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Three Plasmids Constructed for the Production of Monodisperse Semistiff DNA Samples

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Received December 12, 1984

ABSTRACT: We have constructed three plasmids, pLH2311, pLH1010, and pLH762/367, that can be used to produce large amounts of monodisperse DNA fragments by cleavage with the readily available restriction enzymes Pvu II and Hae III. pLH2311 produces a single fragment 2311 base pairs (bp) in length (1.5×10^6 dalton molecular weight) when cut by Pvu II. pLH1010 contains two copies of a fragment 1010 bp in length (6.7×10^5 dalton molecular weight). After cutting by Pvu II these fragments may be easily separated from the other fragments by gel electrophoresis. pLH762/367 contains three copies of a fragment 762 bp in length (5.0×10^5 dalton molecular weight) and six copies of fragments 367 bp or 368 bp in length (2.4×10^5 dalton molecular weight). The first fragments are recovered by Pvu II digestion and the latter by additional digestion of the 762-bp fragment with the enzyme Hae III. Details of the construction of these plasmids and of their use to produce monodisperse semistiff polymer samples are discussed.

Introduction

The polymer chemist who wishes to study the structure and dynamics of wormlike or semistiff molecules is faced with a perplexing situation. The mathematical complexity of the complete problem, including hydrodynamic interactions, the counterion field, etc., is presently insoluble and thus approximations of unknown accuracy are required in order to make any progress whatsoever.[1-4] There now exist a number of theories whose predictions must be tested before it will be known which of the various mathematical approaches is the most valid. For a few specific examples, see ref 3–5. This experimental verification is made significantly more difficult by the polydisperse nature of available polymer systems.[6-8] For example, if a theoretical prediction and experiment disagree, we must determine the source of difference: were the mathematical approximations in the theory invalid or was the sample too polydisperse?

Plasmids, circular pieces of DNA, produce fragments of specific lengths when cut by suitable restriction enzymes[9,10] and the precise lengths of these fragments can be determined from the nucleotide sequence of the DNA and the recognition sequence of the restriction enzyme. A number of reports have appeared in the literature in recent years in which monodisperse restriction fragments of DNA have been used as model polymer systems.[11-14] In order to apply many physical–chemical techniques, such as classical and dynamic light scattering, hundreds of micrograms of polymer are required. In order to produce that quantity of a monodisperse fragment we would need to purify 10 mg of plasmid DNA, cut the DNA with 20000–50000 units of the appropriate restriction enzyme(s), separate the resulting DNA fragments by gel electrophoresis, and then repurify the fragment of interest. If the fragment of interest is 350 bp in length and the original plasmid is 7000 bp in length, then one could obtain less than 500 μg of monodisperse DNA from 10 mg of starting material. We would obtain a low final yield because the fragment of interest represents only 5%, by weight, of the DNA originally produced. Furthermore, the effort and cost of this procedure are substantial when one considers the techniques and equipment necessary to grow 20-L cultures and the current price of restriction enzymes. This approach is therefore impractical for most laboratories. To overcome these difficulties, we have inserted multiple copies of small DNA fragments into plasmids, thus increasing the percentage of the original plasmid that is ultimately useful. In this way, the final yield is increased and the production of monodisperse DNA fragments becomes feasible for a much wider variety of investigators.

In the present work, we have applied the tools of genetic engineering to produce relatively large amounts of monodisperse semistiff polymers in a variety of molecular weights. Our hope is that the plasmids that we have constructed, and specifically the DNA samples that can be easily made from them, will allow investigators to more easily and accurately test models of macromolecular dynamics and structure.

Discussion

In designing plasmid DNAs for the production of monodisperse fragments for physical–chemical study, we followed a number of guidelines. First of all, we used only DNAs for which the complete nucleotide sequence was known. This allows the calculation of molecular weight and contour length for each fragment produced.[15] Secondly, we used only DNAs that were unrestricted by the current NIH recombinant DNA guidelines.[16] A third consideration in plasmid design involved the ends of the fragments that would be produced by cleavage with restriction enzymes.

Restriction enzymes can leave two types of ends when they cut DNA.[9,10] The so-called "sticky ends" have short single-stranded regions because the enzyme involved

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cleaves the DNA in different positions on the two strands. Any two sticky ends created by the same enzyme are complementary and can base pair to each other—hence the term "sticky". In the case of a single molecule, this tendency of the ends to stick together will change the equilibrium configuration of the molecule, making it unsuitable as a model system for polymer studies. Blunt ends, which do not have single-stranded regions and are created by a minority of the known restriction enzymes, do not bind to each other and are therefore appropriate in a model polymer system. The plasmids were constructed so that all of the primary fragments are produced by a single restriction enzyme, Pvu II,[17] which leaves blunt ends. This enzyme is among the least expensive of the restriction enzymes and is supplied by a number of different sources. In one case a second widely available blunt-ended enzyme, Hae III,[18] is used to cleave one of the primary fragments into secondary fragments. In each case the fragments of interest constitute greater than 30% of the plasmid.

We have restriction mapped the plasmids to determine the orientation of the inserted fragments. The reader may refer to Figure 4 for maps of the three plasmids and a table of the restriction fragments produced by Pvu II and Hae III. Agarose gels, documenting the characteristics of these plasmids, are shown in Figure 5.

Methods

pBR322[19-21] and pRI25 in *E. coli* K-37 were obtained from Thomas C. Reynolds in the Department of Pathology at Stanford University. pRI25 was constructed in the laboratory of Paul Berg in the Department of Biochemistry at Stanford. pRLM4[22] was obtained from Tania Pedrini from the Instituto di Genetica CNR, Pavia, Italy. *E. coli* strains HB101 and DH1[10] were obtained from Philip Hanawalt in the Department of Biology at Stanford. Both plasmids were used to transform HB101 by the $CaCl_2$ procedure,[10] grown in amplified culture,[23] and purified by a minor modification of the procedure of Marko, Chipperfield, and Birnboim.[24] In the original plasmid purification procedure,[24] the plasmid DNA is bound to a glass powder and the powder is washed on a suction filter apparatus. We bind the plasmid DNA to the glass powder in an identical manner and then pellet the glass powder by centrifugation at 10000*g* for 10 min. The supernatant is removed and the powder is resuspended in the wash buffer. The powder is then repelleted, and the cycle is repeated 5 or 6 times, washing away contaminating RNA. The powder is then resuspended in the elution buffer and mixed gently at room temperature for 10–15 min. The elution buffer is separated from the glass powder by several cycles of centrifugation as before, followed by careful decanting of the supernatant. The DNA is then recovered by ethanol precipitation. In our hands this procedure results in a larger recovery of DNA than the original procedure.

Restriction enzymes, Klenow fragment of *E. coli* polymerase I,[25] Pvu II linkers, and T4 DNA ligase were obtained from New England Biolabs and used in the buffers suggested in Maniatis.[10] Polynucleotide kinase (New England Biolabs) and calf alkaline phosphatase (Boehringer Mannheim) were kindly donated by Thomas C. Reynolds. Essentially all DNA manipulations and cloning procedures followed the manual of Maniatis.[10] DNA was purified from agarose gels by intercepting the DNA band of interest with Na45 paper from Schleicher and Schuell and then eluting the DNA from the paper using a minor modification of the procedure whose description is included with the Na45 paper.

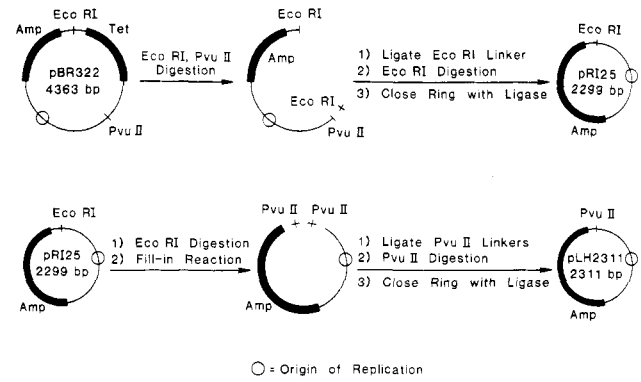

Figure 1. Construction of pLH2311. The top of the figure shows the construction of pRI25 from pBR322 as performed in the laboratory of Paul Berg at Stanford. pBR322 was cut with both Eco RI and Pvu II, generating two fragments. An Eco RI linker, a blunt-ended piece of DNA 8 bp long that contain the recognition site for Eco RI, was then ligated to the blunt ends left by Pvu II. The linkers were cut in half with Eco RI, leaving an Eco RI end that could base pair with the other end of the molecule. The two fragments were then circularized with DNA ligase and the DNA was used to transform *E. coli* using ampicillin resistance as a selective marker. As only the 2299-bp circular piece of DNA had an origin of replication and a gene coding for resistance to ampicillin, only this product was replicated in the bacteria. In our laboratory we took the result of the above work, pRI25, and opened the DNA circle with Eco RI. We then filled in the sticky Eco RI ends with the Klenow fragment of *E. coli* polymerase I, leaving two blunt ends. Pvu II linkers were ligated to each of these ends and cut with Pvu II, leaving half of a Pvu II recognition sequence on each end. We then closed the circle with T4 DNA ligase and used the DNA to transform *E. coli* HB101, using ampicillin resistance for selection. Plasmids isolated from ampicillin-resistant colonies were subsequently screened for the presence of the Pvu II site.

A number of different approaches were taken in the plasmid contruction. In the first case, pRI25 was cut with EcoRI and the two sticky ends filled in with the Klenow fragment of *E. coli* polymerase I.[10] A Pvu II linker (a blunt-ended piece of DNA, 8 bp in length, that contains the recognition sequence for the enzyme Pvu II) was then ligated to both ends of the molecule (see Figure 1). The linkers were cut by Pvu II, leaving half of a Pvu II recognition sequence on each end, and the circle was closed with T4 DNA ligase. The ligation products, which can include multimers of the plasmid or plasmids without complete Pvu II sites, were used to transform HB101 and colonies were selected for ampicillin resistance. A small amount of plasmid DNA was isolated from each colony[26] and tested for the presence of a Pvu II site and proper length by Pvu II digestion followed by agarose gel electrophoresis. pLH2311, a plasmid 2311 bp in length that contains a single Pvu II restriction site, was recovered.

In constructing the second plasmid, a 998-bp fragment from pBR322 was modified to have blunt ends by the previously mentioned Klenow procedure. To these blunt ends were ligated Pvu II linkers that were subsequently cut with Pvu II. Each end of the fragment, now 1010 bp in length, contained half of the Pvu II recognition sequence. This fragment was inserted into the Pvu II site in pBR322 and this intermediate plasmid was used to transform HB101. Small amounts of plasmid, purified from tetracycline-resistant colonies, were tested for the 1010-bp fragment by digestion with Pvu II followed by agarose gel electrophoresis. The recovered intermediate plasmid, containing one copy of the 1010-bp fragment, was grown in large amounts in order to obtain working quantities of the 1010-bp fragment. The 1010-bp fragment was then recovered by digestion with Pvu II followed by sep-

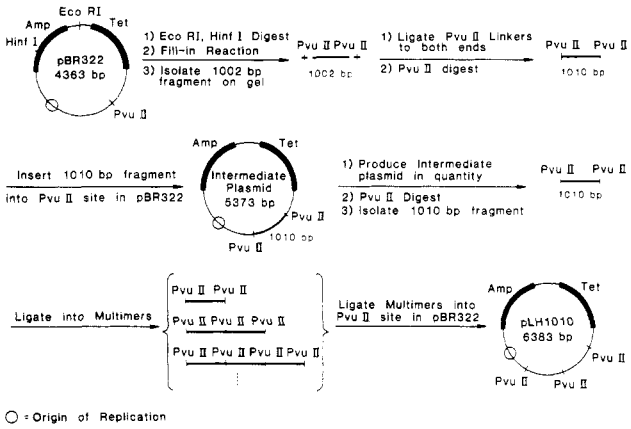

Figure 2. Construction of pLH1010 from pBR322. pBR322 was cut with Eco RI and Hinf I, generating many fragments, the longest of which was 998 bp in length. The sticky ends on these fragments were converted to blunt ends with the Klenow procedure. The band of interest, now 1002 bp in length, was isolated by agarose gel electrophoresis. Pvu II linkers were ligated to both ends and then cut with Pvu II, leaving half of a Pvu II recognition sequence on each end. The resulting 1010-bp fragment was ligated into the Pvu II site in pBR322. The DNA was used to transform *E. coli* HB101 using ampicillin and tetracycline resistance as markers. We then checked for the presence of the 1010-bp insert using Pvu II digestion and agarose gel electrophoresis. Having thus cloned the 1010-bp fragment with the proper Pvu II ends, we then grew this intermediate plasmid in quantity. We cut the 1010-bp fragment out of the plasmid, using Pvu II, and then purified the fragment by agarose gel electrophoresis. In this way we were able to obtain sufficient quantities of the 1010-bp fragment to carry out controlled blunt-end ligations. After ligating the fragments into multimers, we ligated multimers into the Pvu II site in pBR322. The DNA was used to transform *E. coli* DH1 using both tetracycline and ampicillin resistance as markers. Small amounts of plasmid, isolated from each colony, were tested for the presence of the 1010-bp insert and increased overall length.

aration of the fragment from the rest of the plasmid by agarose gel electrophoresis.[10] These 1010-bp fragments were ligated into multimers with T4 DNA ligase and these multimers were inserted into the Pvu II site in pBR322. These plasmids were used to transform DH1 and colonies were selected for resistance to ampicillin and tetracycline. A plasmid that contained two copies of the 1010-bp fragment was recovered from one colony and subsequently named pLH1010. Cutting this plasmid with Pvu II results in the recovery of the original 1010-bp fragments and the 4363-bp pBR322 vector. We originally aimed to incorporate a greater number of 1010-bp fragment copies into the plasmid. Many attempts to accomplish this were unsuccessful and it is likely that plasmids with a greater number of identical inserts are at a biological disadvantage in the transformation procedure. This topic is pursued in greater depth below.

The following procedure was used in constructing the third plasmid. The plasmid pRLM4, which contains the kanamycin resistance gene from Tn5,[22,27] was cut with Pst I (see Figure 3). The 923-bp[28] fragment was isolated by agarose gel electrophoresis and ligated into multimers with T4 DNA ligase. These multimers were inserted into the Pst I site in pBR322 and the resulting plasmids used to transform HB101. Colonies were selected for resistance to tetracycline and sensitivity to ampicillin. A plasmid that contained three copies of the 923-bp fragment was recovered from one colony. Cutting this plasmid, pLH762/367, with Pvu II results in the recovery of three copies of the 762-bp Pvu II fragment that exists within the original Pst I fragment.[28] Also recovered are two larger fragments and a smaller fragment (see Figure 4). The fragments are very different in size, allowing their sepa-

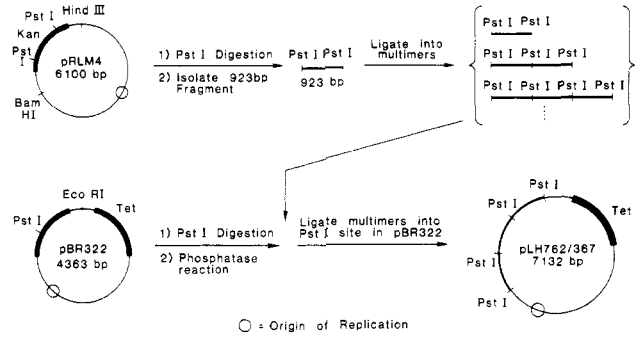

Figure 3. Construction of pLH762/367 from pRLM4 and pBR322. The fragment that we wished to clone in this case was obtained by Pst I digestion of pRLM4, followed by the isolation of the 923-bp fragment by agarose gel electrophoresis. The 923-bp fragment was then ligated into multimers with T4 DNA ligase and then inserted into the Pst I site in pBR322. The DNA was used to transform *E. coli* HB101, using tetracycline resistance as a selective marker. Colonies were then screened for ampicillin sensitivity, since the insertion of any DNA into the Pst I site inactivates the ampicillin resistance gene. Small amounts of plasmid DNA, isolated from colonies with the proper phenotype, were then checked for the presence of the 923-bp insert and increased length.

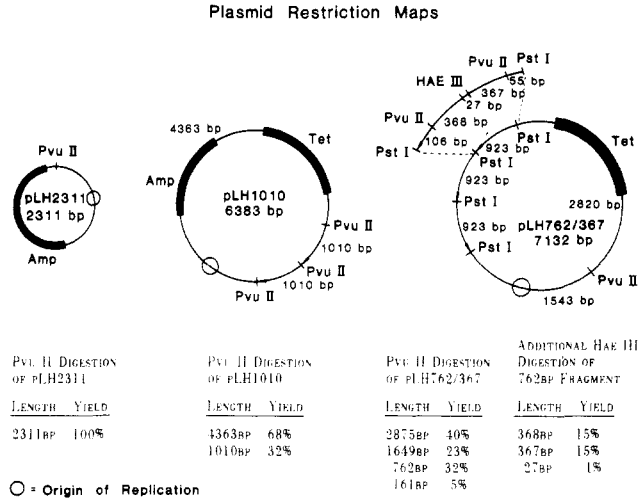

Figure 4. This figure summarizes the structure of the three plasmids and the results of digestion with restriction enzymes. The fragments are present in sufficient number so that the theoretical yield, by weight, is greater than 30% in all cases. The 367- and 368-bp fragments are not meant to be separated from each other but are intended to be used in combination as a single model system. As discussed in the text, the fragment's orientations have been determined by restriction mapping (see Figure 5, panel C). The arrows shown on the inserted fragments show their orientation. In the case of pLH1010 the arrow head is shown on the end of the fragment that was originally cut with Hinf I. In the case of the 923-bp insert of pLH762/367, the arrow points in the direction in which the nucleotide sequence was originally reported.[28]

ration on a preparative agarose gel system, to be described below. After repurification, the 762-bp fragment can be digested with Hae III,[18] yielding fragments 367 bp, 368 bp, and 27 bp in length. For many cases this combination may be used as a model system as is, or the 367- and 368-bp fragments may be repurified together. It is noteworthy that this second digestion yields fragments approximately half as long as the primary fragment without incurring the loss of half of the material.

In both of the latter two cases the inserted fragments have two identical ends but the internal base sequence is not symmetric. Thus each fragment could be inserted into the plasmid vector in either of two orientations. In the

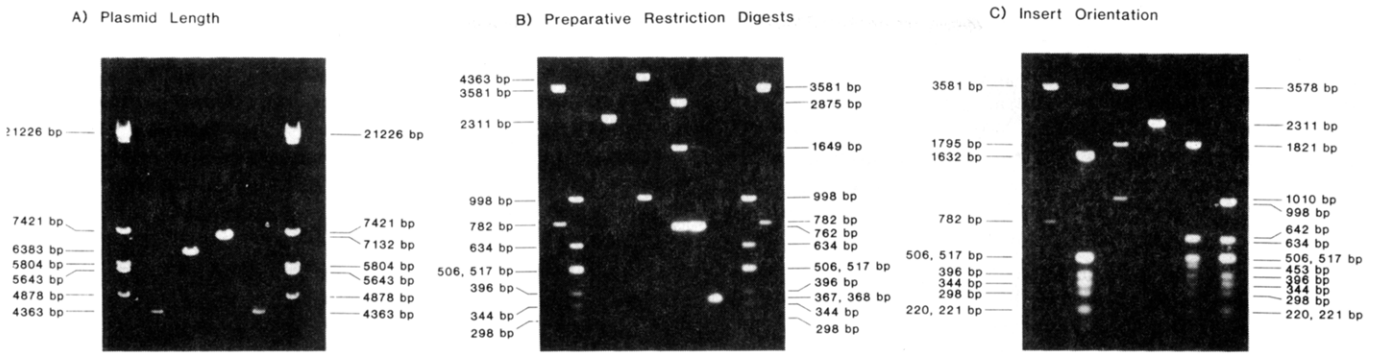

Figure 5. This figure shows agarose gel electrophoresis of restriction digests of the plasmids. Panel A, a 0.7% agarose gel, demonstrates the lengths of the plasmids. The first and last lanes show Eco RI digests of lambda DNA. The second and fifth lanes show pBR322 digested with Pvu II, producing a linear molecule. The third lane, showing pLH1010 cut with Hind III, shows that this plasmid has two copies of the 1010-bp fragment as it is approximately 2000 bp longer than the pBR322 vector. The fourth lane shows pLH762/367 cut with Hind III. In this case the plasmid is about 3000 bp longer than the pBR322 vector, demonstrating the insertion of three copies of the 923-bp fragment. Panel B, a 2% agarose gel, shows the restriction fragments listed in Figure 4. The first and last lanes contain pBR322 that has been digested with Pst I and Hind III. The second and eighth lanes show pBR322 digested with Eco RI and Hinf I. The third lane shows pLH2311 that has been digested with Pvu II, yielding a single fragment 2311 bp in length. The fourth lane shows pLH1010 digested with Pvu II, yielding the 4363-bp vector and the two copies of the 1010-bp fragment. The fifth lane shows pLH762/367 digested with Pvu II. In this case bands at 2875 and 1649 bp are produced in addition to the three copies of the 762-bp fragment. The sixth lane shows the 762-bp fragment of the sample in the previous lane, after it has been isolated from the other bands. The seventh lane shows the results of Hae III digestion of the purified 762-bp fragment, yielding fragments 367 and 368 bp in length. The 27-bp fragment cannot be seen. Panel C, a 1.4% agarose gel, shows the restriction patterns used to determine the fragment orientations. In the first lane is a Pst I and Hind III digestion of pBR322. The second lane contains a Hinf I digestion of pBR322. The third lane shows the results from Pst I digestion of pLH1010. This pattern is consistent only with the fragment orientation shown in Figure 4. Lane 4 shows a Pvu II digestion of pLH2311, used in this case as a marker. Lane 5 shows the results of Hinf I digestion of pLH762/367. Again this pattern is only consistent with the fragment orientation shown in Figure 4. The last lane shows a Hinf I and Eco RI digestion of pBR322.

case of pLH1010, two copies of the fragment were inserted and therefore there were four possible orientations. As three copies of the fragment were inserted into pLH762/367, there were eight possible orientations. Digestion of the plasmids with restriction enzymes (Hinf I in the case of pLH762/367 and Pst I in the case of pLH1010) and agarose gel electrophoresis of the resulting fragments allowed us to determine the orientations of the inserted fragments. Although there are pairs of possible orientations of three fragments that cannot be distinguished by this procedure, we did not obtain such a result. Thus the exact plasmid configuration has been determined (see Figures 4 and 5).

It is interesting to note that in each case the fragments were inserted in a head-to-tail orientation. Although this observation is not statistically significant ($P = 0.125$ by direct calculation), it is consistent with the observations[29,30] that head-to-head and tail-to-tail configurations of DNA sequences are unstable biologically. Presumably this is because of the ability of the two gene copies in these configurations to base pair with each other, forming a hairpin structure that is removed from the plasmid.[29] The difficulty that we encountered in inserting a greater number of fragment copies into the plasmids was due, in part, to the decreased likelihood of constructing the purely head-to-tail configuration as the number of inserted fragments increased. Most importantly, the plasmids created, with their purely head-to-tail orientation, seem to be entirely stable in these bacterial strains.

Production of Purified Fragments

In order to purify the fragment of interest from the other products of restriction enzyme digestion, we use the following procedure. The plasmids are grown and purified from 1- or 2-L cultures of either *E. coli* strain DH1 or HB101 using the modification of the procedures of Marko, Chipperfield, and Birnboim[24] that is described above. The plasmid DNA (either pLH1010 or pLH 762/367; 100–300 μg) is digested by Pvu II using a 2–4-fold excess of enzyme at 37 °C for 3–5 h. The DNA is precipitated with ethanol,

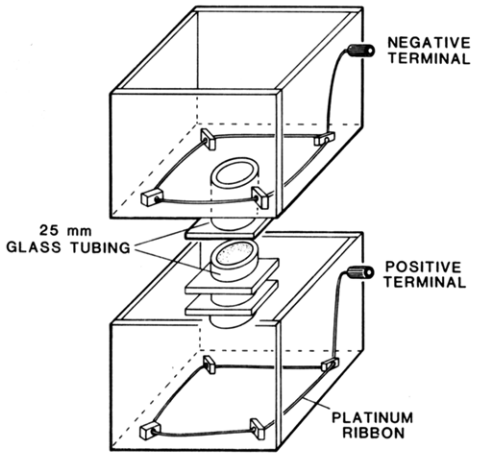

Figure 6. This figure shows the preparative agarose gel electrophoresis rig. The buffer tanks are constructed from $^3/_{16}$-in. plexiglass. The buffer tanks are approximately 10 cm on a side and 8 cm high. The gel tube is 25-mm glass tubing 25.4 mm in length and the electrodes are platinum ribbon. Rubber bands, wrapped around the square flanges on the glass tubes, are used to hold the bottom tube securely against the top tube. Similarly, another square of plexiglass (not shown) is held against the bottom of the bottom tube when pouring an agarose gel.

resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and mixed with one-fifth volume of 40% sucrose in water, to increase its density. The sample is then ready to be loaded on the agarose gel apparatus as described below.

Agarose gel electrophoresis, although a highly developed and powerful technique for the analysis of DNA,[10] is rarely used preparatively with samples containing hundreds of micrograms of DNA. Because of this, we chose to build an apparatus specifically for this purpose (see Figure 6).

The gel apparatus, constructed out of $^3/_{16}$-in.-thick Lucite, consists of two 500-mL buffer tanks which stack on top of one another. A 1-in. section of 25-mm-diameter glass tubing is mounted in an opening on the bottom of the top

tank. A 2-in. square piece of Lucite is mounted near the bottom end of the tubing. A second 1-in piece of 25-mm glass tubing has 2-in. Lucite squares mounted near each end. The ends of the glass tubes have been ground flat with 600-grit abrasive paper. When the second piece of glass tubing is placed with one end against the bottom end of the buffer-tank-mounted tube, the 2-in Lucite squares align, allowing the tubes to be held together with rubber bands. Another 2-in Lucite square is used to seal off the bottom of the bottom tube in a similar manner. This arrangement generates a sealed tube into which the 1–2% agarose gel is poured, usually to within 2 or 3 mm of the top of the tube. After the agarose has solidified, the bottom square of Lucite is removed, exposing the bottom of the gel. The bottom of the gel will be flat and the top will be concave because of the liquid gel's meniscus. Because of this, it is useful to slide the gel out of the tube and turn it over. In this way a very flat band of DNA will form in the gel. A small square of cheesecloth may be placed over the bottom of the gel and held in place with a rubber band in order to prevent the gel from slipping out of the tube. Electrophoresis buffer[10] (80 mM Tris·PO_4, pH 8.0, 2 mM EDTA, 0.5 μg/mL ethidium bromide) is poured into the tanks and the top tank is placed on the bottom one. The DNA sample, in TE and sucrose, is placed on top of the agarose gel and voltage is applied, usually 50–70 V. Periodically, the top tank is lifted up (after the voltage is turned off) and long-wave UV light is shown on the agarose gel through the glass tubing. The fluorescent dye allows the localization of the DNA band of interest. When the band is approaching the break between the two glass tubes, the voltage is turned off and the glass tubes slightly pulled apart. The gel is cut just in front of the DNA band and a 25-mm-diameter circle of Na45 paper is placed between the two pieces of the gel. The gel apparatus is reassembled and the electrophoresis continued. The band of interest continues onto the Na45 paper where it is bound because of the low ionic strength of the electrophoresis buffer. When the DNA has become completely bound to the Na45 paper, as judged by periodically visualizing the DNA with long-wave UV light, the Na45 circle is removed and washed in electrophoresis buffer for a few seconds to remove any adherent agarose. The DNA may then be recovered from the paper as described in the Schleicher and Schuell procedure. When a large amount of DNA is being repurified, it is often useful to elute the Na45 paper twice.

In our experience it is possible to recover approximately 70–80% of the theoretical yield of restriction fragments by following this procedures.

To obtain linear molecules of pLH2311, the plasmid is simply digested with Pvu II, extracted with phenol,[10] and precipitated with ethanol.

Conclusions

We have, through the techniques of genetic engineering and a modest adaptation of existing electrophoresis techniques, given polymer chemists access to a monodisperse, semistiff, model polymer system. We hope this will accelerate the progress that is being made in the experimental testing and verification of theories of polymer structure and dynamics. Investigators interested in using these plasmids may obtain them by writing R. Pecora at the address above.

Acknowledgment. Thomas C. Reynolds and Jeremy Nathans both provided assistance and useful suggestions during the course of this work. This work was supported by NIH Grant 2ROI GM 22517 and NSF Grant CHE 82-00512 and the NSF MRL Program through the Center for Materials Research at Stanford University. R.J.L. was supported by the NIH Medical Scientist Training Program at the Stanford University School of Medicine.

Registry No. Pvu II, 81295-34-3; Hae III, 81295-18-3.

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