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Visualization of Ribosome–Single-Stranded DNA Complexes in the Electron Microscope†

Kathryn Calame and Garret Ihler*,‡

ABSTRACT: A procedure is described which allows ribosomes bound to single-stranded DNA to be visualized in the electron microscope. The number of bound ribosomes may be determined and the position of the bound ribosomes may be readily measured along the DNA. The distribution of ribosomes bound to separated *l* and *r* strands of λ DNA was shown to conform to the pattern predicted for binding at specific sites. The procedure should allow mapping of ribosome binding sites for the determination of genetic maps and may also be useful for studying translational control and relative binding affinities for ribosomes.

Mapping the location of genes, protein binding sites, and other interesting nucleotide sequences along DNA is now commonly done by electron microscopy. Procedures have become very versatile and include DNA–DNA heteroduplex analysis to map deletions and substitutions, RNA–DNA hybridization to map genes directly, visualization of transcription complexes to map promotor and termination sites, and direct observation of proteins bound to the DNA. (For a review, see Younghusband and Inman, 1974.) There is no direct procedure at present for determining the location of ribosome binding sites, although the positions can be inferred, for example, by sequencing the RNA or by assuming that the site lies immediately adjacent to the DNA sequences coding for the protein. We have developed a procedure for visualizing ribosomes bound to single-stranded DNA which we expect to be useful in mapping the location of ribosome binding sites and in investigating the factor requirements and binding affinities of different initiation sites.

Single-stranded DNA is of course not the natural polynucleotide for ribosome binding, but DNA can act as a messenger for polypeptide synthesis in the presence of the antibiotic neomycin (McCarthy and Holland, 1965; Thorpe and Ihler, 1974). The role of neomycin is not understood, but Bretscher (1969) has shown that neomycin is not required for binding of the ribosome or for formation of the first few peptide bonds. Ihler and Nakada (1970) found that ribosomes bound preferentially to the strand of T7 DNA that contained the same sequences as in vivo T7 mRNA. Robertson (1975) studied the specificity of DNA–ribosome interactions and showed that only a small number of specific fragments could be protected by ribosomes from pancreatic DNase digestion within both f1 and φX174 DNAs. Barrell et al. (1975) sequenced the major ribosome protected fragment of φX174 DNA and showed that it corresponded to the initiation region for the spike protein (gene G). This protein had previously been known to comprise more than one-half of the total product of protein synthesis stimulated in vitro by φX174 duplex DNA in a coupled transcription–translation system (Gelfand and Hayashi, 1969).

The use of single-stranded DNA in place of mRNA potentially offers a number of technical advantages in certain kinds of experiments because the DNA is easy to purify and is less susceptible to nucleolytic attack than RNA. For the purposes of mapping, the use of DNA is essential since the mRNA in general would contain the binding sites for only a few of the proteins. The procedure discussed here allows ribosome–DNA complexes to be readily visualized and the locations of the complexes along the DNA to be accurately measured.

Experimental Procedures

Preparation of DNA and Ribosomes. φX174 DNA and [^{3}H]-φX174 DNA (specific activity, 2×10^4 cpm/μg) were obtained by phenol extraction of phage using amber 3 mutants purified by the method of Pagano and Hutchinson (1971). Single-stranded T7 DNA was obtained directly from CsCl banded wild type phage by simultaneous lysis and denaturation with 0.2 M NaOH. Phage proteins were not removed before the denatured DNA was used in binding reactions. Separated strands of $\lambda_{bb\text{-}kan\ 2}$ DNA were prepared from purified phage by the poly(U,G) method of Szybalski et al. (1971); poly(U,G) was removed as described by Ihler and Nakada (1970).

E. coli strain 238 (DNase I$^-$, B1$^-$) were used for the preparation of ribosomes, initiation factors, and charging enzymes, essentially as described by Anderson et al. (1967). Crude and salt washed (1 M NH_4Cl) ribosomes were prepared by the standard alumina grinding procedure except that the addition of pancreatic DNase immediately after cell breakage was omitted. Ribosomes were resuspended in ribosome buffer (100 mM NH_4Cl, 10 mM magnesium acetate, 20 mM Tris[1]-HCl,

† From the Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261. *Received August 4, 1976.* This work was supported by National Institutes of Health Grants CA 16003 and CA 20813. K.C. was the recipient of a National Institutes of Health Research Fellowship, No. 1-F32GM05442-01.

‡ Present address: College of Medicine, Texas A&M University, College Station, Tex. 77843.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

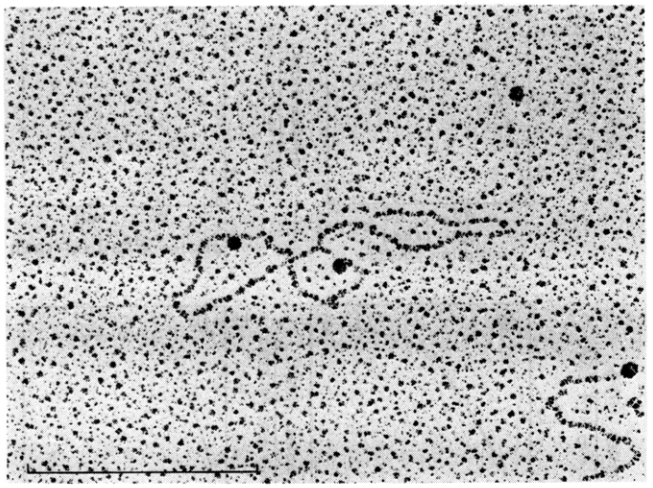

FIGURE 1: ϕX174 DNA, covered with gene 32 protein, which has two ribosomes bound. Bar is 0.5 μm.

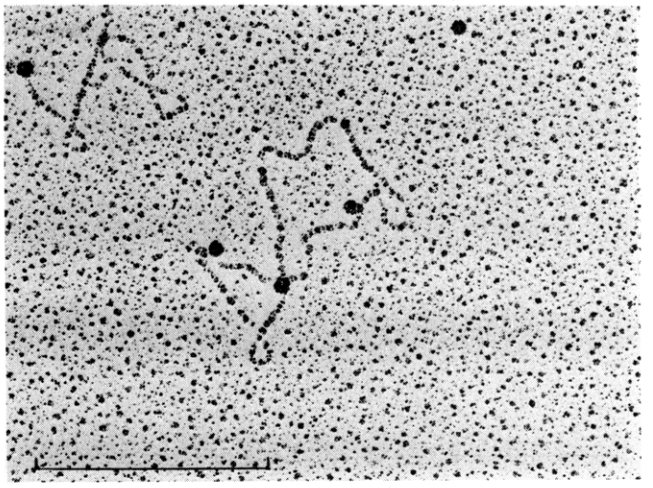

FIGURE 2: ϕX174 DNA, covered with gene 32 protein, which has three ribosomes bound. Bar is 0.5 μm.

pH 7.5, 2 mM mercaptoethanol) and stored at −70 °C. They were diluted with the same buffer and activated by preincubation for 5 min at 37 °C immediately prior to use. Crude initiation factors were isolated from the ribosomal 1 M NH_4Cl wash by precipitation with 70% ammonium sulfate. [^{35}S]-fMet-tRNA was prepared by incubating L-[^{35}S]methionine (380 Ci/mmol) with stripped *E. coli* tRNA and charging enzymes under formylating conditions.

Binding Reaction. The reaction was carried out at 37 °C for 20 min in 0.1 mL of a solution containing 1 mM Tris-HCl, pH 7.2, 5 mM magnesium acetate, 0.25 mM GTP, 100 μg of ribosomes (crude or salt washed), 20 μg of crude initiation factors, 40 pmol of [^{35}S]fMet-tRNA, and varying amounts of single-stranded DNA. When appropriate, the reaction was stopped by adding 20 volumes of cold wash buffer (100 mM Tris-HCl, pH 7.4, 50 mM NH_4Cl, 10 mM magnesium acetate) and filtered through Millipore filters (HA type, 0.45 μm, 2.5 cm diameter) as described by Leder (1968). The filters were air dried and radioactivity was determined in a liquid scintillation spectrometer.

Isolation and Fixation of DNA–Ribosome Complexes. Fifty microliters of a binding mixture as described above were diluted to 0.2 mL with buffer A (10 mM potassium phosphate (pH 7.4)–50 mM KCl–10 mM magnesium acetate), layered on a 5-mL glycerol gradient (30–10% (v/v) in buffer A), and centrifuged at 49 000 rpm for 1 h at 4 °C in swinging bucket rotor. The tubes were punctured at the bottom and 20 fractions were collected from each gradient.

Fifty-microliter aliquots of appropriate gradient fractions were treated with 6 μL of 1% glutaraldehyde (8% EM grade from Polysciences, freshly diluted with buffer A) for 15 min at 37 °C. The samples were quenched with equimolar amounts (6 μL of 0.1 M) glycine at 37 °C for 5 min. Two micrograms of gene 32 protein (contained in a volume of 4–5 μL) was then added and the mixture incubated at 37 °C for 5 min. Final fixation was accomplished by adding 11 μL of 2% glutaraldehyde, incubating for 15 min at 37 °C, and quenching with a tenfold excess (16 μL of 1 M) glycine. The fixed samples were kept in an ice bath and used for electron microscopy within 1 h.

In early experiments we used gene 32 protein which was the generous gift of Dr. Bruce Alberts. For later experiments gene 32 protein was obtained by chromatography of T4 infected *E. coli* cell lysates on a column of denatured calf thymus DNA-cellulose (Alberts and Frey, 1970). It was stored in buffer containing 10 mM Tris-HCl, pH 8.1, 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM mercaptoethanol, and 50% (w/v) glycerol at −20 °C; aliquots were dialyzed against 0.1 M phosphate buffer (pH 7.0)–5 mM magnesium acetate–1 mM mercaptoethanol for 1 h immediately prior to use.

Electron Microscopy. The DNA–ribosome complexes were spread in a cytochrome *c* monolayer essentially following the method of Davis et al. (1971). Routinely, the spreading solution contained 0.1 mg/mL cytochrome *c*, 0.1 M Tris, pH 8.5, 5 mM magnesium acetate, and 30% formamide, and the hypophase contained 10 mM Tris (pH 8.5) and 10% formamide. The cytochrome *c* film was picked up on carbon-coated copper grids, dipped for 5 s into 0.5% Kodak Photoflo solution, air-dried on filter paper, and stained for 30 s in 5×10^{-5} M uranyl acetate (freshly diluted with 90% ethanol from a 5×10^{-3} M stock). After staining, the grids were rotary shadowed with platinum–palladium (80:20) at an angle of 4° and examined in a Phillips 200 electron microscope. For length measurements, the photographed molecules were enlarged approximately 10 to 25 times and tracings were measured with a map measurer. Exact magnifications were determined using a carbon grating replica (54 865 lines per in.).

Results

The procedure for preparing the DNA–ribosome complexes for electron microscopy consists of the following steps:

1. Binding of the ribosomes to single-stranded DNA
2. Purification of the DNA–ribosome complexes on a glycerol gradient
3. Prefixing the complex with 0.1% glutaraldehyde
4. Binding gene 32 protein to the DNA
5. A second fixation with 0.3% glutaraldehyde
6. Spreading using the cytochrome *c*–formamide procedure.

The typical appearance of the DNA–ribosome complexes is shown in Figures 1–4 in which it can be seen that the DNA contour can be readily followed and measured throughout its length and the position of bound ribosomes can also be readily determined. The length of the ϕX174 DNA (1.7×10^6 daltons) as seen in Figures 1 and 2 was found to be 2.28 ± 0.12 μm which agrees closely with the value of 2.38 ± 0.15 μm reported by Wu and Davidson (1975) for single-stranded ϕX174 DNA complexed with gene 32 protein. T7 DNA (12.5×10^6 daltons)

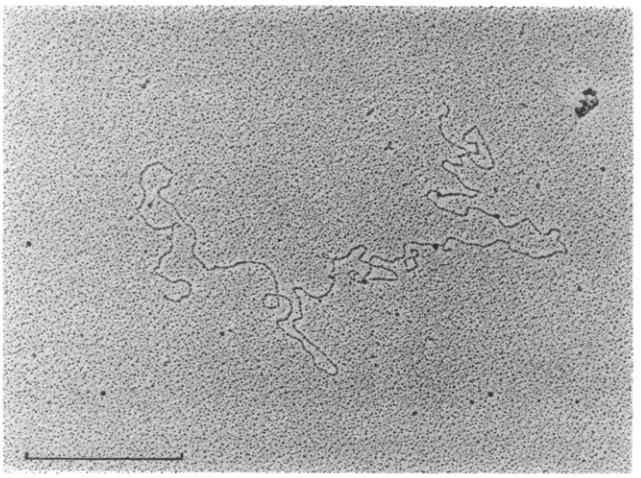

FIGURE 3: Denatured T7 DNA, covered with gene 32 protein, which has four ribosomes bound. Bar is 1.0 μm.

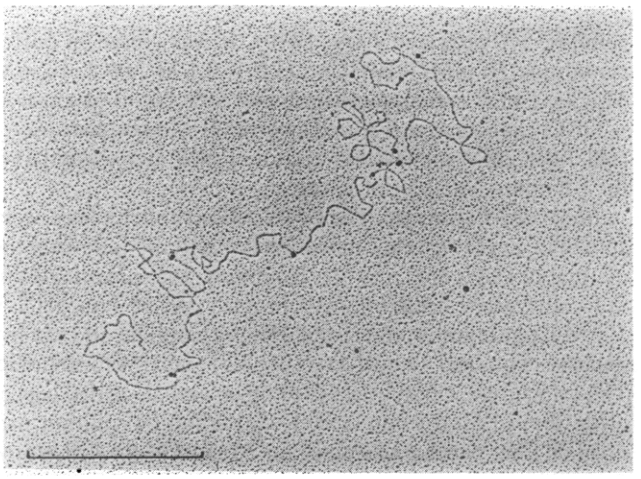

FIGURE 4: Denatured T7 DNA, covered with gene 32 protein, which has six ribosomes bound. Note that one ribosome appears at a point where the DNA is crossed so that its binding site cannot be determined. Bar is 1.0 μm.

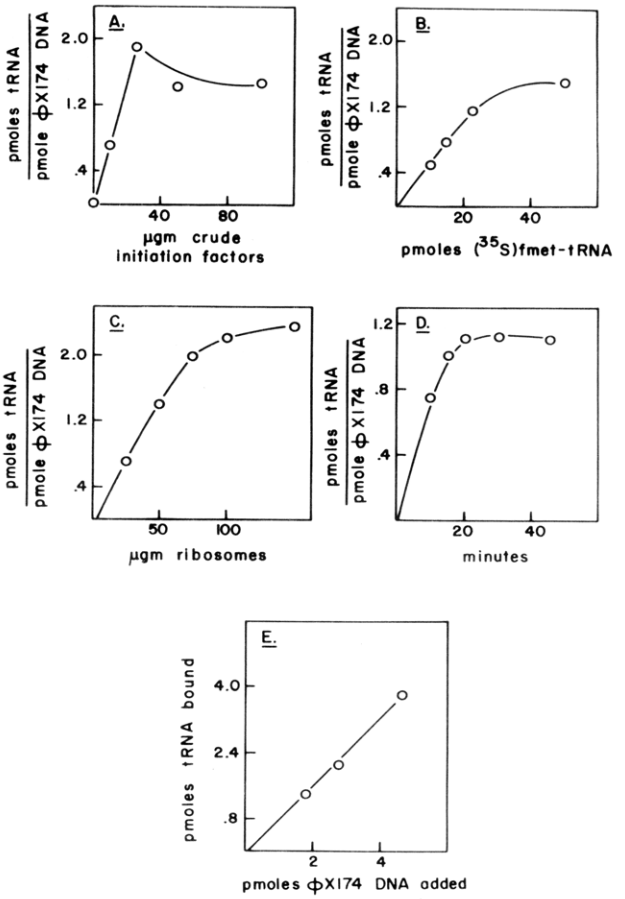

FIGURE 5: Binding of ribosomes to ϕX174 DNA measured by Millipore filter assay. Samples containing 100 μg of salt washed ribosomes, 20 μg of crude initiation factors, 40 pmol of [^{35}S]fMet-tRNA (specific activity, 9450 cpm/pmol), and 4 pmol of ϕX174 DNA in a volume of 0.1 mL were incubated for 20 min at 37 °C. The graph shows the dependence of binding on initiation factors (A), fMet-tRNA (B), ribosomes (C), and time (D). Using optimum conditions, binding is linearly dependent on ϕX174 DNA concentration (E).

is substantially longer and has more crossover points and loops (Figures 3 and 4), but about 75% of the molecules such as those shown in Figures 3 and 4 could be measured unambiguously. The number of ribosomes bound to ϕX174 DNA can be varied by altering the input ratios of ribosomes to DNA. The maximum number we have seen bound to ϕX174 DNA (9 genes) is nine.

Ribosome Binding. Preliminary binding experiments were carried out using a Millipore filter binding assay (Leder, 1968; Ihler and Nakada, 1970) to determine the appropriate reaction conditions. The binding reaction was carried out under conditions (5 mM Mg^{2+}) known to favor specific initiation complexes between ribosomes and mRNA (Kondo et al., 1968) and ribosomes and DNA (Ihler and Nakada, 1970; Robertson, 1975). Either crude ribosomes or salt-washed ribosomes (1 M NH_4Cl) can be used. Using salt-washed ribosomes, the reaction is dependent on the addition of crude initiation factors (Figure 5A). Figures 5B–D demonstrate the dependence of binding on tRNA concentration, ribosomes, and time. Under the conditions used routinely (100 μg of ribosomes, 20 μg of crude initiation factors, 40 pmol of tRNA, 20-min incubation), the amount of $fMet\text{-}tRNA_{fMet}$ bound is dependent on the amount of added DNA (Figure 5E).

Using the Millipore filter assay, we obtain an average of 2.5 ribosomes bound per ϕX174 DNA molecule and about 5 ribosomes bound per T7 DNA molecule. The average number of ribosomes bound as determined by direct count in the electron microscope is 4.1 ribosomes per ϕX174 DNA molecule, nearly twofold higher. The explanation for the lack of precise agreement between the two methods is not known, but may indicate that some of the complexes are not retained by the filter. Similar filter assays using MS2 RNA (Ihler and Nakada, 1970) gave an average of 0.4 binding site per RNA molecule rather than 1 binding site per MS2 molecule. The results show, however, that the ribosomes are not being lost during preparation of the complexes for electron microscopy to a greater extent than for the filter assay.

Glycerol Gradient Purification. Since only a small fraction of the added ribosomes are bound to the DNA, it is necessary to purify the DNA–ribosome complex from the free ribosomes. If this is not done, the background of free ribosomes is sufficiently great that some ribosomes fortuitously appear to be bound to the DNA in the electron microscope and an accurate determination of the number of bound ribosomes becomes impossible. We separate the complexes from free ribosomes on a glycerol velocity gradient so that no free ribosomes are found contaminating the fractions containing DNA–ribosome complexes. Preparation of active ribosomal subunits might be

a viable alternative to gradient sedimentation and would be technically preferable.

The glycerol gradients contain 10 mM Mg^{2+} and 50 mM KCl which we feel stabilizes the initiation complex somewhat. However, the KCl can be omitted from the gradient and the Mg^{2+} concentration reduced to 5 mM with results that are nearly comparable. Complexes may be treated with glutaraldehyde and gene 32 protein prior to the gradient step or fixation may be done after sedimentation. Since fixation is not required prior to sedimentation, ribosomes do not appear to dissociate from the DNA during sedimentation to any significant extent.

Figure 6A shows the polysome profile obtained when a binding mixture containing [^{3}H]-ϕX174 DNA in slight excess over ribosomes and factors was centrifuged in a 30–10% glycerol gradient. Similar gradients using binding mixtures lacking initiation factors or fMet-tRNA showed DNA sedimentation patterns to be the same as that obtained with control ϕX174 DNA. The presence of fast sedimenting ϕX174 DNA seen in Figure 6A indicates that ribosomes are bound to the DNA, and the broad sedimentation profile indicates that there is considerable variation in the number of bound ribosomes, as expected with these binding conditions.

Samples of the various fractions were viewed in the electron microscope and the number of ribosomes per DNA molecule was determined in the absence of gene 32 protein as illustrated in Figure 7A and 7B. The results in Figure 6B show that the fastest sedimenting DNA (fractions 3–5) contains four or more ribosomes per molecule. The area around fraction 7 contains an average of 3 ribosomes per DNA molecule and 2 ribosomes per molecule are found in fraction 10. Monosomes sediment in fraction 13, but the DNA could not be accurately scored in this region because of the large background of free ribosomes. The DNA in fraction 16, which corresponds in position to free DNA, did not have any ribosomes bound and presumably is the zero term in a Poisson distribution.

A similar experiment was performed using a lower DNA to ribosome ratio so that the amount of DNA in the mixture was strictly limiting. Figure 8A shows that this DNA sedimented as a single, more homogeneous peak. Aliquots from this peak were viewed in the electron microscope and the DNA molecules were scored for the number of ribosomes bound. An average value of 4.1 ribosomes per DNA molecule was obtained as shown in Figure 8B, although occasional molecules having as many as 9 ribosomes were observed. The input ratio of DNA to ribosomes determined the average number of ribosomes bound, and the gradient allows the polysomes to be further fractionated according to the number of ribosomes bound. Selection of the polysome class is potentially useful since it becomes more difficult to follow the entire DNA molecule clearly as the number of bound ribosomes increases (Figure 4).

Prefixing with 0.1% Glutaraldehyde. When gene 32 protein binds to the single-stranded DNA–ribosome complex, most of the ribosomes are displaced from the DNA. To demonstrate this, an aliquot from a single glycerol gradient fraction was directly fixed with 0.2% glutaraldehyde. In the electron microscope, this DNA was tangled and the contour impossible to follow (Figures 7A and 7B), but the average number of bound ribosomes could be readily determined and for this aliquot was 3.3 ribosomes per ϕX174 DNA molecule. When gene 32 protein was added first and then the mixture fixed with 0.3% glutaraldehyde, the average number of ribosomes was only 0.2 per DNA molecule. If the ribosome–DNA complexes are prefixed with 0.1% glutaraldehyde, gene 32 protein then

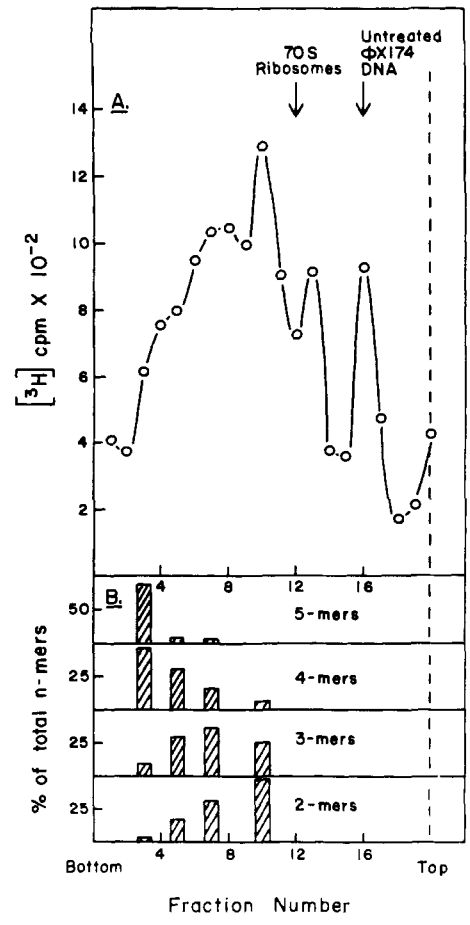

FIGURE 6: Sedimentation of ribosome-ϕX174 DNA complexes in a glycerol gradient. A binding mixture containing 200 μg of ribosomes, 40 μg of crude initiation factors, 80 pmol of fMet-tRNA, and 27 μg [^{3}H]-ϕX174 DNA (2950 cpm/μg) in a total volume of 0.25 mL was incubated for 20 min at 37 °C. Aliquots of the reaction mixture were centrifuged in parallel 30–10% glycerol gradients for 1 h at 49 000 rpm. (A) Fractions from one gradient were placed directly in counting vials for determination of radioactivity. (B) One-hundred-microliter aliquots of appropriate fractions from a parallel gradient were treated with 12 μL of 2% glutaraldehyde for 15 min at 37 °C and subsequently quenched by the addition of 12 μL of 1 M glycine. The samples were spread for electron microscopy from a solution containing 40% formamide, 0.1 M Tris (pH 8.5), 5 mM magnesium acetate, and 0.1 mg/mL cytochrome *c* onto a hypophase containing 10% formamide. Greater than 100 DNA molecules from random fields from each fraction were viewed in the electron microscope and scored for the number of ribosomes bound per DNA molecule. The approximate sedimentation profile for polysomes containing n = 5, 4, 3, or 2 ribosomes was determined by multiplying the percent of molecules in each fraction containing n ribosomes by the relative DNA concentration in that fraction. The results are expressed as percent of the total number of polysomes containing n ribosomes. Fraction 13 was prepared but ribosomes could not be scored accurately due to the large amount of free ribosomes in the background.

added, and then the complexes fixed again with 0.3% glutaraldehyde, the average number of ribosomes bound per DNA is 2.1, about two-thirds of the original number bound. The distribution of the number of ribosomes per DNA molecule in this experiment is shown in Figure 9.

Glutaraldehyde (0.1%) does not fix ribosomes well enough to withstand the subsequent spreading procedure, but it does prevent displacement of ribosomes by gene 32 protein. Also, 0.1% glutaraldehyde does not introduce a significant number of DNA cross-links. The range of glutaraldehyde concentrations that can be used is rather narrow since 0.1% is insufficient to give good fixation of ribosomes and 0.3% results in unac-

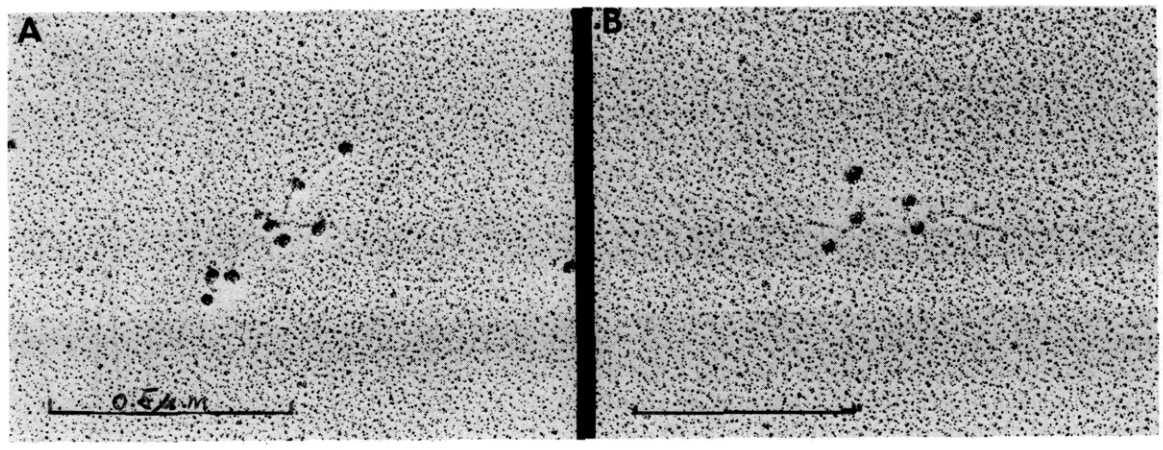

FIGURE 7: (A and B) ϕX174 DNA molecules with ribosomes bound but not treated with gene 32 protein prior to fixation of the complexes with 0.2% glutaraldehyde for 15 min at 37 °C. Spreading solution contained 40% formamide and 5 mM Mg^{2+}. Bar is 0.5 μm.

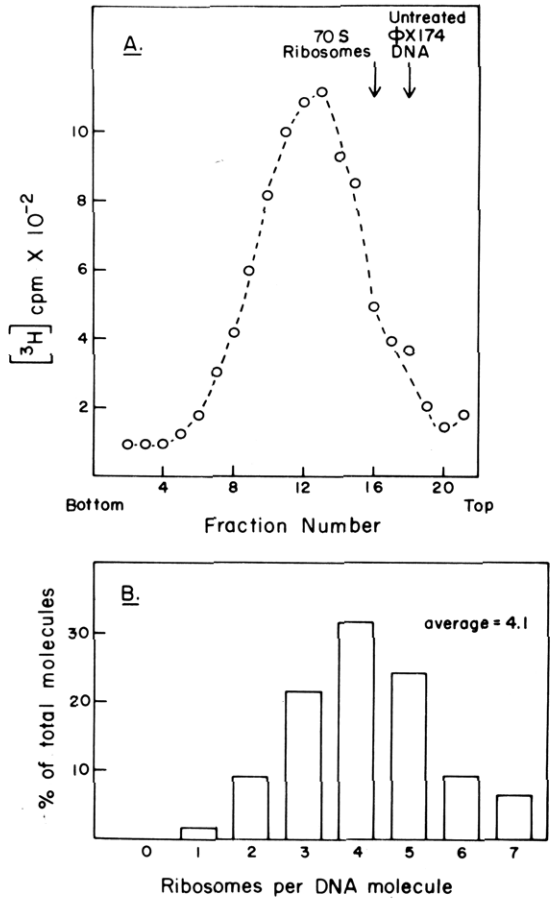

FIGURE 8: Sedimentation in a glycerol gradient of ribosome-ϕX174 DNA complexes formed under conditions of limiting DNA. This experiment was performed essentially as described for Figure 6, except that an aliquot of a binding reaction containing 5.8 μg of [^{3}H]-ϕX174 DNA (6900 cpm/μg) in a total volume of 0.1 mL was sedimented in a gradient at 49 000 rpm for 40 min. (A) Aliquots of 100 μL from the gradient fractions were used for determination of radioactivity. (B) Fifty-microliter aliquots of peak fractions (No. 12 and 13) were prepared for electron microscopy by fixation in 0.2% glutaraldehyde. Random fields of over 100 molecules were scored for the number of ribosomes bound per DNA molecule.

ceptable DNA morphology. In fact we were unable to find any glutaraldehyde concentration between 0.1 and 0.3% which would fix ribosomes without cross-linking the DNA. Thus the use of gene 32 protein became mandatory.

Binding of Gene 32 Protein. Omission of gene 32 protein from the procedure results in tangled and kinky DNA at glutaraldehyde concentrations higher than 0.1% (Figures 7A and 7B). Random base interactions, especially as stabilized by 5 mM Mg^{2+}, result in short regions of duplex DNA. If this DNA is cross-linked, the duplex regions would not be disrupted by formamide in the spreading procedure. Random base interactions of the reverse repeat type would be expected to shorten the overall length of the molecule and perhaps to give the morphology a wavy appearance. Long range interactions between different regions of accidental homology should result in crossing and tangling. These features are observed in molecules such as those shown in Figures 7A and 7B and the morphology cannot be improved by using formamide concentrations as high as 80% or by treating the DNA with gene 32 protein after glutaraldehyde fixation.

We were unsuccessful in finding a procedure other than gene 32 protein to solve this problem. Among the procedures tried were heating the ribosome–DNA complex to 43 °C during fixation, using various formaldehyde concentrations alone or with glutaraldehyde, and treatment with ethidium bromide.

Fixation of the Complexes. Glutaraldehyde has been used to fix both 30S and 50S ribosomal subunits (Subramanian, 1972; Sun and Traut, 1974) and also for fixing gene 32 protein to DNA (Delius et al., 1972). It effectively cross-linked ribosomes to the DNA and fixed the ribosomes to render them resistant to the spreading conditions whereas other cross-linking agents we tried did not. 1,2-Phenylenedimaleimide which reacts with sulfhydryl groups was unable to prevent ribosome–DNA dissociation in formamide, probably because it cannot cross-link the 30S and the 50S ribosomal subunits (Chang and Flaks, 1972).

Even ribosomes fixed in 0.3% glutaraldehyde are not completely resistant to disruption. If spread in 10 mM EDTA–40% formamide, they unfold and show an extended appearance in the electron microscope (Figure 10A). Fortunately the addition of 5 mM Mg^{2+} to the spreading solution allowed the ribosomes to maintain their integrity (Figure 10B) with good DNA morphology. Raising the Mg^{2+} to 10 mM did not further improve the ribosome morphology but gave poorer DNA morphology. Ribosomes spread in formamide with 5 mM Mg^{2+} had the same morphology as ribosomes dropped onto the grid intact (Figure 10C). Apparently even 0.3% glutaraldehyde is not sufficient to cross-link the rRNA and ribosomal proteins into a completely stable structure.

Preparation for Electron Microscopy. We have used the

TABLE I: Distribution of Ribosomes Bound to Separated *l* and *r* Strands of $\lambda_{bb\text{-}kan2}$ DNA.[a]

	No. of DNA Molecules	No. of Ribosomes in Region I	No. of Ribosomes in Region II
r strands	50	73	3
l strands	68	22	108

[a] Separated *l* and *r* strands were heated at 68 °C for 30 s just prior to addition of ribosomes and other components of the binding mixture. The ribosome–DNA complexes were sedimented on glycerol gradients and the complexes were located by microscopy. The loop (2.3 kbases) was used as a length standard for single-stranded DNA.

cytochrome *c*–formamide procedure essentially as described by Davis et al. (1971) since this procedure is not standard. However, since gene 32 protein is also used, presumably an alternative procedure of spreading such as the ethidium bromide procedure of Wu and Davidson (1975) would also be successful.

Specificity. We have used DNA from bacteriophage λ to determine whether the ribosomes we observe bound to DNA in the electron microscope are bound randomly or at specific ribosome binding sites. The pattern of transcription in λ has been extensively studied and is shown in Figure 11a. (For a review, see Herskowitz, 1973). Since ribosome binding sites should occur on the strand complementary to the transcribed strand, we can predict that the pattern for specific ribosome binding to lambda DNA should be similar to that shown in Figure 11b. *l* strands should have ribosomes bound on both ends—on the right between *cro* and the end and on the left between J and the end. The region from c_1 to att should be devoid of ribosomes. (Since translation of the b2 region is not well characterized, we have not considered that segment in the design of this experiment.) *r* strands would be expected to have the opposite pattern, that is, ribosomes bound only in the c_1 to att region.

In order to identify the right and left ends of the DNA molecules, we employed a hybrid phage, $\lambda_{bb\text{-}kan\,2}$, which contains a kanamycin resistance factor inserted in the *rex* gene at 76.1% of λ (Berg et al., 1975). A long inverted repeat sequence in the inserted DNA forms a stem (1.46 kbase) and loop (2.32 kbase) structure which serves as an internal marker on the single-stranded DNA.

The distribution of ribosomes bound to separated *l* and *r* strands of this hybrid λ DNA was determined and the results are shown in Table I. We tabulated the number of ribosomes observed in the N-att region (57.3–76.1% of λ, designated I in Figure 11b) located from the inverted repeat marker and extending to the attachment site and the *cro*-R region (78.5–100% of λ, designated II in Figure 11b) located from a point to the right of the inverted repeat marker to the right end of the molecule. We did not consider the region between 76.1 and 78.5% because c_1 and *cro* are located adjacent to each other and are transcribed off opposite strands. The length of region I is 18.8% of λ (8.7 kbases) and the length of region II is 21.5% of λ (10 kbases).

On *r* strands, 73 ribosomes were found in region I and only 3 ribosomes were found in region II. On *l* strands 22 ribosomes were found in region I and 108 were found in region II (Table I). Of the 22 ribosomes in the "wrong" region of *l* strands, 15 occurred on DNA molecules which had a ribosome distribution consistent with their being contaminating *r* strands. (The presence of some contaminating *r* strands in the *l* strand preparation was shown by hybridization.) These results indicate that the separated strands have ribosome binding sites clustered in exactly the predicted manner. Figure 12 shows a typical *l* strand with ribosomes bound to both right and left ends and which is devoid of ribosomes in region I.

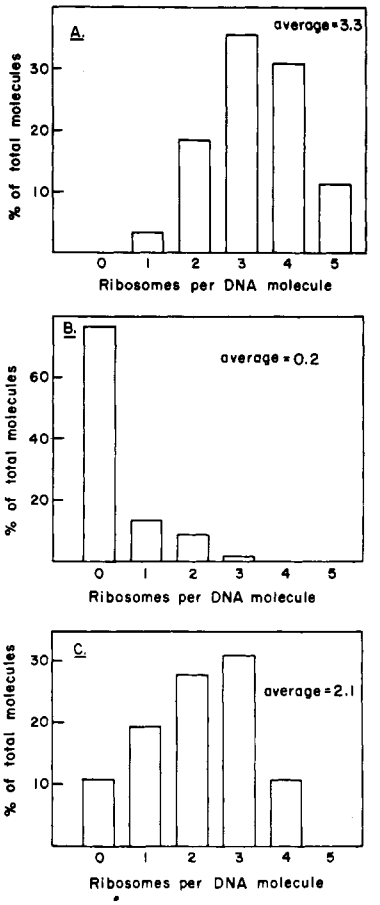

FIGURE 9: Number of bound ribosomes per molecule of φX174 DNA observed in the electron microscope after various treatments. A standard binding mixture was sedimented in a glycerol gradient as described in Experimental Procedures. Fractions 6–10 from a gradient similar to Figure 6 were pooled and 50-μL aliquots were treated as indicated. (A) Fixed with 0.2% glutaraldehyde for 15 min at 37 °C. (B) Treated with gene 32 protein (15:1 weight ratio of gene 32 protein to DNA) for 5 min at 37 °C and then fixed with 0.3% glutaraldehyde for 15 min at 37 °C. (C) Prefixed with 0.1% glutaraldehyde for 15 min at 37 °C, treated with gene 32 protein (15:1 weight ratio) for 5 min at 37 °C, and fixed with 0.3% glutaraldehyde for 15 min at 37 °C. All samples were spread in 30% formamide and 5 mM magnesium acetate onto a hypophase containing 10% formamide. Over 100 molecules from random fields were viewed in the electron microscope and scored for the number of ribosomes bound per DNA molecule.

Discussion

In order to map accurately the positions at which ribosomes bind to single-stranded DNA, it is necessary for the DNA to be extended by eliminating random base interactions and to be untangled with a minimum of crossovers. Unfortunately denaturing agents such as formamide which eliminate random base interactions in single-stranded DNA also eliminate base interactions in rRNA and cause the ribosomes to unravel and to fall off the DNA. Moreover, the requirement for a minimum of 5 mM Mg^{2+} in the binding reaction markedly enhances and stabilizes random base interactions in the DNA.

We had initially expected that it would be easy to avoid this problem by cross-linking ribosomes to DNA with a bifunctional cross-linking reagent so that the complex would be re-

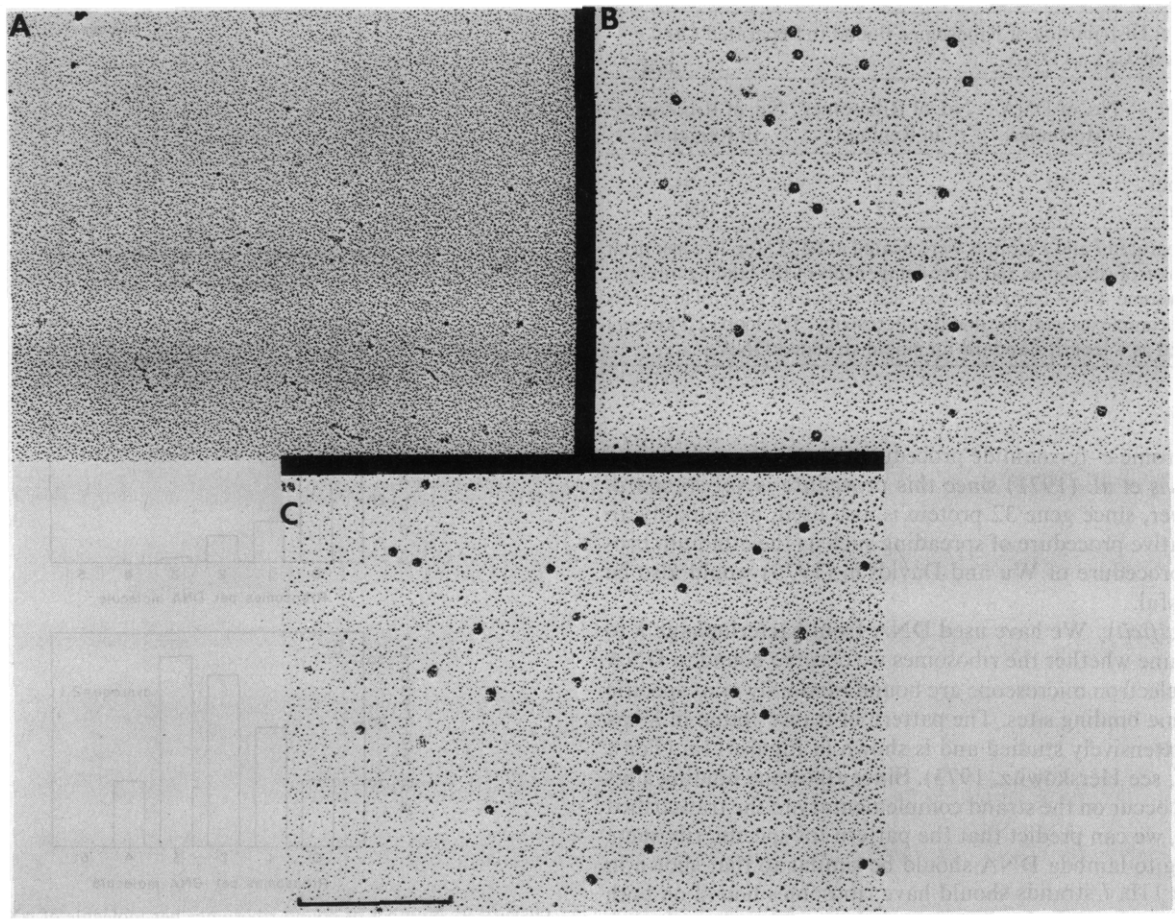

FIGURE 10: *E. coli* ribosomes. (A) Fixed with 0.3% glutaraldehyde for 15 min at 37 °C and spread from a solution containing 40% formamide and 10 mM EDTA. (B) Fixed with 0.3% glutaraldehyde and spread from a solution containing 40% formamide and 5 mM magnesium acetate. (C) A droplet of ribosomes in ribosome buffer was placed directly on the carbon-coated grid. In all three cases, the ribosome preparations were stained and shadowed as described for DNA preparations. Bar is 0.5 μm.

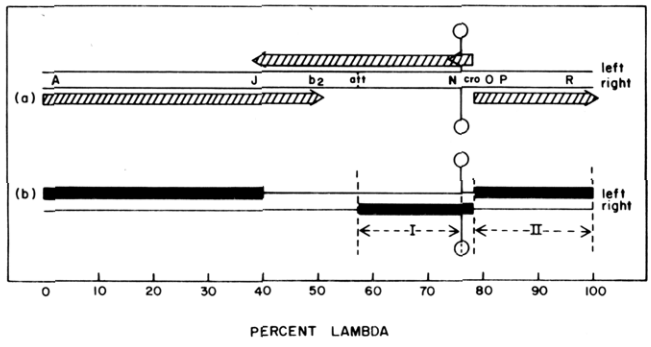

FIGURE 11: Patterns of transcription and ribosome binding on λ DNA. (a) Pattern of transcription of *l* and *r* strands of λ DNA. (b) Shaded areas indicate where ribosomes are expected to bind. Stem and loop indicates the kanamycin resistance factor at 76.1% of λ.

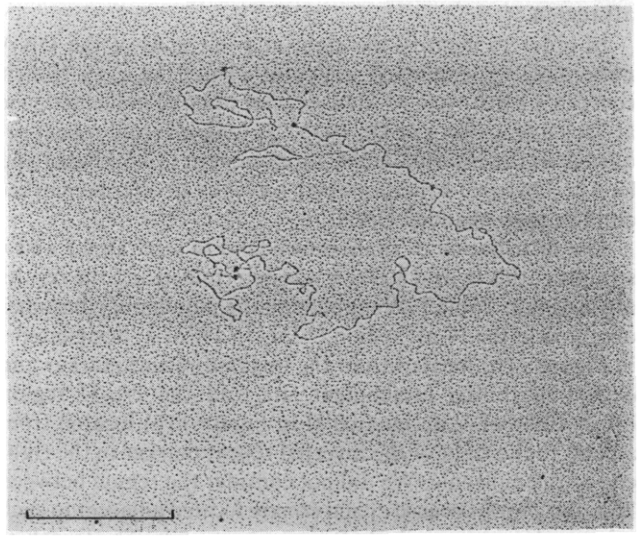

FIGURE 12: *l* strand of $\lambda_{bb\text{-}kan2}$ DNA with four ribosomes bound. Bar is 1 μm.

sistant to subsequent formamide treatment. We were unable, however, to find a cross-linking reagent which would sufficiently stabilize ribosomes to formamide without also cross-linking the DNA. Once cross-linked, the DNA was tangled and kinky and its morphology could not be significantly improved by high formamide concentrations or by gene 32 protein. We attempted to find a cross-linking reagent which would react only with protein, but 1,2-phenylenedimaleimide which reacts with sulfhydryl groups does not cross-link 30S subunits to 50S subunits (Chang and Flaks, 1972) and dimethyl suberimidate (Slobin, 1972) also does not sufficiently stabilize the ribosome (R. Traut, personal communication). Using glutaraldehyde or formaldehyde, we were able to cross-link ribosomes satisfactorily, but the minimum concentration or time we could use successfully resulted in concomitant cross-linking of the DNA.

T4 gene 32 protein binds strongly and cooperatively to sin-

gle-stranded DNA, eliminating random base interactions (Alberts and Frey, 1970). Moreover, Delius et al. (1972) and Wu and Davidson (1975) have shown that single-stranded DNA covered with gene 32 protein can be readily visualized in the electron microscope. By first treating the DNA–ribosome complexes with gene 32 protein to eliminate random base interactions, we were than able to fix the ribosomes to the DNA with glutaraldehyde without forming covalent cross-links between different regions of the DNA. The fixed complexes could then be prepared for electron microscopy by a formamide-cytochrome *c* spreading procedure. Gene 32 protein had the additional advantage of increasing the thickness of the single-stranded DNA so that its contour was easy to follow and measure.

A complication developed when we found that gene 32 protein itself causes displacement of some of the bound ribosomes. Possibly gene 32 protein destabilizes the interaction between the mRNA sequence on the 5′ side of the initiation AUG codon and the rRNA sequence at the 3′ end of the 16S rRNA which is postulated to be important in the formation of the ribosome–messenger complexes (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). To prevent displacement of the ribosomes, the ribosome–DNA complexes were treated briefly with a low concentration of glutaraldehyde prior to the addition of gene 32 protein. These conditions apparently stabilize the DNA–ribosome complexes without introducing a significant number of DNA cross-links.

The DNA molecules visualized by this procedure are about 20-nm thick and, in general, are untangled and easy to follow so that accurate distance measurements are possible. In general, there is no ambiguity as to whether a ribosome is bound to the DNA, although sometimes the DNA is crossed at the ribosome, leaving doubt as to which segment of the DNA the ribosome is bound. The position at which the ribosome is bound cannot be determined exactly due to the width of the ribosome, but this amounts only to the equivalent of about 75–80 nucleotides.

An internal marker on the single-stranded DNA is quite useful in order to orient the right and left ends of the molecules and to avoid uncertainties about whether molecules are broken or unbroken. In addition to reverse duplications, specific RNA–DNA or DNA–DNA duplexes or heteroduplex molecules composed of one normal and one deleted strand of DNA may be used to orient the binding sites.

The distribution of ribosomes which we observed on separated *l* and *r* strands of λ DNA corresponded to the pattern predicted for specific binding. This experiment demonstrates that, in our electron microscopic system, as in other systems reported earlier by Ihler and Nakada (1970) and Robertson (1975), most of the bound ribosomes occur at sites corresponding to those used in vivo for binding ribosomes to mRNA. We are presently mapping the binding sites on λ DNA which are in close proximity to the internal marker, in order to compare the pattern with the known physical map. We expect that this procedure will also be useful in investigation of translational control of protein synthesis and will allow the relative ribosome binding affinities of different genes to be determined.

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