of 2 μCi/ml. At intervals 50-μliter samples were collected on 3 MM Whatman filter disks. The filters were washed for 20 min in 10% trichloroacetic acid at 85 C and then processed as described in the legend to Fig. 1. Chloramphenicol, when present, was added at the start of the preincubation in a final concentration of 100 μg/ml. Incorporation of [^{14}C]AA by minicells produced by P678-54(Clo DF13) in the absence (●) and presence (■) of chloramphenicol. Incorporation of [^{14}C]AA by plasmidless minicells isolated from P678-54 (○) and by cells of P678-54(Clo DF13) at a concentration of 7,000 cells/ml (▲). These cells were treated as described in the legend to Fig. 1.*

ration of [^{14}C]AA is almost completely inhibited (Fig. 2). From these results we conclude that the Clo DF13-containing minicells are able to synthesize protein and that the proteins synthesized in these minicells are Clo DF13 specific.

Storage of minicell in liquid nitrogen. The minicell purification procedure described in Materials and Methods is rather simple. However, there are some disadvantages. The whole procedure is rather time consuming, and the yield of purified minicells is low. For these reasons, we wanted to develop a method for the large-scale purification of minicells. However, before developing such a procedure, one should know how to store the minicells in such a way that they retain their ability to synthesize DNA, RNA, and protein.

According to J. W. Black (M. A. thesis, Univ. of Tennessee, 1967), minicells remain biologically active (measured as capacity for respiration) for 24 h when they are stored at 4 C. We found that Clo DF13 minicells that were stored for 2 days at 2 C had lost up to 80% of their capacity to synthesize RNA and protein. Therefore, purified Clo DF13 minicells suspended in minimal medium were stored in liquid nitrogen, and their activity was determined after several days and weeks of storage. This activity was determined by measuring the incorporation of radioactive [^{3}H]thymidine, [^{3}H]uridine, and [^{14}C]AA into DNA, RNA, and protein, respectively.

Figure 3B shows that the synthesis of RNA remains constant after 4 days of storage at −190 C, but longer storage causes a strong decrease in activity. After 2 weeks, minicells can no longer synthesize RNA. DNA synthesis (Fig. 3C) is reduced by 50% within 4 days. After 2 weeks of storage, no DNA synthesis can be detected in these minicells. For these reasons, we always used freshly purified minicells for DNA and RNA experiments. Since protein synthesis (Fig. 3A) is unaffected and DNA synthesis is completely abolished after 14 days of storage, the Clo DF13 minicells must not be able to synthesize all the enzymes necessary for plasmid replication. This conclusion is supported by other data from our laboratory that show that the Clo DF13 replication is dependent on at least some host proteins (32). Because replication and transcription of the Clo DF13 factor do take place in minicells, a number of chromosomal products necessary for replication and transcription of this factor must segregate into minicells. Probably one or more of these products are inactivated by prolonged storage at −190 C.

Although RNA synthesis does not take place after 14 days of storage, protein synthesis still occurs at the original rate. This means that, after 14 days of storage, only translation of the pre-existing mRNAs of the Clo DF13 factor takes place and that the Clo DF13 mRNAs in purified minicells are rather stable.

The observed unusual stability of Clo DF13 mRNAs might be a property of mRNAs in minicells, because it has been stated by Heppel (cited in reference 27) that the relative activity of RNase I in minicells isolated from cultures in the stationary phase of growth corresponds to only half the activity of RNase I found in cells.

Analysis of RNA synthesized in purified

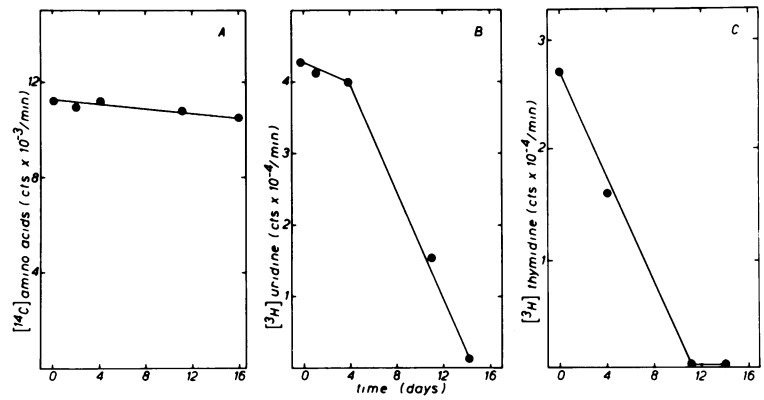

FIG. 3. *Effect of storage at −190 C on the ability of plasmid-containing minicells to synthesize DNA, RNA, and protein. Purified minicells isolated from P678-54(Clo DF13) and suspended in the appropriate medium were divided into 1-ml portions. Some portions were used immediately for incorporation experiments (indicated in Fig. 3 as time zero), and the other protions were quickly freezed and stored in liquid nitrogen. After the indicated periods of storage, minicells were thawed and examined for ability to synthesize protein, RNA and DNA. (A) Minicells were incubated with 1.5 μCi of [^{14}C]AA per ml for 120 min at 37 C. Then, duplicate 200-μliter samples were taken and radioactivity was determined as described in the legend to Fig. 2. (B) RNA synthesis was measured. Minicells were incubated with 40 μCi of [^{3}H]uridine per ml for 30 min at 37 C. Then duplicate 200-μliter samples were taken and the incorporation of [^{3}H]uridine into cold trichloroacetic acid-insoluble material was determined as described in the legend to Fig. 1. (C) DNA synthesis was determined. Minicells were incubated with 50 μCi of [^{3}H]thymidine per ml. After 60 min at 37 C, duplicate 200-μliter samples were collected onto 3 MM Whatman filters and further treated as described (Fig. 1).*

minicells containing a repressed Clo DF13 factor. To examine the Clo DF13-specific RNA synthesized by Clo DF13-containing minicells more extensively, the RNA was extracted from these minicells and applied on 2.1% polyacrylamide gels containing 0.5% agarose. After electrophoresis, the gels were prepared for autoradiography, and microdensitometer scans of these autoradiographs are presented in Fig. 4. RNA isolated from plasmidless minicells is shown in Fig. 4B; only labeled 16*S* and 23*S* rRNA synthesized by the contaminating cells can be observed. In this experiment we used less purified minicells (1 cell per 5×10^4 minicells) to obtain internal markers for the calculation of *S* values. When the purified minicell fraction contained less than one cell per 10^6 minicells, the synthesis of these labeled rRNA species was almost abolished (see Fig. 8B). The same rRNA species can be observed in Fig. 4A, which represents RNA extracted from Clo DF13-containing minicells. However, there is one striking difference. A very sharp peak is present, migrating between the 16*S* and 23*S* peaks. This peak represents neither a precursor of the rRNA species nor a complex between rRNA and protein or DNA, because in that case this peak should also be present in the gel containing rRNA from P678-54 minicells. This RNA species is only synthesized in Clo DF13-containing minicells, and RNA isolated from these minicells hybridizes only with Clo DF13 DNA (see Table 1). From these results we concluded that this labeled RNA peak represents a Clo DF13-specific mRNA synthesized by minicells containing a repressed Clo DF13 factor. This mRNA is designated as R.1. The prefix R is used to designate an mRNA synthesized by the Clo DF13 factor present in minicells. The average *S* value of this messenger, which was estimated from the plot in Fig. 5A, is 21.3, which corresponds to a molecular weight of 950,000. This implies that the 21.3*S* messenger can code for proteins with a maximal molecular weight of 114,000 (Table 2).

Analysis of proteins synthesized in purified minicells containing a repressed Clo DF13 factor. To determine which Clo DF13-specific proteins are directed by the Clo DF13 factor in repressed state, labeled proteins were isolated from Clo DF13-containing minicells as described and subjected to polyacrylamide gel electrophoresis. Densitometer scans of the autoradiographs of the resulting gels are shown in Fig. 6. Three distinct peaks can be identified on the gel that represent proteins isolated from P678-54(Clo DF13) minicells (Fig. 6A). These three peaks (P.1, P.2, and P.3) are numbered from the origin. The prefix P is used to designate a polypeptide synthesized by the Clo DF13 minicells. The three polypeptides are only synthesized in Clo DF13-containing minicells and not in plasmidless minicells (Fig. 6B). From

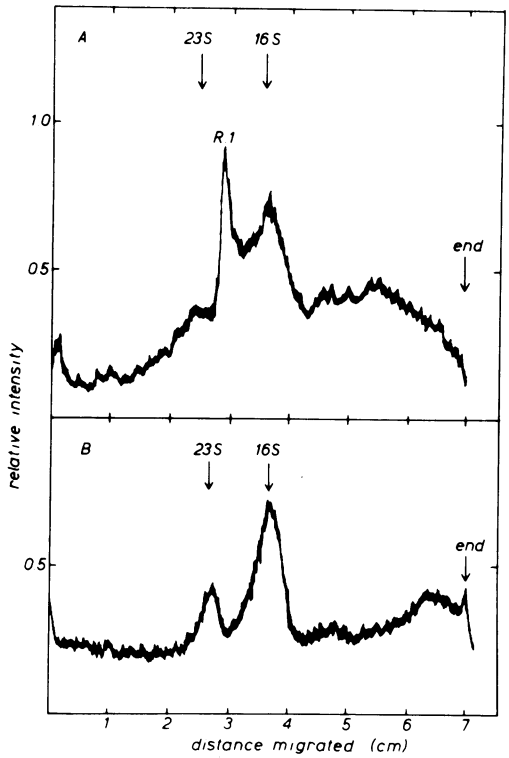

FIG. 4. *Gel electrophoresis of labeled RNA extracted from minicells. The positions at which the 23S and 16S rRNA from E. coli migrated are indicated on the tracings with arrows. (A) ^{14}C-labeled RNA extracted from minicells containing the Clo DF13 factor. (B) ^{14}C-labeled RNA extracted from plasmid-free minicells (degree of purification: one cell per 5 × 10^4 minicells).*

these results we concluded that the repressed Clo DF13 factor directs the synthesis of at least three polypeptides. The molecular weights of the polypeptides can be estimated from the plot of the molecular weights of protein markers versus their electrophoretic mobilities (32). Figure 7A shows the estimated molecular weights of the polypeptide species synthesized in Clo

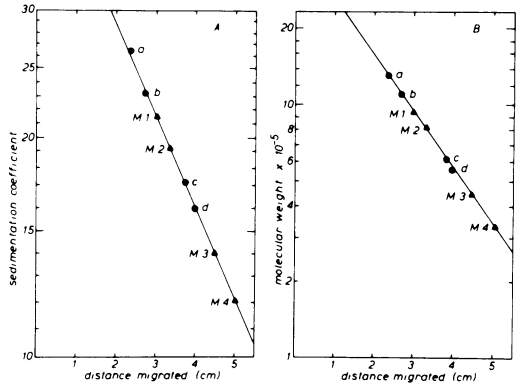

FIG. 5. *Plot of the distance migrated of RNA species in Fig. 4 and Fig. 8 and rRNA markers versus log sedimentation coefficients (Fig. 5A) or log molecular weights (Fig. 5B). The distance migrated was measured directly from the autoradiographs. The molecular weights of the optical markers (●) are 11.0 × 10^5 and 5.4 × 10^5 for 23S (b) and 16S (d) rRNA from E. coli (26) and 12.4 × 10^5 and 6.0 × 10^5 for 26S (a) and 17S (c) rRNA from yeast (6). The Clo DF13-specific mRNAs (▲) are indicated as M.1 (Clo DF13 minicell RNA) and M.2, M.3 and M.4 (RNA from minicells harboring the mutant Clo DF13 plasmid).*

TABLE 2. *Schema of identified Clo DF13-specific mRNAs and polypeptides synthesized in Clo DF13 minicells and minicells harboring the mutant Clo DF13 factor*

S values of Identified mRNAs			Mol wt[c] of mRNAs	Coding capacity (mol wt)[d]	Mol wt of identified polypeptides		
Code	Clo DF13[a]	Clo DF13[b] mutant			Clo DF13[a]	Clo DF13[b] mutant	Code
R.1	21.3	21.3	95. × 10^5	114,000	70,000	70,000	P.1
					20,000	20,000	P.2
					11,000	11,000	P.3
R.2		19.5	8.2 × 10^5	98,000		58,000	P.4
						44,000	P.5
R.3		14.0	4.5 × 10^5	54,000		28,000	P.6
						16,000	P.7
R.4		12.0	3.3 × 10^5	40,000		14,000	P.8
Total			25.5 × 10^5	306,000		261,000	

[a] mRNAs or polypeptides synthesized in Clo DF13 minicells.
[b] mRNAs or polypeptides synthesized in minicells harboring the mutant Clo DF13 factor.
[c] Molecular weights of the mRNA species were estimated from the plot in Fig. 5B.
[d] The coding capacity for protein of an mRNA was estimated from the molecular weight of the mRNA by assuming that a triplet has an average molecular weight of about 1,000 and that an amino acid has an average molecular weight of about 120.

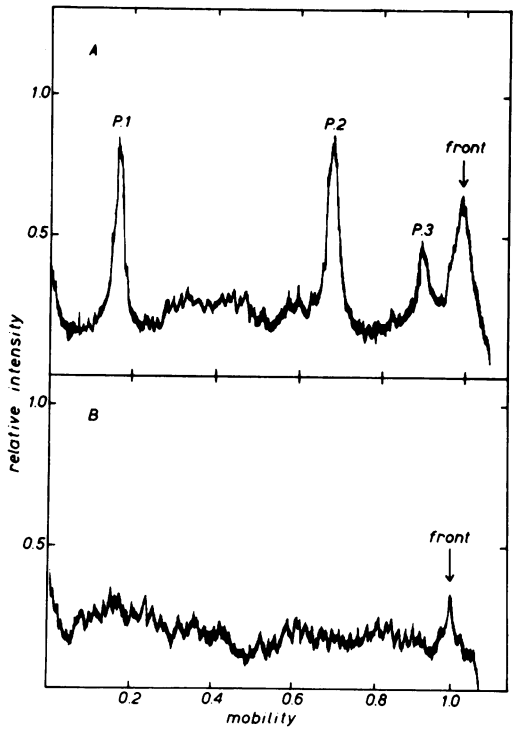

FIG. 6. *SDS-polyacrylamide gel electrophoresis of labeled proteins extracted from minicells. (A) ^{14}C-labeled proteins extracted from Clo DF13 minicells. (B) ^{14}C-labeled proteins isolated from plasmid-free minicells.*

DF13 minicells. The molecular weights are about 70,000, 20,000, and 11,000 for polypeptide P.1, P.2, and P.3, respectively.

The total molecular weight is about 101,000. The 21.3*S* mRNA synthesized by the Clo DF13 minicells is large enough to code for these three polypeptides.

Induction of the Clo DF13 factor. We have been unable to detect induction of cloacin DF13 synthesis in minicells treated with mitomycin C. Therefore, we attempted to isolate a thermoinducible Clo DF13 mutant. Such a mutant should show enhanced cloacin production at the restrictive temperature (42 C). After mutagenesis with *N*-methyl-*N*-nitroso-*N'*-nitroquanidine we were able to isolate a Clo DF13-containing mutant strain that produces about eightfold more cloacin than the parent strain. The mutation is located on the Clo DF13 factor, since enhanced cloacin production is observed in strains into which the plasmid has been transferred. This enhanced production is observed at all temperatures; therefore, this mutant is not thermoinducible for cloacin production. Cells containing this mutant plasmid contain, on the average, 70 Clo DF13 copies per cell, whereas wild-type cloacinogenic cells contain only 10 Clo DF13 copies per cell. Minicells harboring this mutant plasmid synthesize about six times more RNA and protein than minicells with a wild-type Clo DF13 factor (Kool and Nijkamp, manuscript in preparation). Furthermore, cloacin production in whole cells con-

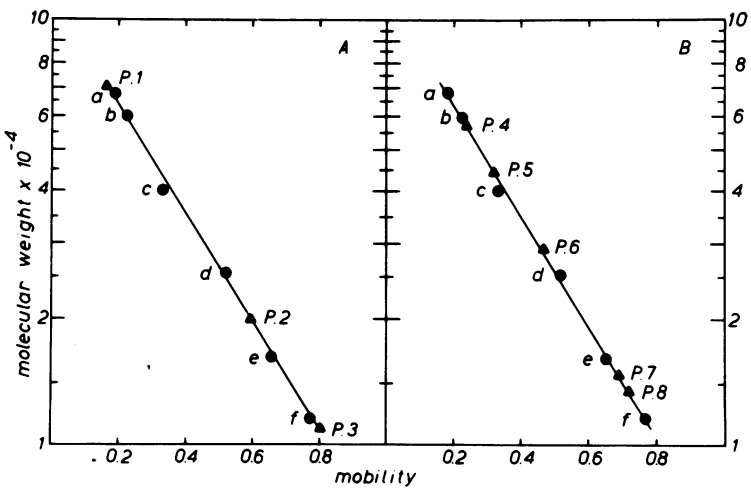

FIG. 7. *Estimation of the molecular weights of Clo DF13-specific polypeptides from the linear plot of log molecular weight versus mobility. The mobility of the polypeptide bands was determined by the method of Weber and Osborn (33). The standard molecular weight values (●) of the protein markers used were (a) 68,000 for serum albumine, (b) 60,000 for catalase, (c) 40,000 for aldolase, (d) 25,700 for chymotrypsinogen, (e) 17,200 for myoglobin, and (f) 11,700 for cytochrome c. (A) Molecular weights of the polypeptides synthesized by Clo DF13 minicells, indicated as P.1, P.2, P.3. (B) Molecular weights of the additional polypeptides species (P.4, P.5, P.6, P.7, and P.8) synthesized by minicells harboring the Clo DF13 mutant.*

taining this mutant plasmid can be further enhanced about 10-fold by mitomycin C, suggesting that this mutant plasmid is still partially repressed.

Species of RNA synthesized in minicells harboring the mutant Clo DF13 factor. Minicells of strain P678-54 and P678-54 harboring the mutant Clo DF13 factor were purified and incubated with [^{14}C]uridine. ^{14}C-labeled RNA was extracted from these minicells and subjected to polyacrylamide gel electrophoresis. The densitometer traces of the autoradiographs of the resulting gels are shown in Fig. 8. These minicell preparations were highly pure, and there is almost no 16*S* and 23*S* rRNA synthesized by the contaminating cells in the purified minicells of strain P678-54 (Fig. 8B). The RNA species synthesized by the mutant Clo DF13 factor minicells are presented in Fig. 8A. Besides the 21.3*S* mRNA, which is also synthesized by the wild-type Clo DF13 minicells but which is now present in larger amounts, three additional RNA species are present (R.2, R.3, and R.4). Hybridization experiments (Table 1) showed that RNA from these minicells represents Clo DF13-specific RNA. Since the RNA species are not synthesized in plasmid-free minicells (Fig. 8B) or in wild-type Clo DF13 minicells (Fig. 4A), we conclude that these three additional RNA species probably represent Clo DF13-specific mRNAs that are transcribed from the part of the Clo DF13 plasmid that is usually repressed. The average *S* values of these three mRNAs, estimated from the plot of the rRNA markers in Fig. 5A, are about 19.5, 14, and 12 for messengers R.2, R.3, and R.4, respectively.

Species of protein synthesized in minicells harboring the mutant Clo DF13 factor. ^{14}C-labeled proteins were isolated from minicells harboring the mutant Clo DF13 factor and subjected to polyacrylamide gel electrophoresis. Microdensitometer traces of the autoradiographs of the gels are shown in Fig. 9. The primary polypeptide species (P.1, P.2, and P.3) observed are the same as those synthesized in the wild-type Clo DF13 minicells. However, these three polypeptides are now synthesized in larger amounts. Additionally, five other polypeptides (P.4, P.5, P.6, P.7, and P.8) are present. We think that the synthesis of these five polypeptides by minicells harboring the mutant Clo DF13 factor is directed by the usually repressed part of the plasmid.

The molecular weights of these five additional polypeptides are estimated from the plot of the molecular weight of protein markers versus their mobility (Fig. 7B). The estimated molecular weights are about 58,000, 44,000, 28,000, 16,000, and 14,000 for polypeptide P.4, P.5, P.6, P.7, and P.8, respectively.

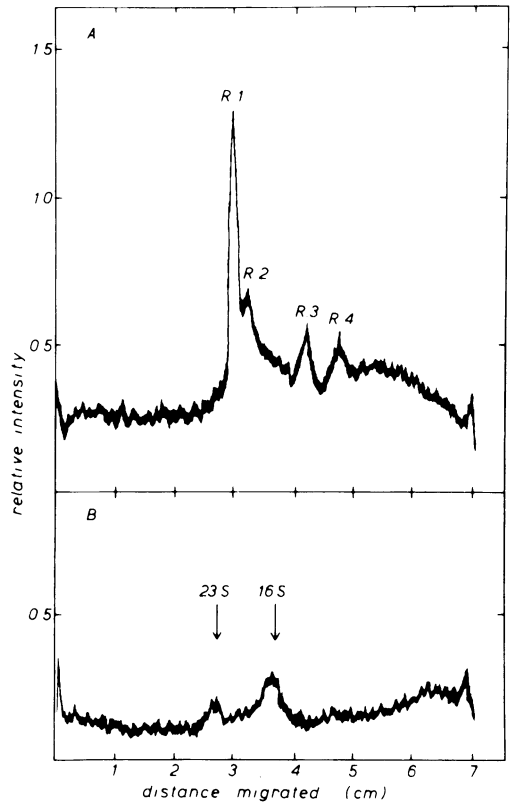

FIG. 8. *Gel electrophoresis of labeled RNA extracted from minicells. (A) ^{14}C-labeled RNA synthesized in minicells harboring the mutant Clo DF13 factor. (B) ^{14}C-labeled RNA extracted from plasmid-free minicells (degree of purification: less than one cell per 10^6 minicells).*

DISCUSSION

In this paper we present data that show that Clo DF13-containing minicells are able to incorporate radioactive precursors into RNA and protein. This incorporation represents mainly the synthesis of Clo DF13-specific mRNA and protein species. Plasmid-free minicells are able to synthesize a small amount of RNA and protein, which is not due to the presence of contaminating cells in the minicell preparation. Analysis on polyacrylamide gels of this synthesized RNA and protein reveals no distinct radioactive peaks. From these results we conclude that the RNA and protein synthesized in plasmid-free minicells must be due to transcription and translation of random chromosomal

DNA fragments and host RNAs trapped in these minicells.

The Clo DF13-specific mRNAs synthesized in minicells are rather stable, since protein synthesis in Clo DF13 minicells occurs at the original rate after 14 days of storage in liquid nitrogen, although DNA and RNA syntheses are not observed.

The Clo DF13-specific polypeptides and RNAs, likely mRNAs, synthesized by minicells harboring the Clo DF13 factor or the mutant Clo DF13 factor are summarized in Table 2. The wild-type Clo DF13 factor in minicells directs the synthesis of one mRNA (R.1) and three polypeptide species (P.1, P.2, and P.3). The total molecular weight of these three polypeptides is about 101,000. The 21.3*S* mRNA could code for polypeptide(s) with a maximal molecular weight of 114,000 (Table 2), so this mRNA is large enough to account for translation of these three polypeptides. Minicells harboring the mutant Clo DF13 factor synthesize about six times more RNA and protein than minicells with the wild-type Clo DF13 factor, and analysis of this RNA and protein shows mainly the same RNA and polypeptides species as are synthesized by the wild-type Clo DF13 minicells. However, the amount synthesized is greater, and, besides these species, three additional RNA species (R.2, R.3, and R.4) and five additional polypeptide species (P.4, P.5, P.6, P.7, and P.8) are synthesized. Only the 19.5*S* mRNA (R.2) is large enough to code for polypeptide P.4. We think that polypeptide P.4 represents the cloacin DF13, since its molecular weight of 58,000 is in good agreement with the molecular weight of cloacin, which was estimated earlier at 61,000 ± 4,000 by de Graaf et al. (13).

The total molecular weight of all eight polypeptides corresponds to 85% of the maximal coding capacity of the identified mRNAs. This could mean that we either did not observe one or more polypeptides, or the remaining parts of the identified mRNAs are not translated into protein. The total molecular weight of the four identified mRNAs is about 25.5×10^5. The total molecular weight of RNA that could be transcribed from the Clo DF13 genome is about 30×10^5 (i.e., half the molecular weight of the Clo DF13 DNA). This could mean that we could not identify one or more small mRNA species (small RNA species in Col factor-containing minicells were recently detected by Roozen et al. [27]). However, it may be that a part of the Clo DF13 DNA is not transcribed into RNA but serves as regulatory sites. Evidence that the identified mRNAs represent four different mRNAs can be obtained from DNA-RNA hybridization competition experiments, which rule out the possibility that the same nucleotide sequences are present in two or more RNA bands.

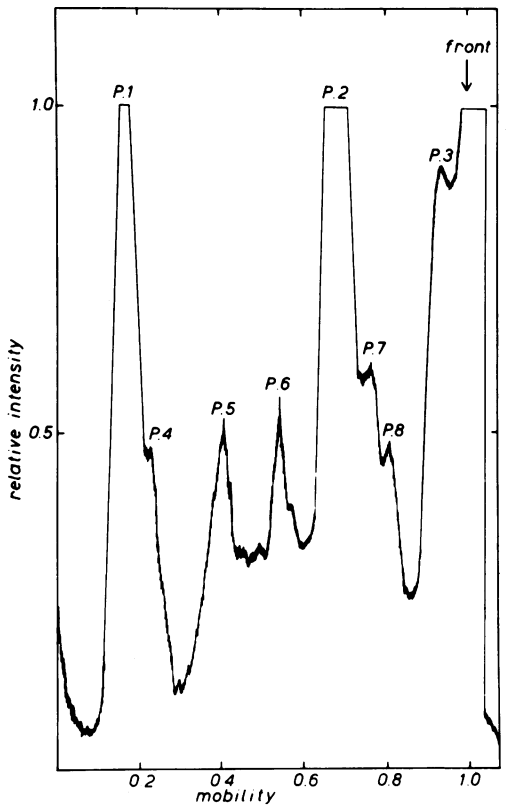

FIG. 9. *SDS-polyacrylamide gel electrophoresis of ^{14}C-labeled proteins extracted from minicells harboring the mutant Clo DF13 plasmid. The polypeptide species P.4, P.7, and P.8 appear as "shoulders" on this microdensitometer tracing, but were clearly discernible "bands" on the original autoradiographs.*

Analysis of RNA and proteins synthesized by minicells harboring the mutant Clo DF13 factor shows that the synthesized amount of the additional RNAs (R.2 to R.4) and polypeptides (P.4 to P.8) is very low, so a clearly observable induction of the usually repressed part of the plasmid apparently does not take place, and mutant cloacinogenic cells probably contain mutant Clo DF13 plasmids that are still partially repressed. The observed enhancement of cloacin production in cloacinogenic cells and the small synthesis of additional RNA and polypeptide species in mutant Clo DF13 minicells could be the result of either the increased gene dosage or the threefold increase of Clo DF13 plasmids in cells harboring the mutant plasmids, enhancing the chance per cell on spontaneous induction. That the spontaneous

induction is influenced by the number of plasmids per cell has been shown for Col E2 by Hardy and Meynell (15).

We have shown in this paper that the minicell system is an extremely useful tool for the identification and characterization of bacteriocinogenic factor Clo DF13-specific gene products. Furthermore, we think that the minicell system will enable us to purify and elucidate the function of bacteriocinogenic factor-specific gene products.

In general, the minicell system should be a very useful tool for studying the gene products of plasmids, of phage genomes, and of those bacterial genes that are integrated into plasmid or phage DNA, if such species can segregate into minicells.

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