**In Vitro Synthesis of the E3 Immunity Protein Directed by Col E3 Plasmid Deoxyribonucleic Acid***

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JOSEPH SIDIKARO AND MASAYASU NOMURA

From the Institute for Enzyme Research, and Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706

**SUMMARY**

E3 colicinogenic cells are immune to colicin E3. A protein called "E3 immunity protein" was previously isolated from E3 colicinogenic cells and shown to prevent the E3-induced in vitro inactivation of ribosomes. We now show that the structural gene for E3 immunity protein resides on the Col E3 plasmid, a plasmid which is present in E3 colicinogenic cells and carries the structural gene for colicin E3 production. For this purpose, Col E3 plasmid DNA was purified and characterized. The DNA preparation was shown to be homogeneous as judged by electron microscopy as well as agarose gel electrophoresis and has the ability to transform noncolicinogenic *Escherichia coli* cells into E3 colicinogenic cells with a high efficiency. The Col E3 DNA was then used as a template in a DNA-dependent in vitro protein-synthesizing system, and protein products were characterized. A radioactive protein product was detected which co-migrates with reference E3 immunity protein in urea-polyacrylamide gel electrophoresis at pH 8.7 and urea-sodium dodecyl sulfate polyacrylamide gel electrophoresis at pH 7.6. This protein was produced only in the presence of Col E3 plasmid DNA. No such protein was produced when Col E3 plasmid DNA was omitted or replaced with chromosomal DNA. The radioactive protein was isolated and shown to be very homogeneous as judged by the correspondence of tryptic peptides. The synthesis of immunity protein in vitro was also shown by radioimmunodiffusion. Thus, the structural gene for E3 immunity protein resides on the Col E3 plasmid. In addition, we have shown that colicin E3 protein is also synthesized in the same in vitro system using Col E3 plasmid DNA as a template, confirming the previous notion that the structural gene for colicin E3 protein resides on the Col E3 plasmid.

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of chloramphenicol, the cultures were further shaken for 8 to 12 hours. The cultures were then chilled to 4°C and harvested by centrifugation on a Sorvall GSA rotor at 7000 rpm for 5 min. A second procedure, which did not affect the properties of the plasmid DNA but increased the yield considerably, was used in later experiments. This procedure was a modification of the above procedure. When the cell culture reached a density of 140 Klett units, mitomycin C was added at a final concentration of 0.5 μg per ml. The culture was shaken for 30 min, and chloramphenicol then was added. The culture was then treated as described in the first procedure.

Two techniques were used for preparation of DNA. In early experiments the "cleared lysate" technique of Clewell and Helinski (13) was used. However, this technique was not suitable for large scale preparations because the cleared lysate contains relatively large amounts of chromosomal DNA and was quite viscous. The second technique used was a modification of the method of Guerry et al. (14). This technique, which is described below, gave better yields.

Plasmid DNA was routinely prepared from 10-liter cultures. The cell pellet (thawed if stored frozen) was resuspended in 150 ml of 0.04 M Tris (pH 7.9), 0.02 M blue, and applied to agarose gels. Weisblum (University of Wisconsin). A typical reaction mixture contained 0.2 ml of the cell suspension (10^9 cells per ml) in 0.03 M CaCl_2 or, as a control, 0.2 ml of 0.03 M CaCl_2 and the appropriate amounts of DNA in 0.1 ml of 0.05 M MgCl_2 in Buffer A (0.02 M Tris (pH 8.0), 1 mM EDTA (pH 8.0), and 0.02 M NaCl). The transformation mixtures were incubated for 60 min at 0°C, transferred to 43°C for 3 min, and chilled on ice. Cold L broth (2.7 ml) was added to each tube, and the tubes were incubated for 75 min at 37°C to allow expression of immunity. The tubes were then chilled to 0°C and 0.3-ml samples were mixed with an equal volume of E3 solution (0.5 μg per ml; 5 × 10^6 killing units per μg) in DE-BSA. The mixtures were incubated at 37°C for 30 min to allow maximum colicin E3 adsorption, and chilled to 0°C again. Survivors were scored by plating 0.2-ml duplicates on TM-agar plates and incubating overnight at 37°C.

Colonies of survivor cells were tested for colicin E3 production and E3 immunity. Single colonies from the TM-agar plates were suspended in mastic drops of DE medium. Samples from the cell suspensions were spotted on TM plates overlayed with 2 ml of TM-soft agar, and TM plates overlayed with 10^9 NO26 cells in 2 ml of TM-soft agar. Colicin production was scored by formation of an inhibition zone on the NO26 indicator lawn. Immunity was scored by cross-streaking the cell suspensions with purified colicin E3 and colony hybridization of colonies which did not contain colicin E3 transforms. All of these were immune to E3. The nutritional requirements of the transforms were tested. When plasmid DNA from NO543 was used for transformation, transforms were checked for immunity to colicin Iα as well as Iα production. Iα production was tested by cross-streaking the lysates of small cultures of transforms with NO1170 (resistant to.colicin E1, E2, E3, and sensitive to Iα). All of the transforms which received Col E3 DNA did not receive Col Iα as judged from the above tests.

EcoRI Cleavage of Plasmid DNA—Escherichia coli restriction endonuclease I was prepared from E. coli K123 according to the method of Wessman and Dusl (17), and was a gift from Dr. B. Weisblum (University of Wisconsin). A typical reaction mixture contained 1 to 2 μg of DNA (DNA was in 0.1 X SSC) and 5 μl of the restriction enzyme in a total volume of 50 μl of 0.1 M MgCl_2 and 0.1 M Tris, HCl (pH 7.4). The reaction mixture was incubated for 30 min at 37°C, mixed with 25 μl of 30% sucrose-0.05% bromphenol blue, and applied to agarose gels.

A gel electrophoresis was done essentially according to the method of Sharp et al. (18). The agarose concentration was used was 1.5% (w/v). Agarose (Seakem Marine Colloids, Inc., Springfield, N. J.) was dissolved in a hot solution of 0.4 M Tris (pH 7.9), 0.02 mM sodium acetate, 0.001 M EDTA (pH 7.9), and 0.5 μg per ml of ethidium bromide (Sigma) (Eth buffer), and autoclaved. Gels were run in Eth buffer at 3 mA per gel for 16 hours at room temperature. DNA bands could be visualized during the run with a long wave ultraviolet light.
violet light (Ultra-Violet Products, San Gabriel, California). At
the end of a run the gels were gently blown out of the tubes and
photographed using Polaroid type 57 film and a Kodak No. 22
yellow-orange wratten filter.

In Vitro Synthesis of E3 and E3 Immunity Protein—In vitro
plasmid-DNA-dependent protein synthesis was essentially carried
out according to the method of Kaltschmidt et al. (19) which is a
method adapted from the methods described by Zubay et al. (20)
and Miller (21). Ribosomes, S100DE fraction, RNA polymerase,
and partially purified initiation factors were a gift of Dr. L. Lind-
dahl in our laboratory. All of the above components were pre-
pared from NO1310 cells. To reduce the level of endogenous
mRNA, cells were starved for uracil for 90 min and harvested, and
the various components were then prepared (19, 22). S100DE
fraction was prepared by removing nucleic acids from ribosome-
free S100 fraction as previously described (22).

A standard reaction mixture contained the following compo-
nents: RNA polymerase, 2 ~1 (about 30 ~g); initiation factor, 5 ~l
(containing about 50 ~g of protein); S100DE, 10 ~l (containing
about 250 ~g of protein); 70 S ribosomes, 2 ~l (2.4 A260 units);
“chemicals,” 10 ~l (“chemicals” are identical to the “reaction
mixture” as described by Miller (21) and includes tRNA, amino
acids, and cofactors); cyclic AMP (70 mM), 1 ~l; NaPO4 (50 mM),
1 ~l; pyruvate kinase (6.3 mg per ml), 0.5 ~l; 70 m& Tris-acetate buffer (pH 7.8), 3 ~l; and 30
~l of DNA solution (50 ~g per ml) in 10 mM Tris-acetate buffer
(pH 7.8). When ~C-labeled L-amino acids were used, the chemi-
cals used contained only the amino acids not present in the radio-
active mixture. The ~C-labeled mixture of amino acids contained
15 amino acids with specific activities ranging from 93 mCi per nm
for glycine to 387 mCi per nm for tyrosine. The concentration of
radioactivity was 0.1 mCi per ml. In reaction mixtures that con-
tained ~C-labeled L-amino acids, 5 ~l of the radioactive amino
cids mixture were used in a standard reaction mixture. Reaction
mixtures were incubated at 37° for 2 hours. Five-microliter sam-
ple solutions were taken out and the amount of acid-precipitable
~S- or
~C-protein was measured. The amounts of ribosomes, S100DE,
RNA polymerase, and initiation factor were predetermined to give
maximum incorporation. Cyclic AMP was always included, al-
though we have not examined its necessity for colicin or immunity
protein synthesis.

Reductive Methylation of E3 Immunity Protein—In order to label
purified immunity protein with ~H, the reductive methylation
of 3H-labeled immunity protein was 2900 cpm per ~g.

Urea-SDS-Polyacrylamide Gel Electrophoresis—Urea-SDS-poly-
acrylamide gel electrophoresis was carried out according to a
modification of the method of Strauss and Kaesberg (24). The
urea-SDS (pH 7.6)-Polyacrylamide gels contained 0.1% SDS
(Sigma), 12% acrylamide (Eastman Organic Chemicals), 0.24%
N-acrylamide (Sigma), 6 M urea, and 0.1 M sodium phosphate
buffer, pH 7.6. After polymerization the gels were prerun for 30
min at 3 ma per gel with a buffer solution containing 0.1% SDS,
5 mM 3-mercaptoethanol, and 0.1% phosphate (pH 7.6) at
the cathode chamber, and a buffer containing 0.1% SDS, 6 M urea,
and 0.1 M phosphate (pH 7.6) at the anode chamber. Samples
(65 ~l) were mixed with reference ~H-labeled immunity protein
(3.6 ~g) in 30 ~l of dissociation mixture (0.125 M EDTA,
0.1 M NaOAc, 0.1 M NaOAc, pH 8.0 (5% w/v), casein amino acids, and 50 ~g per ml of RNase
(protease-free, Sigma) (25)). The mixtures were then incubated
10 min at 37°. Solid urea (to give 6 to 8 M), 2 ~l of 2-mercaptoethanol,
and 20 ~l of 10% SDS were added, and samples were incubated at
60° for 15 min. Samples were then dialyzed against 6 M urea, 0.1% SDS,
and 0.1 M sodium phosphate buffer, pH 7.6. Dialysis was
carried out at room temperature for 24 hours against three to four
changes of 5000 volumes of dialysis buffer. Five microliters of
0.05% bromphenol blue were added to each sample before elec-
trophoresis. Electrophoresis was carried out at room temperature
at 3 ma per gel for 18 hours. The gels were fractionated on a Gil-

![Fig. 1. Partially denatured Col F3 DNA. Isolation of DNA and preparation of samples for electron microscopy were described under “Experimental Procedures.” The bar represents 1.0 μm.](http://www.jbc.org/abstract.png)
samples were counted. The vials were capped and incubated for 1 hour at 60°C; 6 ml
son automated fractionator. Slices of 1 mm were crushed to-
frames without DNA.
urea (10). At the end of the run, gels were removed from the
tubes and stained with Amido black (Calbiochem) in 7.5% acetic acid. Gels were destained electrophoretically. After destaining, the gels were photographed. They were then sliced longitudi-
Extraction of in Vitro Products from Gels and Peptide Mapping—
ference was calculated from the ratio of the number of transformants to the number of viable cells in control cultures without DNA.
<table>
<thead>
<tr>
<th>DNA</th>
<th>Frequency of transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col E3 DNA</td>
<td>0.10</td>
</tr>
<tr>
<td>Col E3 DNA (+ DNase)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chromosomal DNA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No DNA</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a Concentration of DNA was 3 µg per ml in all cases. When indicated, DNA was treated with pancreatic DNase (15 µg per ml) at 37°C for 5 min before mixing with recipient cells.

b Frequency was calculated from the ratio of the number of transformants to the number of viable cells in control cultures without DNA.

Urea-Polyacrylamide Gel Electrophoresis of in Vitro Products—

<table>
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</tr>
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<tr>
<td>Complete system</td>
<td>100.0</td>
</tr>
<tr>
<td>- DNA</td>
<td>9.8</td>
</tr>
<tr>
<td>+ Rifampicin (70 µg per ml)</td>
<td>7.2</td>
</tr>
<tr>
<td>+ DNase (70 µg per ml)</td>
<td>8.3</td>
</tr>
</tbody>
</table>

FIG. 2. Agarose gel electrophoresis of DNA. Electrophoresis was carried out for 18 hours in 1.5% agarose gels containing 0.5

TABLE II
Transformation of noncoliogenic bacilli with Col E3 DNA

1 Eco.RI

2

3

E3 Plasmid

Fragments

TABLE III
Incorporation of 14C-labeled amino acids into proteins directed by

Col E3 DNA

Reaction mixtures containing 1.5 µg of DNA were incubated for
2 hours at 37°C and acid-precipitable 14C material was determined. The final volume of the reaction mixtures was 70 µl. DNA was always added last. When DNase was present, the DNA was first preincubated with the indicated amount of the enzyme for 5 min at 37°C, then chilled and added to the rest of the reaction mixture.

<table>
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teins, it was first necessary to obtain sufficient amounts of pure
Col E3 DNA. This was achieved by growing colicinogenic cells
in rich medium, inducing the cells with mitomycin C for a short
time, and then adding chloramphenicol to the cultures. DNA
was isolated by the cleared lysate method or the SDS-NaCl
method (see "Experimental Procedures").

Fig. 1 shows an electron micrograph of Col E3 DNA. From
measurements of the contour of the circular DNA molecules in
Fig. 1, a length of approximately 2.5 μm was obtained. This
value is in good agreement with the value of 2.4 μm reported by
Inselburg (30). This is also approximately the expected length
for a circular DNA molecule with a molecular weight of $5 \times 10^6$
(31). The homogeneity of the Col E3 preparation was further
examined by the technique of agarose gel electrophoresis. As
shown in Fig. 2, a single DNA band was observed. Treatment
of the Col E3 DNA with EcoRI restriction endonuclease gave rise
to two distinct bands which migrated faster than the original
DNA. These results strongly support the conclusion that the
preparation is homogeneous.

The biological activity of the Col E3 DNA was demonstrated
by its ability to transform noncolicinogenic Escherichia coli cells
into E3 colicinogenic cells. The method used was similar to that
used for the transformation system using a drug-resistant factor
DNA (16). At a concentration of 3 μg per ml of the Col E3
DNA, as much as 0.1% of the treated cells was transformed into
E3 colicinogenic cells (Table II). Omission of the Col E3 DNA,
as well as pretreatment of plasmid DNA with DNase or use of
the "chromosomal" DNA, did not result in transformation. We
conclude that the Col E3 DNA preparation is biologically active.

In Vitro Protein Synthesis Directed by Col E3 DNA—We have
previously described a DNA-dependent in vitro protein-synthes-
ing system and shown synthesis of a number of ribosomal pro-
teins directed by E. coli DNA (19). This in vitro protein-syn-
thesizing system was used to examine proteins coded for by
genes on Col E3 DNA. Specifically, we have asked whether E3
immunity protein is synthesized in vitro under the direction of
Col E3 DNA.

Table III shows that the addition of 1.5 μg of Col E3 DNA
stimulates incorporation of ¹⁴C-labeled amino acids into acid-
precipitable material by a factor of about 10. As can be seen,
this stimulation is abolished by the addition of DNase or rifampi-
cin, a specific inhibitor of DNA-dependent RNA polymerase.

The radioactive protein products synthesized in this system

![Fig. 4. Detection of immunity protein synthesized in vitro by radioimmunodiffusion. Immunodiffusion was done in 1% agar
gels in T1 buffer. I²S-proteins (Gels 1 and 2) or I²C-proteins (Gels
3 and 4) synthesized in vitro using either chromosomal DNA
(Gels 2 and 4) or Col E3 DNA (Gels 1 and 3) as a template were
placed together with carrier immunity protein (10 pg per ml) in
each center well of the four sets, respectively. The outer wells
received antiserum against purified immunity protein (AT); anti-
senin and an excess of immunity protein (10 pg per ml) (AT +
Im); antiserum and purified E3 (5 pg per ml) (AT + E3); and
preimmune serum (PI) as a control. (The amount of E3 added
was in excess of any amount of anti-E3 antibody which might con-
taminate the anti-immunity protein antiserum. This was tested
by the reaction of the mixture (AT + E3) with anti-E3 antiserum
in a separate immunodiffusion experiment.) After 3 days at room
temperature the gels were photographed (a). The gels were
washed extensively by repeated soaking in T1 buffer and finally
in distilled water. The gels were covered with a piece of filter paper,
dried, and exposed to x-ray film. The autoradiograms obtained
are shown (b).](http://www.jbc.org/)

![Fig. 3. Urea-SDS polyacrylamide gel electrophoresis of in
vitro synthesized proteins. The proteins synthesized in the in
vitro protein-synthesizing system under the direction of chromo-
osomal DNA (1.5 μg) (B), and Col E3 DNA (1.5 μg) (C) or without
DNA (A) were analyzed on 12% acrylamide, 6 m urea, 0.1% SDS
gels. Samples were subjected to electrophoresis with (B and C)
or without (A) 12 μg of I²H-labeled immunity protein (---).
Fractions were collected and counted as described under "Experi-
mental Procedures." The direction of migration is from right to
left.](http://www.jbc.org/)
FIG. 7. Tryptic peptide maps of E3 immunity protein synthesized in vitro. Proteins co-migrating with the carrier E3 immunity protein were extracted from the gels after staining (cf. Figs. 5 and 6) and mixed with purified immunity protein as described under "Experimental Procedures." The line drawing of the peptide map is shown in c. Peptides are numbered as described in the previous paper (10). ——, incompletely digested peptides. The direction of chromatography is upward. The origin is marked with an arrow.

FIG. 6. Gel electrophoresis of proteins synthesized in vitro. Samples were prepared and subjected to electrophoresis in the same manner as described in Fig. 5. The staining pattern (A) and autoradiogram of the same gels (B) are presented. Carrier E3 immunity protein (7.5 μg) was added to all samples. Gels 2 and 3 contained 14C-proteins synthesized in the systems containing chromosomal DNA (1.5 μg) (Gel 2) and the same amount of Col E3 DNA (Gel 3), respectively. Gels 4 and 5 are similar to 2 and 3, respectively, except that [35S]methionine was used instead of 14C-labeled amino acid mixture. Gels 1 and 6 contained only carrier E3 immunity protein. The arrows indicate the position of the immunity protein band.
tein which co-migrates with the reference E3 immunity protein was extracted from the polyacrylamide gels after electrophoresis and mixed with excess carrier E3 immunity protein, and the mixture was digested with trypsin. The tryptic peptides were separated by a two-dimensional fingerprinting technique on silica gel thin layer plates. The separated peptides were stained with ninhydrin and the radioactive peptides were detected by autoradiography. The results are shown in Fig. 7.

The peptide pattern as revealed by ninhydrin staining (Fig. 7a) was very similar to that reported previously (10) except for the presence of larger amounts of peptides 8 and 9, both of which are incomplete digestion products. As can be seen in Fig. 7b, the pattern of radioactive peptides which come from the \textit{in vitro} product is almost identical to the ninhydrin-stained pattern, except that peptide 7 was not visible in the autoradiogram. This peptide is the NH$_2$ terminus peptide, and its sequence is Gly-Leu-Lys (10). There are several possible explanations for the absence of this peptide in the \textit{in vitro} product. The most likely possibility is that the synthesis of the E3 immunity protein starts with formyl methionine and the present \textit{in vitro} system lacks the enzyme which removes the formylmethionine, leading to the absence of tryptic peptide that coincides with peptide 7. However, there are no "new" radioactive peptides that do not correspond to ninhydrin-reactive peptides. The "new" radioactive NH$_2$ terminus peptide might co-migrate with one of the other peptides. Although the correct explanation for the absence of peptide 7 in the \textit{in vitro} protein must await further experiments, it is safe to conclude that the \textit{in vitro} synthesized protein is nearly identical to the reference E3 immunity protein, and therefore the primary structure of this protein is coded for by a part of the \textit{Col} E3 DNA used as a template in the \textit{in vitro} system.

\textbf{Synthesis of Colicin E3 Protein \textit{in Vitro}}—In addition to the E3 immunity protein, synthesis of the colicin E3 protein was also examined in the same DNA-directed protein-synthesizing system. Immunochemical methods using antisera against purified colicin E3 protein were used for detection of products similar to colicin E3. Radioactive proteins synthesized in the \textit{in vitro} system were mixed with a carrier nonradioactive colicin E3 and the presence of radioactive colicin E3 protein was detected by autoradiography of the immunodiffusion plates. The results presented in Fig. 8 show that a protein which reacts with anti-E3 antiserum was produced only when \textit{Col} E3 DNA was used as a template DNA. No such protein was produced when chromosomal DNA was used as a template. It should be noted that the observed cross-reaction is not due to possible contamination of E3 immunity protein in the colicin E3 preparation used for preparation of the anti-E3 antiserum; pretreatment of the anti-E3 antiserum with excess E3 immunity protein did not affect the results (cf. Fig. 8). These results strongly indicate that the structural gene for colicin E3 protein is, in fact, on the \textit{Col} E3 factor DNA as expected. However, no further studies were done on the exact chemical and biological properties of the "colicin E3" molecules synthesized in the present \textit{in vitro} system.

\textbf{DISCUSSION}

In our previous papers (7, 10), we have already discussed evidence to support the conclusion that the E3 immunity protein is responsible for the immunity of E3 colicinogenic cells to the homologous colicin. The results described in the present paper prove that the structural gene for the E3 immunity protein is, in fact, on the \textit{Col} E3 plasmid. Such a simple situation found in the colicin E3 system may apply to other colicin systems as well.
Thus it is likely that colicin plasmids in general carry both the structural gene for colicin and another gene for a protein ("immunity protein"); the latter protein would inhibit the biochemical reaction carried out by the colicin.

DNA-dependent in vitro protein-synthesizing systems such as the one described here may be very useful for study of the genetic organization and expression of genes on a plasmid DNA. In the present system, we have shown that at least two proteins, the E3 immunity protein and the colicin E3, are produced in the presence of the Col E3 DNA. The pattern of proteins synthesized is relatively simple with this DNA as a template. In the absence of DNA, virtually no protein is synthesized (cf. Fig. 3A).

Thus, characterization of other protein products coded for by the Col E3 plasmid DNA may be possible. Regulation of synthesis of these proteins both at the transcriptional and translational levels could also be studied. In addition, we have already shown that a restriction enzyme, EcoRI, cleaves the Col E3 DNA into two fragments (Fig. 2), and that the fragmented DNA retains the activity to produce both E3 immunity protein and colicin E3.2 Thus, it would be possible to map the genes, that is, to separate the two fragments of Col E3 DNA and find out which fragment carries one or both of the genes for these proteins.

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J Sidikaro and N Masayasu


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