Hybridization of labeled RNA to DNA in agarose gels

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ABSTRACT

Specific DNA restriction endonuclease fragments can be identified after electrophoresis in agarose gels by hybridization in the gel (*in situ*) to radioactive homologous RNA. RNA-DNA hybrids are detected by autoradiography of the gel. Comparison of band patterns of the autoradiogram and the ethidium bromide stained gel allows the identification of the DNA fragment which is complementary to the RNA probe. The technique is rapid, easy and inexpensive. It is sensitive enough to detect individual genes in a mixture of fragments produced by restriction enzyme digestion of complex cellular DNA.

We have used this technique to determine which of the *Hin* III and *Eco* RI fragments of $\phi 80d, ilv^+su^+$ and *E. coli* DNAs contain the 5S, 16S and 23S ribosomal RNA (rRNA) genes of *E. coli*.

INTRODUCTION

Restriction endonucleases make specific double-stranded breaks in DNA (1). These enzymes are widely used to dissect high molecular weight DNA genomes into fragments of a manageable size, which can be separated by gel electrophoresis. After purification the individual fragments may be identified by specific RNA-DNA or DNA-DNA hybridization. However, it has been rather laborious to elute, purify and hybridize the often numerous fragments on a gel. Furthermore the occurrence of a very large number of individual fragments in digests of complex DNAs makes the analysis of these DNAs by such methods almost impossible.

Here we describe a technique which is designed to overcome these problems. Specific DNA fragments are identified after electrophoresis through agarose gels by hybridization in the gel (*in situ*) to a ³²P RNA probe. The hybrid containing fragments are detected directly by autoradiography of the gel.

We have applied this technique to restriction enzyme digests of both viral ($\lambda, \phi 80$) and cellular (*E. coli*) genomes. In each case we are able to detect specific fragments corresponding to single genes. For RNA probes we have used 5S, 16S, and 23S rRNA of *E. coli* and *in vitro* transcribed *p_L* and *p_R* RNA of the two viruses.

MATERIALS AND METHODS

DNA Preparations

A mixture of $\phi 80d_3ilv^+su^+7$ ($\phi 80d3$) DNA and $\phi 80$ wild type helper was a gift of Dr. R. Jaskunas. Purified $\phi 80d_3ilv^+su^+7$ phage (without helper) was generously provided by Dr. L. Soll. E. coli DNA samples were prepared by a modification of the procedure of Berns and Thomas (2) or obtained as a gift from Ms. B. G. Sahagan. λ phages and derivatives were grown lytically. The lysates were concentrated by vacuum evaporation and purified by low speed centrifugation, polyethylene glycol precipitation and two steps of CsCl gradient centrifugation. Phage DNA was purified by 3 phenol extractions. Calf thymus DNA was purchased from Worthington Corporation and was also purified by 3 phenol extractions.

RNA Preparations

A mixture of p_L and p_R RNA was synthesized in vitro using λcb_2 DNA as a template and a $\alpha^{32}P$ UTP (New England Nuclear, Inc.) as a precursor as previously described (3) except the time of incubation was 125 seconds allowing the RNA chains to grow to about 750 nucleotides in length. Under these conditions more than 97 percent of the transcript was p_L and p_R RNA as determined by analytical hybridization.

Labelled E. coli RNA was prepared from 10 ml of E. coli B AS 19 ($OD_{450}=1.00$) after labelling for 2 hrs at $37^\circ C$ with 2 mCi of ^{32}P orthophosphate (10 mCi/mmol) (4). 16S and 23S rRNA were separated by two successive centrifugations in 5-20% linear sucrose gradients at $4^\circ C$, 27,000 rpm for 20 hrs in a Beckman SW27 rotor. 5S rRNA was purified by two-dimensional polyacrylamide gel electrophoresis according to Ikemura and Dahlberg (4). Carrier RNA was prepared from Sigma Type VI RNA by 2 phenol extractions followed by ethanol precipitation (Sigma Chemical Corporation, St. Louis, Missouri).

Sonification of RNA

^{32}P labelled RNA preparations were dissolved in water and sonicated with a standard microtip driven by a type W 350 driving unit (Heat Systems Ultrasonics, Plainview, Long Island, New York). Lowest background results were obtained with ten successive treatments of five seconds each at 255 watts. Less satisfactory but acceptable results were obtained with ten treatments of 15 seconds each at 40 watts using a lower power driving unit.

Digestion of DNA

The restriction endonucleases Eco R1 and Hin III were generously provided by Dr. B. Weisblum, or purified by the method of Tanaka and Weisblum (5). Digestion was carried out according to Griffin et al. (6) and Danna and Nathans (7).

Gel Electrophoresis of DNA Fragments

Restriction fragments were separated by horizontal agarose gel electrophoresis. A clean, dry 8x10 cm. slide (for example, Kodak Projector Slide Cover glass B324) was placed on a support block as shown in the lower part of Figure 1. Approximately 25 ml of a melted 1% agarose solution ("Seakem", Bausch and Lomb, New York, New York) in buffer A (0.16 M Tris-acetate, pH 8.0, 0.08 M NaCl, 0.08 M Na₂Acetate, 0.008 M EDTA) at 70-80°C was pipetted onto the slide, forming a layer 3-4 mm deep. Buffer A is four times the concentration of the buffer used by Helling et al. (8). While the agarose was still liquid a slot-former with a row of 1 mm x 3 mm or 2 mm x 10 mm plastic teeth was placed in the agarose so that it rested on the slide. The slots were positioned 2 cm from the 8 cm side of the slide. After the gel had set (10-15 minutes) the slot-former was removed and wells were completely filled immediately with samples or buffer A. In the case of λ DNA, samples were heated briefly to 85°C before loading to disaggregate the "cohesive ends". After all samples had been loaded the gel was sealed with petroleum jelly (Amojel Snow White, Vaseline or Petrolatum P66 (Fisher Chemical Company)). Melted jelly (75-85°C) was first spread over the row of sample slots, using a warm eyedropper held nearly horizontally and then over the entire surface except for about 1 cm at each end, where contact was to be made with the electrodes.

The electrophoresis apparatus is shown in the upper part of Figure 1. Current flows in and out of the gel through wicks saturated with buffer A. The two compartment, platinum-wire electrode chambers which minimize pH changes during the run should be nearly full. The chambers and slotformers are available from Otto Hiller, Inc., Box 1294, Madison, Wisconsin, 53701.

Normally, a potential gradient of 1.0 v/cm was applied to the gel. This was measured by placing the probes of a volt meter in contact with the gel. Electrophoresis of Eco R1 and Hin III digests of λ , ϕ 80 and E. coli DNAs was for 14-18 hours at room temperature. The current was about 35 milliamperes. The apparatus was covered with Saran Wrap to retard the evaporation from the wicks during the run. To resolve high molecular

weight fragments (6 to 30 million daltons) electrophoresis was in 0.5% agarose gels at 0.5 v/cm for up to 72 hours. Shorter DNA fragments could be rapidly separated using a higher voltage gradient, with the gel placed on a cooling plate.

After electrophoresis the petroleum jelly was stripped off (it adheres nicely to Saran Wrap) and the gel was stained in ethidium bromide (0.1 µg/ml in 1/4 x buffer A) for 30 minutes. The gel was dislodged from the glass to ensure faster staining. Gels were photographed with Polaroid type 57 film through a Wratten #29 (red) filter under longwave U.V. illumination (Ultra Violet Products type B100A Black-ray lamp).

The horizontal gel system described above can also be scaled up for preparative separations. A larger piece of glass was used to support the gel (15 x 30 cm) and plastic rails were placed around the edges held in place by modeling clay so that a thicker gel (0.5 to 1 cm) could be poured. Correspondingly larger slot formers were used. After the gel had set the rails were removed. Samples were loaded in melted 0.5% agarose (70°C) which was allowed to set. The gel was then coated with petroleum jelly and run as described. The DNA capacity was increased in proportion to the gel cross section. A battery charger was used as a high current low voltage power supply. For extended runs the electrode chamber buffer was changed to avoid excessive pH changes.

Fixation of Gels for in situ Hybridization

Depending on the experiment, the gel was used intact or cut into strips for hybridization. Care was exercised to avoid RNase contamination. The strips were cut with a scalpel with reference to the photograph of the stained gel. For denaturation of the DNA, the gel was soaked for 15-30 minutes at room temperature in 1 M KOH. The gel was placed on a piece of cellulose acetate (Oxoid membrane filter sheet, Wilson Diagnostics, Chicago or Millipore type HAWP00010) which was placed on 3-4 layers of absorbent Whatman 3MM paper on a glass plate. The gel was covered with a sheet of 10 mil polyethylene or mylar plastic under a sheet of glass and weighted to provide about 20 gm/cm² of pressure. The absorbent paper was changed 2-3 times over a period of 1 to 1 1/2 hours. This dries the gel to a paper thin slice which sticks to the cellulose acetate.

It was then neutralized by soaking for 10-15 minutes in 50 ml of buffer B (0.020 M Tris-HCl, pH 7.6, 0.3 M KCl, 0.001 M EDTA) (9). The gel was carefully removed from the cellulose acetate and neutralization was continued for at

least another 30 minutes in another 100 ml of buffer B. At this stage the gel could be stored at 4°C for up to 48 hours.

In situ Hybridization

Hybridization was carried out in 1-5 ml of buffer B containing 50 percent (v/v) formamide (stabilized, Fisher Cat. #F95), 10 µg/ml carrier RNA and sonicated, ³²P labelled RNA usually 10⁴-10⁶ dpm. Since the hybridization was done during dialysis the initial salt concentration of the RNA solution was relatively unimportant.

The gel strip was placed in a dialysis bag wide enough to accommodate it without folding by gently pulling the strip into the middle of the tubing. If the gel was too fragile it was placed on a "conveyer" piece of dialysis tubing which was pulled into the end of the open hybridization bag. One end was tied shut and the hybridization mixture added. Air bubbles were removed before closing the other end of the bag. The hybridization mixture was carefully distributed around the gel strip(s), and the bag placed in a flask with 100 ml of buffer B, containing 50% formamide. Hybridization was usually for 10-40 hrs. at 37°C in the dark to prevent degradation of formamide and the concomitant fall in pH. If desired, the concentration of RNA around the gel was increased osmotically by adding 5 grams of sucrose to the solution outside the bag.

RNase Treatment

After hybridization the gel was removed from the dialysis bag, rinsed in two changes of buffer B for 30 minutes each and incubated with buffer B containing 20-100 µg/ml of Ribonuclease A (Worthington Biochemicals, Inc., Freehold, N. J.). After RNase treatment for 3-5 hours at 37°C the gels were rinsed for an additional 2-3 hours at 37°C in 2-3 changes of buffer B.

Autoradiography

The rinsed gel was placed on a sheet of polyethylene plastic and excess liquid was removed by blotting with 3MM paper. The slightly moist gel adhered to the plastic. After covering with another piece of plastic or Saran Wrap the top and bottom of the gel was marked with radioactive (³⁵S) ink and the gel was placed in close contact with X-ray film (Kodak "No screen" NS 54-T) for exposure for an appropriate time (12 hours to two weeks).

Photography of Autoradiograms

After development, autoradiographs were photographed at the same magnification as the original gel. It was convenient to place the autoradiograph on a sheet of good quality bond (fluorescent) paper and illuminate it from above with the ultraviolet lamp. This method of illumination increased the effective film contrast since silver grains shielded the underlying fluorescent paper from ultraviolet excitation.

Figure 1. Gel electrophoresis apparatus.

Restaining of Gels

After autoradiography the dried gels could be carefully removed from the plastic, placed in ethidium bromide staining solution for 30 minutes, and rephotographed, if desired.

RESULTS AND DISCUSSION

Gel Electrophoresis of DNA

The gel electrophoresis system described in Materials and Methods and Figure 1 provides a very convenient way to separate DNA fragments according to size. To characterize the separation, DNA fragments of known size, obtained by Hin III digestion of λ DNA, were electrophoresed under a variety of conditions beginning with those recommended by Helling *et al.* (8). We examined the effects of agarose gel concentration, buffer-salt concentration and voltage gradient, keeping the product of voltage gradient and time constant. The distance of migration of each fragment was plotted vs the log of fragment size (data not shown). In agreement with Helling *et al.* we found that for fragments in the range 1.5 to 7 million daltons the plot was linear, whereas for larger fragments the curve rose sharply indicating anomalously high mobility for high molecular weight DNA (6 to 30 million daltons). We found, however, that the upward curvature could be reduced by lowering the voltage gradient and raising the buffer-salt concentration. Lowering the agarose concentration also improved the linearity slightly, although the looser gels were fragile.

We do not know the reason for the strong voltage dependent component of electrophoretic mobility but it may be due to a tendency of longer DNA fragments to align with the applied field and run endways through the gel. For most fragments we used a 1% agarose gel in buffer A at voltage gradient of 1 volt/cm at room temperature. For the largest DNAs we recommend lower gel concentration and as low a voltage gradient as possible. As an example, in a run of 72 hours at 0.5 v/cm in a 0.5% gel we observed 5 mm separation between intact λ DNA (30.1×10^6 Daltons) and λ cb₂ DNA (26.4×10^6 Daltons) which had migrated approximately 30 mm.

Capacity of agarose gels for restriction enzyme digests of DNA

As discussed in a subsequent section, the gel system must have a high DNA capacity for identification of a single gene or integrated virus in a complex genome. The experiment shown in Figure 2 was designed to test the capacity of gels for restriction enzyme digests of simple (phage) and complex (calf thymus) DNAs. In the case of simple (ie phage) DNA the capacity is quite low. For example in slot 5 of Figure 2, the band pattern was considerably distorted with only 0.15 micrograms of DNA per square millimeter of gel cross section. To test the capacity for complex (ie mammalian) DNA, increasing amounts of calf thymus DNA were mixed with a

Figure 2. Capacity of agarose gels for digests of simple and complex DNA. Each pair of photographs 1-4 shows an ethidium bromide stained gel (left) and an autoradiogram of the same gel (right). A fixed amount (0.4 µg) of ^{32}P λ cb_2 DNA was mixed with various amounts of calf thymus DNA: (1) 0 µg, (2) 5 µg, (3) 20 µg and (4) 50 µg corresponding to 0, 0.5, 2.0, and 5.0 µg per square millimeter of gel cross section respectively. The mixtures were digested with Eco RI and electrophoresed. Slot 5 shows a stained and photographed gel that has been overloaded with 1.5 µg (0.15 µg/mm²) of λ $\text{b}_2\text{KH100}$ DNA. Slot 6 shows calf thymus DNA without digestion.

fixed small amount of ^{32}P labelled λ DNA. The mixture was then digested with Eco RI, electrophoresed in parallel with an Eco RI digest of pure λ DNA and examined by autoradiography (slots 1-4). We found that 50 µg of calf thymus DNA (5 µg/mm²) could be applied to a single slot (slot 4) without distorting the resolution of individual fragments.

Characterization of the in situ hybridization system

The in situ hybridization technique described in Materials and Methods was designed to detect very low concentrations of specific DNA sequences in a complex mixture of DNA molecules produced by cleavage with restriction endonucleases and separated by gel electrophoresis. In developing the technique we found that the most effective medium for supporting separated

denatured DNA fragments during hybridization was the dried agarose slab gel itself. We have also been able to apply our method to longitudinal slices from cylindrical agarose gels.

The low temperature formamide hybridization conditions (9) were selected after we found that the high temperature-phenol (10) and SDS (11) methods did unacceptable damage to the gel. Dialysis tubing proved to be the most convenient container for hybridization. We found that the gels sometimes adhered to the glass containers leaving spots. In addition, the RNA concentration in the bags can be adjusted osmotically with sucrose.

In order to optimize the sensitivity of the method we examined the effects of RNA and DNA concentration and the time of hybridization. In these experiments, ^{32}P labelled 16S RNA with a specific activity of 3×10^6 dpm per microgram was hybridized to complementary DNA (rDNA). The source of the rDNA was bacteriophage $\phi 80\text{d}3$ (see below) which carries one set of 16S and 23S and RNA genes (12). Several gels containing 1.25 μg , 0.25 μg or 0.05 μg of a 3:5 mixture of $\phi 80\text{d}3$ and $\phi 80$ DNAs were prepared. These amounts of DNA correspond to 20, 4, and 0.8 nanograms of 16S rDNA per gel respectively. Representative photographs of the ethidium stained gels before hybridization are shown in panels 1-4 and 12 of Figure 3. In the first experiment of Series A (panels 5-8) the effect of time (10-70 hours) on the hybridization of 16S RNA (0.1 $\mu\text{g}/\text{ml}$) to a constant amount of DNA (20 nanograms of rDNA) was studied. Hybridization increased with time. This effect was confirmed in panels 9 and 10 (15 and 70 hours hybridization to 4 nanograms rDNA). In panel 11 hybridization to 0.8 nanograms of rDNA was still detectable after 70 hours. This approaches the sensitivity needed to detect single copy genes in a eukaryotic DNA. In series B of Figure 3 the effect of varying RNA concentration was examined in a 15 hour hybridization. In this case the gels contained 20 nanograms of rDNA per slot. The intensity of radioactive bands was roughly proportional to the concentration of 16S RNA between 2.00 and 0.02 micrograms per ml (slots 13, 14, and 15). Several radioactive bands from the above experiment were cut out and counted in a Geiger counter. We found the maximum efficiency of hybridization to be 0.5 to 1%. From this experiment we conclude that neither DNA concentration nor RNA concentration is limiting in our system, and for best results both RNA and DNA concentrations as well as time should be increased as far as practicable when sensitive detection is required.

When the 16S RNA concentration in the experiment of Figure 3 exceeded about 2×10^6 cpm/ml of hybridization mixture, the background irradiation

Figure 3. Effect of nucleic acid concentrations and time on *in situ* hybridization. The two experiments discussed in the text are denoted A and B. Slots 1-4 and 12 show photographs of stained gels. The remaining slots show autoradiograms after hybridization *in situ*. All DNA samples were digested with *Eco* RI and electrophoresed as described in Materials and Methods. Slot 1 contains digested $\phi 80$ DNA (0.75 μ g). The remaining slots contain digests of a 3:5 mixture of $\phi 80d3$ and $\phi 80$ DNA. The total amounts added to the gel slots were (2) 1.25 μ g; (3) 0.25 μ g; (4) 0.05 μ g; (5-8) same as slot 2; (9-10) same as slot 3; (11) same as slot 4; (12-15) 1.25 μ g. Gels 5-11 were hybridized with 32 P labeled 16S RNA at 0.1 μ g/ml. The times of hybridization were 10, 20, 40, 70, 15, 70 and 70 hours, respectively. Gels 13-15 were hybridized for 15 hours with the same RNA at 2.0, 0.1 and 0.02 μ g/ml, respectively.

from RNA sticking nonspecifically in the gels made it difficult, if not impossible to detect specific hybrids. We found that this problem was more serious with longer RNAs. For example 5S RNA gave less and 23S RNA more background than 16S RNA. (See slots 4, 5, and 6 of Figure 4). Fragmentation of the RNA by RNase, alkali or sonication before hybridization was very helpful in reducing the background. Best results were obtained with very powerful sonication (see Materials and Methods). Sonication did not appear to have any adverse effect on efficiency of hybridization and thus the signal to noise ratio was considerably improved.

We have detected specific hybridization to all size classes of DNA fragments from 1000 to 20,000 basepairs in length. However, we observe

Figure 4. Hybridization of 5S, 16S, and 23S Ribosomal RNAs to *Hin* III digests of $\phi 80$ d3DNA. Each panel shows a photograph of an ethidium bromide stained gel (left) and an autoradiogram of the same gel (right). Each gel contained approximately 1 μ g of *Hin* III digested DNA. Two analogous experiments are shown in panels 1-3, and 4-6. The DNAs were pure $\phi 80$ d3 (panels 1-3); a 3:5 mixture of $\phi 80$ d3 and $\phi 80$ DNA (panels 4-6) and pure $\phi 80$ DNA (panel 7). Hybridization was with 32 P labeled 5S (panels 1 and 4), 16S (panels 2 and 5), or 23S (panels 3, 6, 7) RNAs having specific activities of approximately 10^6 cpm/ μ g. RNA concentration during hybridization was approximately 1 μ g/ml for 16 and 23S and 0.1 μ g/ml for 5S RNA. Radioautograms were exposed for from 24 to 72 hours. The time of electrophoresis was slightly longer for panels 5 and 6 than for the others. The RNA in panels 4, 5, and 6 was carefully prepared to minimize breakage. Note the tendency of background irradiation to increase with the size of RNA.

lower hybridization efficiency with decreased DNA fragment size. For example, in the case of $\phi 80$ d3 DNA (see following section) the intensity of 5S rRNA hybrids to the specific *Hin* III fragment (6500 basepairs) was 2-3 fold higher than that found in the *Eco* RI fragment which is 1900 basepairs in length. It is not clear whether this is due to a preferential loss of the smaller fragments by diffusion or to an increase in the rate of DNA-DNA renaturation which would prevent RNA-DNA annealing. By comparing stained gels before and after hybridization we have noted that DNA fragments less than 1000 base pairs in length tend to be lost in our procedure. The use of more concentrated agarose gels, e.g., 1.5% instead of 1%, reduces the rate of diffusion of the DNA fragments but also decreases the rate of diffusion of the RNA probe.

Attempts were made to cross link the denatured DNA to the agarose by heavily irradiating the dried, alkaline gel, with a germicidal lamp but this step severely inhibited the formation of RNA-DNA hybrids. We also noted that prolonged exposure of the ethidium bromide stained gel to Ultraviolet light resulted in more diffuse hybrid-containing bands. It does appear that the normal exposure to long wave ultraviolet light during photography is beneficial since complete elimination of the photography step resulted in substantial losses of DNA from the gels during hybridization in situ.

In summary, despite the low efficiency of the in situ hybridization, the method is extremely sensitive. Autoradiography using X-ray film allows the detection of 5-10 dpm of ^{32}P in a 1 x 8 mm hybrid band within 8-10 days of exposure. The technique permits the identification of as little as 0.8 nanograms of specific DNA provided the RNA probe has a specific activity not less than about $3\text{--}5 \times 10^6$ dpm per microgram. With probes of higher specific activity considerably smaller amounts of DNA should be detectable provided the RNA is partially degraded to minimize background irradiation. This level of sensitivity can lead to the detection of partial digestion products (see below). Furthermore, it can reveal unexpected impurities in the RNA probe. For example, we found that "5S rRNA" prepared from a $\phi 80$ infected culture of E. coli hybridized to $\phi 80$ -specific DNA fragments due to contamination with very low levels of $\phi 80$ mRNAs.

Identification of rRNA genes of E. Coli

E. coli DNA contains about six rRNA cistrons with different locations on the chromosome (see recent review by Jaskunas et al., (13). $\phi 80\text{d}3$ (14) is a defective transducing phage which carries the 16S and 23S rRNA genes of one of these cistrons, the rrnB locus (12). In the experiments illustrated in Figures 4 and 5 we examined the hybridization of 5S, 16S and 23S rRNA to gels containing Hin III and Eco RI digests of $\phi 80\text{d}3$ DNA and Eco RI digests of total E. coli DNA.

Figure 4 shows the identification of these genes on the Hin III fragments of $\phi 80\text{d}3$ DNA. Two different experiments are shown, one in which the $\phi 80\text{d}3$ DNA was separated from helper phage (slots 1, 2, and 3) and the other in which a 3:5 mixture of $\phi 80$ and $\phi 80\text{d}3$ DNA was used (slots 4, 5, and 6). The ^{32}P RNAs used in the second experiment were not fragmented. Hybridization was with ^{32}P labelled 5S (slots 1, 4), 16S (slots 2, 5) and 23S (slots 3 and 6) RNAs. The results of the two experiments are in

agreement except that in slots 1, 2, and 3 there was hybridization to a minor band that was barely visible in the photograph. Since the other experiments did not show this band and because this fragment was longer than the major fragment it probably was a partial Hin III digestion product present in very low amounts in the digest for slots 1-3. In all cases the major radioactive band corresponded to the same $\phi 80d3$ specific fragment, which was about 6500 basepairs long. We therefore conclude that all three ribosomal RNAs, including the 5S RNA which had previously not been tested, are in a single contiguous segment on the *rrnB* locus. None of the rRNAs hybridized to $\phi 80$ DNA fragments (slot 7, data for 5S and 16S rRNA are not shown).

Figure 5 shows experiments in which each of the three ribosomal RNAs was hybridized to the Eco RI fragments of $\phi 80d3$ and of total E. coli DNA. In $\phi 80d3$ DNA the rRNAs hybridized to 3 specific fragments about 1750, 1900 and 2100 basepairs long indicated by dots next to panel 1 of Figure 5. Both the 16S and 23S RNAs, whose lengths are about 1500 and 3000 basepairs respectively, hybridized to two of the bands whereas 5S RNA hybridized to a single one (the autoradiogram in panel 3 of Figure 5 is overexposed, but 16S RNA clearly hybridized to a doublet in Figure 3). Although we were not able to determine precisely which of the RNAs hybridized to which DNA band because the bands are so close together, we interpret the results as showing that both 16S and 23S DNAs of the *rrn-B* cistron have Eco RI cleavage sites within them since each hybridizes to two bands, and in the case of 23S RNA both fragments are considerably smaller than the RNA itself. In the case of 16S DNA this cleavage site was predicted from the nucleotide sequence (17) which has an Eco RI recognition sequence (16 and H. M. Goodman, personal communication) located about 720 nucleotides from the 5' end. Sequence information on 23S DNA is not available at present.

In the Eco RI fragments of E. coli DNA (Figure 5) we found 5S, 16S, and 23S genes on several large fragments. This was also expected since E. coli contains about six rRNA cistrons with different locations on the chromosome, (13) although we cannot rule out the possibility that some of the fragments may be partial digestion products. We also observed 16S and 23S RNA hybridizing to what appears to be a single fragment in the 2000 basepair region but no hybridization of 5S RNA was seen to small fragments. Otherwise 5S RNA hybridized to the same size fragments as 23S RNA whereas 16S RNA hybridized to a different set.

Although these observations do not prove any model for the structure

Figure 5. Hybridization of 5S, 16S and 23S ribosomal RNAs to *Eco* RI digests of *E. coli* and ϕ 80d3 DNA. Each panel shows two gels, one containing 20 μ g of *Eco* RI digest of *E. coli* K12 DNA (left) and the other containing 1.25 μ g of digested ϕ 80d3 DNA. Panel 1 shows the ethidium bromide stained band patterns of these DNAs. Panels 2, 3, and 4 show autoradiograms after hybridization with 5S, 16S, and 23S ribosomal RNAs, respectively. Hybridization conditions were as described in the legend to Figure 4 except incubation was for 60 hours and the ϕ 80d3 DNA gel strips used for hybridization were cut in half, corresponding to a 1 x 5 mm gel slot with 0.6 μ g of DNA. The autoradiograms were exposed for 5 to 10 days.

of rRNA cistrons, they are consistent with the simple picture in which each rRNA cistron is divided into three fragments by *Eco* RI cuts in 16S and 23S DNA sequences. On this hypothesis, only the central fragment would have a consistent size (around 2000 basepairs in length), whereas the other fragments would vary in size from cistron to cistron depending on the location of *Eco* RI sites in adjacent sequences. The *rrnB* cistron would also present a different pattern in ϕ 80d3' from that seen in *E. coli* because the major chromosomal rearrangements involved in the phage construction altered the

flanking sequences on each side of the rRNA genes (14). The finding that 5S RNA hybridizes to the same fragments as 23S RNA indicates that the 5S RNA is located on the side of 23S DNA distal to the spacer.

In summary, our analysis of E. coli digests suggests tentatively that the several E. coli rRNA cistrons are probably organized similarly, although each gives a different R1 fragment pattern due to differences in flanking sequences. The location of the rRNA genes on the Hin III and Eco R1 DNA fragments of $\phi 80d3$ (Figures 3, 4, and 5) is consistent with the structure of the phage as determined by electronmicroscopic analysis (12), which localized the 16S and 23S genes within a single 5700 basepairs region of DNA. The identification of all three rRNA genes on a single Hin III fragment and the distribution of 5S, 16S, and 23S rRNA sequences on the Eco R1 fragments of E. coli and $\phi 80d3$ is in agreement with the gene order in the *rrnB* locus (5') 16S-23S-5S (3') as previously suggested (15). A more thorough analysis is in progress and will be published elsewhere (E. L. manuscript in preparation).

Detection of the λ prophage in E. coli DNA

One of the purposes for developing this technique was to study the structure of integrated viral genomes. As an example, Figure 6 shows the detection of λ specific DNA sequences in the chromosome of a monolysogenic strain of E. coli. A mixture of ^{32}P labelled λp_L and λp_R RNAs of 750 nucleotide chain length containing less than 2% of other λ transcripts (See Materials and Methods) was hybridized to the Hin III digested DNA of E. coli W3102 (λ)₁ (slot 2 of Figure 6). As a control for this experiment E. coli DNA admixed with ^{32}P labelled λ DNA was digested in parallel with that used for hybridization. The autoradiograph of this gel showed no partial digestion products of the λ sequences (data not shown). Thirty micrograms of E. coli DNA was applied to the gel slot, corresponding to about 4 nanograms of each of the 750 base pair segments homologous to p_L and p_R RNA. After hybridization, two radioactive bands corresponding to the two components of the probe were observed. The lower of the bands corresponded in position with band f in the comparison digest of λ DNA (compare slot 1 with slot 2). However, the upper one was between bands a and d and had no counterpart in the λ digest. No bands were detected with DNA from a non-lysogenic E. coli control (data not shown).

The Hin III cleavage map of λ which we determined by digestion of the DNA of various deletion and substitution mutants of λ (data not shown) is

Figure 6. Detection of integrated λ prophage DNA within the *E. coli* chromosome. Panel 1 shows the band pattern of λ DNA digested with *Hin* III. The bands are labeled a-e in accordance with Figure 7. Note that the three smallest fragments ran off the gels. Panel 2 shows a photograph of the ethidium bromide stained gel (left) and the autoradiogram of the same gel after hybridization (right). A *Hin* III digest of 34 μ g of DNA from *E. coli* W3102 lysogenic for a single copy of the lambda prophage. The completeness of digestion was monitored with admixed radioactive DNA in parallel digests (data not shown). Hybridization was with ^{32}P labeled λ RNA prepared *in vitro* using λ RNA as template (see methods). The time of hybridization was 14 hours. Autoradiographs were exposed for 10 days.

presented in Figure 7. An important feature is the fact that the λ attachment site is on fragment d which also carries p_L , whereas p_R is on fragment f. Therefore as shown in Figure 7, the integration of λ into the *E. coli* genome by the Campbell mechanism (18) should result in a change in the size of the p_L containing fragment but not the p_R fragment. This is precisely what was observed. We conclude therefore that the technique not only is capable of detecting the presence of an integrated viral genome but it can be used to investigate the mechanism of its integration.

Figure 7. Rearrangement of Hin III fragments of λ as a result of prophage integration. The upper panel shows the λ vegetative chromosome including the positions of Hin III cuts, the attachment site and two major phage promoters p_L and p_R. The approximately 750 nucleotide RNA transcripts used for hybridization in situ (Figure 6) are drawn roughly to scale. Phage integration proceeds via a circular intermediate (center) which is inserted into the E. coli chromosome between gal and bio by reciprocal site specific recombination at att. As a result (lower) the λ chromosome is rearranged and the pattern of Hin III fragmentation is altered.

SUMMARY AND CONCLUSIONS

A horizontal slab gel system for electrophoretic separation of DNA fragments has been developed including the use of high salt low voltage conditions which result in excellent separations of high molecular weight fragments. These gels have a capacity for resolving 0.1 micrograms per square millimeter of restriction enzyme digested phage DNA and more than 5 micrograms per square millimeter of digested complex DNAs.

An in situ RNA-DNA hybridization technique is described that allows the identification of specific DNA fragments in situ in agarose gels. Using this technique it is unnecessary to purify individual fragments from the gel before identification and it is possible to detect unique fragments in a complex mixture of DNA restriction fragments of a cellular DNA. The method is easy, rapid, sensitive and inexpensive.

The technique has been used to identify ribosomal RNA cistrons in E. coli and φ80d3, and to identify the prophage in a λ lysogen. Additional information reported includes the Hin III restriction site map of phage λ and the finding that a 5S RNA gene is closely linked to the 16S and 23S genes of φ80d3. This method can be extended to study the integration of viral genomes into eukaryotic hosts as well as to identify restriction endonuclease fragments containing eukaryotic genes for which RNA probes are

available. Progress has already been made in the successful identification of several tRNA and the 5S rRNA genes in chicken cell DNA (Gordon Peters, personal communication). We anticipate that the technique will also be useful for identification of genes for DNA cloning.

A similar hybridization technique has recently been developed by Dr. E. M. Southern (19). His method differs from ours in that DNA fragments are transferred from the agarose gel to a nitrocellulose filter before hybridization.

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