THE 3'-TERMINUS OF 16 S RIBOSOMAL RNA OF *ESCHERICHIA COLI.* ISOLATION AND PURIFICATION OF THE TERMINAL 49-NUCLEOTIDE FRAGMENT AT A MILLIGRAM SCALE

R. A. BAAN, R. VAN CHARLDORP, E. VAN LEERDAM, P. H. VAN KNIPPENBERG and L. BOSCH

Department of Biochemistry, State University, Wassenaarseweg 64,

and

J. F. M. DE ROOIJ and J. H. VAN BOOM

Department of Organic Chemistry, State University, Wassenaarseweg 76, Leiden, The Netherlands

1. Introduction

Recently special interest has been focussed upon the important role of the 3'-terminus of 16 S ribosomal RNA of *Escherichia coli* in protein synthesis. A number of proteins which are required for initiation of protein synthesis, like S1 [1,2], S12 [3], IF-2 and IF-3 [4], are located at or near the 3'-end of 16 S RNA [5–8]. Furthermore, cleavage of the 16 S RNA at a site which is 49 nucleotides from the 3'-end by colicin E3 [9] or cloacin DF13 [10] blocks protein synthesis completely [11]. We found it to impair the response of the ribosome to the initiation factor IF-1 [12]. Finally, it has been proposed that a sequence of nucleotides near the 3'-end of 16 S RNA base-pairs during initiation with ribosomal binding sites on messenger RNA [13–14].

In order to obtain a more detailed insight in how this end of 16 S RNA is involved in protein biosynthesis, it is important to study its structure and the way in which this structure is influenced by ribosomal proteins and initiation factors. As a first approach we isolated the RNA fragment which is formed after cloacin treatment of ribosomes in large amounts in order to

study its structure by physical, chemical and enzymatic methods. This paper describes the isolation of the pure fragment in milligram amounts.

2. Materials

Ribosomes were isolated from *E. coli*, strain MRE 600. Cloacin DF13 was a gift from Dr F. K. de Graaf, Microbiology Department, Free University, Amsterdam.

To prevent degradation of RNA by RNAase contamination all glassware was dry heated at 140–150°C for 15 h. Buffers were made in demineralized, distilled water and sterilized at 110°C for 1 h. Dialysis tubing, magnetic stirrer bars, pipette tips and other plastic utensils were immersed in a solution of 0.6% diethylpyrocarbonate in sterile water for at least 3 h. Sucrose solutions were prepared with sterile water, filtered through millipore membranes (pore size 0.45 μ) and treated with bentonite (0.2 mg/ml of solution) before use.

3. Methods and results

3.1. *Isolation of the 3'-terminal fragment*

Ribosomes (70 S tight couples) were isolated from *E. coli* and purified by zonal centrifugation at 6 mM

Abbreviations: IF, initiation factor; MBA, methylene bisacrylamide; TEMED, *NNN'N'*-tetramethylethylenediamine; SSC, standard saline citrate.

Mg^{2+} as described [12]. About 8000 A_{260} units of
those ribosomes were incubated in standard buffer
(10 mM Tris–HCl, pH 7.6, 60 mM NH_4Cl, 10 mM Mg
acetate, 6 mM β-mercaptoethanol) with cloacin DF13
(0.02 μg of bacteriocin/A_{260} unit of ribosomes) for
30 min at 43°C. Before use, the bacteriocin was activat-
ed by heat treatment for 20 min at 95°C, according
to Oudega et al. [15]. Isolation of 30 S subunits from
cloacin treated ribosomes – hereafter referred to as
30 S clo – occurred by centrifugation at 5°C in a
BXIV zonal rotor of an MSE 65 centrifuge on a 10–45%
sucrose gradient in standard buffer, containing 1 mM
Mg acetate. Fractions containing 30 S clo were pooled,
the Mg^{2+} concentration adjusted to 10 mM and the
subunits precipitated by addition of two volumes of
cold absolute ethanol. After at least 2 h at –20°C the
precipitate was collected by centrifugation, the pellets
suspended in standard buffer and dialyzed against the
same buffer. Usually about 1500 A_{260} units of pure
30 S clo were obtained from each zonal run. RNA
was obtained from 30 S clo subunits by phenol
extraction in the presence of 1% SDS. Precipitation of
the RNA with ethanol occurred twice and after cen-
trifugation the RNA pellets were dissolved in 0.1 M
sodium acetate, pH 5.5.

In the earlier experiments isolation of the 49-nucleo-
tide fragment was achieved on 37.5 ml 5–20% sucrose
gradients in the same buffer. Centrifugation was at
22 000 rev/min for 40 h in an SW27 rotor of a Beck-
man L2-65 centrifuge. About 10 mg of RNA was
fractionated in each gradient. The gradients were
monitored at 260 nm with the aid of a Gilford 2400
spectrophotometer equipped with a flow cell. A
typical absorbance profile is shown in fig.1. Fractions
containing the 49-nucleotide fragment were collected,
pooled (pool II) and dialyzed against sterile water. The
residue obtained after lyophilization was dissolved in a
small volume of water. Salts and other low molecular
weight contaminations were removed by chromato-
graphy on a Sephadex G-50 column, eluted with water.
Fractions containing the void volume were pooled and
lyophilized. Finally, the isolated 49-nucleotide fragment
was converted into the Na^+-form by passing it through
a column (10 × 0.5 cm) filled with Dowex-50W
cation-exchange resin (Na^+-form, 100/200 mesh). The
final yield of purified fragment (pool II of fig.1) was
approximately 20 A_{units} from 1500 A_{260} units of 30 S
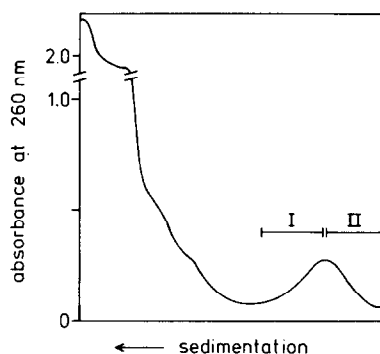clo ribosomes. The yield could be increased by running

Fig.1. Sucrose gradient centrifugation of RNA from 30 S clo.
RNA from 30 S clo (1 ml containing about 10 mg) was layered
on 37.5 ml 5–20% sucrose gradients and centrifuged as describ-
ed in the text. Usually two fractions containing the 49-nucleo-
tide fragment were collected, as indicated.

pool I (cf. fig.1) on a second sucrose gradient (not
shown). More recently fractionation of the RNA from
30 S clo occurred on a 5–30% sucrose gradient in a B
XIV zonal rotor. Essentially the same results were
obtained.

3.2. Purity and preliminary characterization of the fragment

In order to judge the extent of purification of the
fragment two procedures were followed:
(a) Electrophoresis on 15% polyacrylamide gels
    containing 0.1% SDS as described (ref. [12] and
    legend to fig.2). This method enables the detection
    of contaminating polynucleotides varying in size
    from 30–150 nucleotides.
(b) High-pressure liquid chromatography on a Perma-
    phase AAX anion-exchange column which permits
    a fractionation in the range from mononucleotides
    up to polynucleotides about 80 nucleotides long.

The electrophoretic method was used in each stage
of the purification. A typical set of gels, stained with
0.2% methylene blue in 0.2 M sodium acetate, pH 4.0,
is shown in fig.2. Where indicated, the presence of
protein has been detected by staining with Coomassie
Brilliant Blue. The final product only shows one band
after staining with methylene blue. No detectable
protein is present. From extrapolation with markers
5 S RNA and tRNA the size of the fragment is estimat-
ed to be approximately 50 nucleotides.

HIGH MW RNA →

5 S →

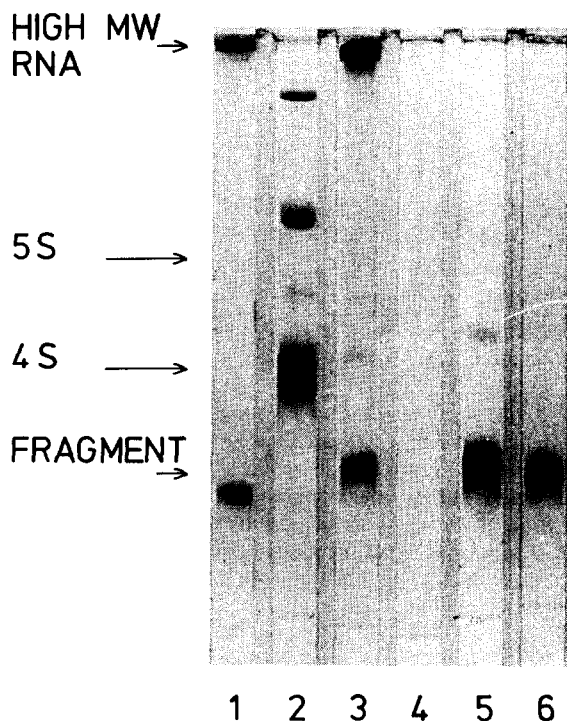4 S →

FRAGMENT →

1   2   3   4   5   6

Fig.2. Analysis of purification on polyacrylamide gels. Gels contained 15% acrylamide/MBA (19.5:0.5), 0.045% (w/v) ammonium persulphate and 0.17% (v/v) TEMED in a buffer containing 20 mM sodium acetate, 40 mM Tris–HAc, pH 7.6, 2 mM EDTA, 0.1% SDS. Pre-electrophoresis was performed for 4–5 h at 5 mA/gel in the same buffer, pH 6.0. The buffer was circulated between the upper and lower compartment. Samples were prepared in the same buffer containing 2.5% sucrose. Electrophoresis occurred at 4°C for 15–16 h at 2 mA/gel. Samples containing 3 A_{260} units of 30 S clo (gels 1 and 2) or RNA from 30 S clo (gels 3 and 4) or 0.2–0.4 A_{260} units of purified 49-nucleotide fragment (gels 5 and 6) were analyzed. Staining occurred with Methylene Blue (gels 1,3,5 and 6) or with Coomassie Brilliant Blue (gels 2 and 4). Gels 5 and 6 show typical gel patterns of fragment containing fractions I and II, respectively (compare fig.1). Positions of high molecular weight RNA, 5 S, 4 S RNA and 49-nucleotide fragment are indicated on the left.

The result of the analysis of the RNA fragment in the low molecular weight range with the HPLC technique is shown in fig.3A. The fragment emerges from the column after about 25 min of elution with the salt gradient. No ultraviolet absorbing material is found in the low molecular weight range. To analyze the base composition of the fragment it was treated with snake venom phosphodiesterase and the digestion products

were separated on the HPLC column (fig.3B). Separation of the four common ribonucleoside-5′-phosphates was achieved by eluting the column under isocratic conditions, i.e. with only the low-salt buffer. The nucleotides are well separated and their identity can easily be determined from their retention times. When the digestion was complete the relative amounts of the four mononucleotides corresponded quite well with the total base composition of the fragment (table 1).
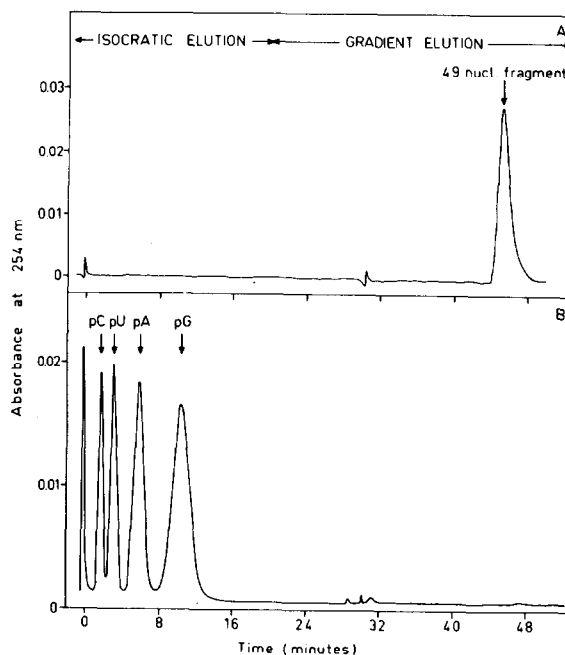
←ISOCRATIC ELUTION→ ←—— GRADIENT ELUTION ——→   A

49 nucl fragment

0.03

0.02

0.01

0

Absorbance at 254 nm

B

pC pU pA   pG

0.02

0.01

0

0   8   16   24   32   40   48

Time (minutes)

Fig.3. Purity and base composition of the fragment as determined by HPLC analysis. A sample – 0.1–0.2 A_{260} unit – of purified 49-nucleotide fragment in 5 μl buffer, which contained 0.025 M Tris–HCl, pH 9.0, 0.005 M MgCl$_2$, was injected on a 100 × 0.21 cm column, dry-packed with superficially porous Permaphase AAX anion-exchange resin (Dupont de Nemours). A Hupe & Busch UFC 1000 liquid chromatograph equipped with a Hewlett & Packard gradient programmer, was used. Column pressure was 70 kp/cm^2 at a flow rate of 1 ml/min. During the first 20 min of elution the mobile phase contained 5 × 10^{-3} M KH$_2$PO$_4$, pH 4.5 (isocratic elution). Thereafter a gradient was applied by mixing a second buffer, containing 5 × 10^{-3} KH$_2$PO$_4$/1 M KCl, pH 4.5 into the eluens at a rate of 3%/min (gradient elution). The eluate was monitored at 254 nm. Panel A: Elution pattern of the 49-nucleotide fragment. Panel B: Elution pattern of the enzymatic products obtained after treatment of the 49-nucleotide fragment with snake venom phosphodiesterase (1 μg/A_{260} unit of fragment) for 24 h at 30°C.

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Table 1
Amounts of the nucleoside-5'-monophosphates after
complete digestion of the fragment with snake venom
phosphodiesterase (pH 9.0, 30°C)

Nucleotide	Found	Expected
pC	11.8	12
pU	10.5	11
pA	9.8	10
pG	15.3	15

Peak areas from panel B of fig.3 were calculated and correct-
ed for specific absorption for each of the nucleotide mono-
phosphates [16]. The theoretical values were calculated from
the known sequence of the rRNA fragment [6]. Under the
conditions used, dimethyladenosine monophosphate showed
the same retention time on the HPLC column as 5'-GMP. In
our calculation we attributed the same specific absorption
coefficient to these two compounds, because the exact
$\in$-value of di-CH_3-5'-AMP is not known. Since the 5'-terminus
of the fragment is HO–GpUp . . . (the presence of the free
5'-OH is indicated by digestion with spleen phosphodiesterase
(not shown)) this terminal G residue appears as the nucleoside
and is not retained on the column. Therefore the total number
of nucleoside-5'-monophosphates to be expected is 48.

HPLC analysis of the products obtained after RNase T1
treatment of the 49-nucleotide fragment yielded the
set of oligonucleotides to be expected on the basis of
the primary nucleotide sequence (not shown). On the
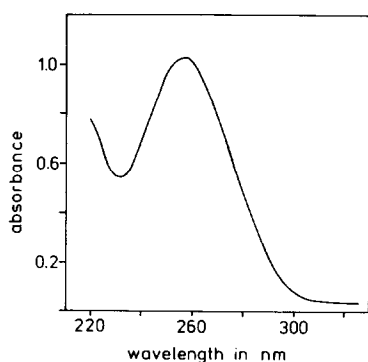basis of these analyses the isolated fragment appeared
to be virtually pure.

Fig.4. Ultraviolet spectrum of the 49-nucleotide fragment.
About 1 A_{260} unit of purified 49-nucleotide fragment was
dialyzed against 0.1 × SSC, pH 7.0. The ultraviolet spectrum
was recorded with a Unicam SP 1800 double beam UV spec-
trophotometer. From the spectrum the following data were
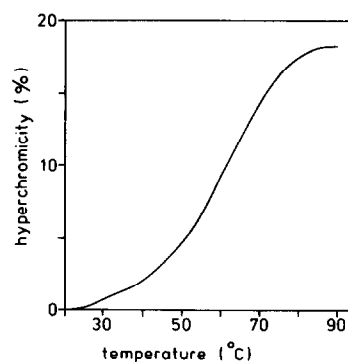obtained: A_{260}/A_{230} = 1.85; A_{260}/A_{280} = 1.96.

Fig.5. Melting curve of the 49-nucleotide fragment. About
1 A_{260}^- unit of purified 49-nucleotide fragment was dialyzed
against 0.1 × SSC. A melting curve was recorded with a
Unicam SP 1800 UV spectrophotometer equipped with an
SP 876 series temperature programme controller, at a heating
rate of 1°C/min. Hyperchromicity is expressed as percentage
of A_{260} at 20°C.

Figure 4 shows the ultraviolet spectrum of the
purified fragment dissolved in 0.1 × SSC (0.0015 M
Na citrate, 0.015 M NaCl, pH 7.0), fig.5 the melting
curve of the same solution measured by reading optical
densities at 260 nm. A hyperchromicity of 18.0% was
found in the range from 20–87°C, and a T_m of
59–60°C. Upon cooling to room temperature no
hyperchromicity remained. Addition of Mg acetate to
a final concentration of 10 mM yielded a slightly
higher hyperchromicity (19.5%) and T_m (60–61°C).
After the melting experiment the ultraviolet spectrum
of the fragment was unchanged.

When the fragment is submitted to molecular sieving
on a Sephadex G-50 column, equilibrated with distilled
water, two or more peaks can be observed suggesting an
opening of the structure upon lowering of the ionic
strength (unpublished results).

4. Conclusions

The isolation and purification procedure described
above allows the isolation at a milligram scale of the
3'-terminal 49-nucleotide fragment of 16 S rRNA,
virtually free of contaminating mono-, oligo- and poly-
nucleotides and proteins. Sufficient amounts have thus
become available for physico–chemical characteriza-
tions.

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