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Preparation of Milligram Amounts of 21 Deoxyribonucleic Acid Restriction Fragments†

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ABSTRACT: Twenty-one DNA restriction fragments ranging in size from 12 to 880 base pairs (bp) were purified to homogeneity in milligram amounts. The developments which facilitated this work were (a) procedures for the rapid preparation of gram quantities of pure recombinant plasmid DNAs, (b) selective poly(ethylene glycol) (PEG) precipitation of DNAs according to broad classes of lengths, and (c) large-scale high-pressure liquid chromatography on RPC-5 for the purification of fragments to homogeneity. The 95- and 301-bp sequences from the lactose control region of *Escherichia coli* were cloned into the single *Eco*RI site of pVH51 in up to four copies per plasmid. These tandem inserts are separated by *Eco*RI sites and have a head to tail orientation in all cases. A total of 50 and 90 mg of the 95- and 301-bp fragments, respectively, were prepared from 300-L fermentations of *E. coli* cells transformed with these plasmids. A rapid and improved method, which can easily be scaled up, for the purification of plasmids and DNA restriction fragments was developed. Also, the linear pVH51 vector DNA was digested with *Hae*III to yield fragments ranging in size from 12 to 880 bp. The five smaller fragments (from 12 to 180 bp) were purified quantitatively by a selective PEG precipitation enrichment step followed by RPC-5 column fractionation. The larger fragments (245-880 bp) were prepared in milligram amounts. Ten subfragments from the 301-bp *lac* fragment were prepared by *Hpa*II, *Hin*fI, or *Hae*III/*Alu*I digestions followed by separation of the reaction products on RPC-5.

The role of the physical properties of DNA in biological regulation has been studied principally with large chromosomal DNAs (or heterogeneous populations of broken chromosomal DNAs) or with biosynthetic DNA polymers containing defined and repeated nucleotide sequences (reviewed in Wells et al., 1977, 1980b; von Hippel, 1979). The existence of sequence-dependent structural differences was demonstrated by a variety of physical and spectroscopic studies on the DNA polymers (Wells et al., 1970, 1977, 1980b). Extension of this approach to natural DNA molecules requires the development of methodology for the isolation of milligram quantities of small (10-200 base pairs) and homogeneous DNA sequences. Four developments in the past several years have laid the groundwork for this methodology: (i) the characterization of a large number of restriction endonucleases with precise specificities, (ii) the development of gene cloning with multicopy plasmid vectors, (iii) the utilization of rapid and reliable sequencing methods for characterizing fragments, and (iv) high-pressure liquid chromatography (HPLC)[1] on RPC-5 for the fractionation of milligram amounts of fragments.

An alternate approach is the chemical synthesis of oligonucleotides which correspond to a DNA sequence of interest (reviewed in Wu et al., 1978). Whereas this approach is useful for relatively small molecules (less than 20 bp in length), it is much more difficult to synthesize milligram amounts of homogeneous duplex DNAs containing 40-100 bp.

Recombinant DNA methodology has been utilized previously for the preparation of homogeneous DNA sequences. Plasmids were constructed containing the desired sequences which can be easily excised with restriction endonucleases and then purified from the vector DNA. Most of the plasmids studied to date contain fragments derived from the lactose control region of *Escherichia coli* (Hardies et al., 1979a; Hardies & Wells, 1979; Sadler et al., 1979; Kallai et al., 1980). The amount of fragments prepared by standard procedures (Hardies & Wells, 1979) was sufficient for optical studies (i.e., DNA melting) but was inadequate for other types of physical

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[1] Abbreviations used: bp, base pairs; PEG, poly(ethylene glycol); DOC, deoxycholate; UV, ultraviolet; HPLC, high-pressure liquid chromatography; *Hae*III/*Alu*I digest, the slash indicates a double digest of the DNA with the two endonucleases indicated; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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analyses such as X-ray crystallography and NMR and Raman spectroscopy. These standard procedures utilize CsCl–ethidium bromide gradients or column chromatography steps (Kallai et al., 1980) which limit the amount of DNA that can be conveniently purified in a single step.

This paper describes the use of recombinant methods to construct plasmids with multiple inserts of a desired fragment together with an improved procedure for the rapid and efficient selective precipitation of DNA restriction fragments from gram amounts of plasmid DNA with the final purification by RPC-5 column chromatography. A total of 21 fragments was purified to homogeneity and range in size from 12 to 880 bp. The final yields from 1 kg of wet packed cells varied from 0.6 mg for a 12-bp fragment to 87.5 mg for a 301-bp fragment.

Materials and Methods

General Methods. *Alu*I and *Hin*fI were purchased from BRL (Bethesda, MD); *Eco*RI, *Hpa*II, and *Hae*III were prepared by using published procedures (Greene et al., 1978); *Hpa*II was a gift from T. C. Goodman (this lab). Restriction enzyme reactions were performed as described previously (Hardies & Wells, 1979; Blakesley & Wells, 1975; Wells et al., 1980a; Hardies et al., 1979a) unless otherwise specified herein. DNA ligase reactions, cellular transformation, and the screening of transformed cells were described previously (Hardies et al., 1979a). RPC-5 column chromatography (Wells et al., 1980a) and gel electrophoresis (Hardies et al., 1979a; Helling et al., 1974; Blakesley & Wells, 1975) were by published procedures.

All plasmids were prepared by using *E. coli* MO (Struhl et al., 1976) as the host cell and pVH51 (Hershfield et al., 1976; Hardies et al., 1979a) as the plasmid vector for inserted DNA fragments. Bacterial cells were grown in M9 minimal media (Clewell, 1972) in a 300-L fermentor (Fermentation Designs, Inc.) in the pilot plant of this Department. Chloramphenicol (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 150 μg/mL when the cell suspension had an A_{550} of 1.5, and plasmid amplification was allowed to continue overnight (Clewell, 1972). Cells were harvested by using a Sharples centrifuge Type 06X with yields ranging between 900 and 1000 g of wet packed cells.

PEG[1] (Carbowax 6000) was purchased from Fisher Scientific Co., Brij 58 from Ruger Chemical Co., Inc., Irvington-on-the-Hudson, NY, and DOC from Sigma.

The size designations of the fragments only include paired bases; sticky ends are not counted.

Cell Lysis. The wet packed cells were suspended with a Waring blender in 25% sucrose and 50 mM Tris-HCl (pH 8.0) to give a final volume of 2.5 L. Cell lysis was by a modification of a published procedure (Clewell & Helinski, 1970); 18.6 mL of the cell suspension along with 4 mL of 0.5 M EDTA (pH 8.0) was placed in polycarbonate tubes for use in a Spinco Ti 42.1 rotor. Solid lysozyme (Sigma) was added to a final concentration of approximately 2 mg/mL. The mixture was kept on ice for 15 min, and then 2.5 mL of a 2:1 (v/v) mixture of 10% Brij–10% deoxycholate was added. The mixture was kept at 0 °C for 10 min. The suspension was centrifuged at 38 000 rpm for 45 min in a Spinco 42.1 rotor. The supernatant was decanted and saved while the pellet was washed with 1 volume of 50 mM Tris-HCl (pH 8.0) and 0.1 M EDTA and recentrifuged as above. The wash was performed in order to extract any trapped plasmid DNA. The wash from the pellet was processed in parallel with the supernatant of the original lysis until it was demonstrated that it was not contaminated with degradative enzymes or chromosomal DNA. When larger volumes of cell lysate were used, centrifugation was performed with a Spinco Type 21 rotor at 18 000 rpm for 14 h.

The plasmid DNA was concentrated by PEG precipitation (Hardies & Wells, 1979; Humphreys et al., 1975). The pellets were resuspended in a total volume of 520 mL of 10 mM Tris-HCl (pH 8.0) and 0.1 M EDTA, overlayed with 0.1 volume of phenol which had been preequilibrated with 100 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, and agitated on a reciprocating shaker at room temperature overnight (T. Harrell and R. Wartell, personal communication).

Phenol Extraction. The resuspended pellets were extracted 5 times with an equal volume of buffer-equilibrated phenol. The phases separate slowly after the first extraction; thus centrifugation in a Sorvall GS3 rotor at 3000 rpm for 10 min may be necessary to break the emulsion. The phenol phases from the first two extractions were back extracted with 0.5 volume of 10 mM Tris-HCl (pH 8.0) and 0.1 M EDTA. The aqueous layers from the back-extraction were combined and extracted 4 more times with phenol and then combined with the aqueous layer from the original extraction. Residual phenol was removed by five extractions with ether, 2 volumes for the first and 1 volume for the next four extractions.

RNase Treatment. Prior to RNase treatment, the nucleic acids were concentrated by PEG precipitation as described above. The pellet was resuspended in 300 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 M EDTA. RNase A [Sigma; boiled for 10 min in 0.1 M sodium acetate (pH 5.0) (Zasloff et al., 1978)] was added to a final concentration of 100 μg/mL. The reaction mixture was incubated at 37 °C for 1 h and then extracted 5 times with 0.25 volume of buffer-equilibrated phenol and 5 times with 0.5 volume of ether. The residual ether was removed from the aqueous phase in vacuo.

Ribonucleotides and ribooligonucleotides were removed by PEG precipitation; 0.25 volume of 1.5 M NaCl and 0.25 volume of 30% PEG in 1.5 M NaCl were added to the solution which was mixed, kept at 0 °C for 15 min, and then centrifuged in a GS3 rotor at 4000 rpm for 15 min. The pellet contained the plasmid DNA whereas the RNA remained in the supernatant. Occasionally, a small amount of plasmid DNA remained in the supernatant and was removed by increasing the PEG concentration to 10% and recentrifuging the mixture. The pellets were resuspended in 400 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA and stored at −20 °C. The yield of plasmid DNA can be accurately determined by UV spectroscopy at this point and was found to vary between 350 and 1600 mg.

RPC-5 Batch Elution. A 200-mL solution of plasmid DNA (1 mg/mL) in 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA was made 0.2 M in NaCl, mixed with 100 mL of RPC-5 resin, and shaken for 30 min. The mixture was centrifuged in a GS3 rotor for 5 min at 3000 rpm. This supernatant was saved, and the resin was washed 3 times in a similar way with 100 mL of 1.5 M NaCl, 0.01 M Tris-HCl (pH 8.0), and 0.1 mM EDTA. The A_{260} of the supernatants was taken to determine plasmid DNA recovery. Small scale *Eco*RI reactions revealed that all DNA fractions could be cleaved to completion at normal enzyme concentrations. Therefore, the 1.5 M NaCl washes were combined with the load supernatant, made 10% in PEG by the addition of 0.5 volume of 30% PEG in 1.5 M NaCl, kept at 0 °C for 30 min, and centrifuged at 6000 rpm for 20 min in a GS3 rotor. The pellets were dissolved in a total of 400 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA. The total recovery of DNA from this procedure was 90 ± 5%.

EcoRI Cleavage of Plasmid and Selective Precipitation of the Insert. An *Eco*RI digest of 250 mg of plasmid DNA in 300 mL was performed as described (Hardies & Wells, 1979). Following digestion, 83 mL of 2% gelatin (previously autoclaved) and 92 mL of 1.5 M NaCl were added with shaking to the reaction mixture. Next, 92 mL of 30% PEG in 1.5 M NaCl was added, and the solution was kept at room temperature for 10 min and then centrifuged in a GS3 rotor at 4000 rpm for 15 min. The supernatant was decanted (the pellet saved), made 10% in PEG by adding 183 mL of 30% PEG in 1.5 M NaCl, kept at 4 °C overnight, and centrifuged in a GS3 rotor at 6000 rpm for 30 min. The pellet from the first precipitation was dissolved in 300 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA while the second pellet was dissolved in 50 mL of the same buffer. These separate solutions were extracted 5 times with 0.5 volume of buffer-equilibrated phenol and 5 times with 1 volume of ether. Residual ether in the aqueous layer was removed in vacuo. The pellet from the first precipitation contained linearized vector DNA with a trace of insert, and the pellet from the second precipitation contained the insert fragment with only a small amount (~10% of original amount) of the vector (see Results).

Selective PEG Precipitation of the HaeIII Digest of pVH51. A *Hae*III digest was performed on 310 mg of *Eco*RI-linearized pVH51 DNA in 500 mL as described (Blakesley & Wells, 1975). After completion, the reaction mixture was extracted 5 times with buffer-equilibrated phenol and 3 times with 1 volume of ether. Residual ether was removed in vacuo. A total of 145 mL of 30% PEG in 1.5 M NaCl was added, and the solution was shaken and centrifuged immediately in a GS3 rotor at 3000 rpm for 15 min (Lis & Schleif, 1975). The supernatant was decanted, and the pellet was dissolved in 50 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA and stored at −20 °C. A total of 250 mL of 30% PEG in 1.5 M NaCl was added to the supernatant and kept at 4 °C overnight. The solution was then centrifuged at 6000 rpm for 30 min in a GS3 rotor. The supernatant was decanted, and the pellet was resuspended in 20 mL of 0.01 M Tris-HCl (pH 8.0) and 0.1 mM EDTA and stored at −20 °C. The supernatant was extracted 5 times with 95% chloroform and 5% 1-propanol (v/v) to remove PEG. The aqueous phase was then concentrated by using a rotory evaporator and dialyzed against RPC-5 load buffer (Wells et al., 1980a).

Results

Construction and Characterization of Plasmids Containing Multiple Inserts of Fragments from the lac Control Region. For increased yields of desired sequences of DNA, recombinant plasmids containing multiple copies of fragments derived from the *lac* region were constructed by the general procedures which were previously described (Hardies et al., 1979a; Sadler et al., 1979). Figure 1 summarizes the results of our cloning experiments with the 95-bp and the 301-bp fragments. Both contain the *lac* operator–promoter, and the sequence of the 95-bp fragment is embodied within the 301-bp DNA. The 95-bp fragment was prepared from pRW4 (Hardies et al., 1979a) and the 301-bp fragment from pRW501 (Hardies et al., 1979b). Each of these fragments, now having *Eco*RI ends, was ligated to give a family of multimers. Multimers of the 95-bp fragment were separated on 5% polyacrylamide gels, and the individual multimers were eluted and ligated into the *Eco*RI site of pVH51. Alternatively, the ligation products from the 301-bp fragment were ligated into the *Eco*RI site of pVH51 without previous separation of the multimers (Hardies et al., 1979a). After selection and screening for *lac* constitutivity (Hardies et al., 1979a), the size of recombinant

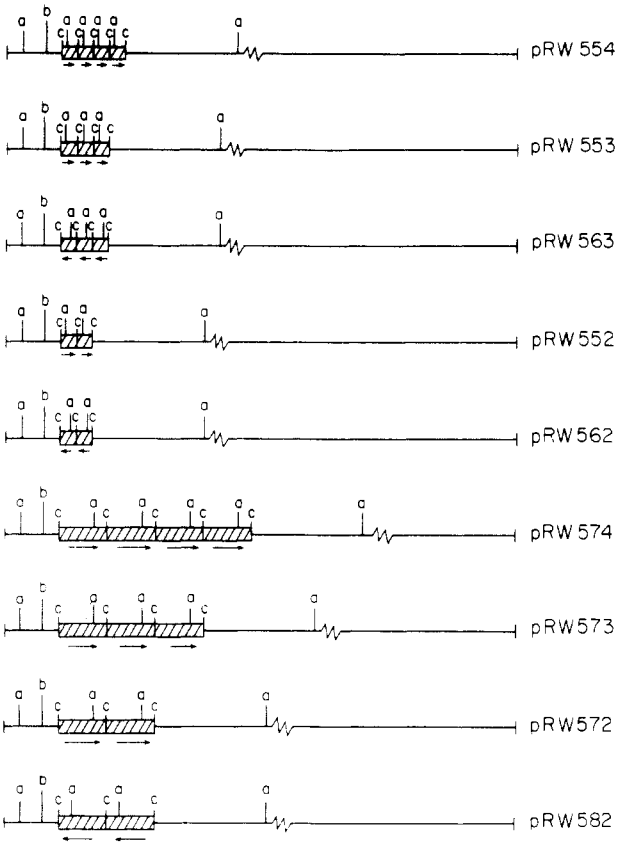

FIGURE 1: Recombinant plasmids with multiple inserts of the *E. coli* lactose control region. The first five panels show the multiple insertion clones obtained from the 95-bp fragment (prepared from pRW4; Hardies et al., 1979a); the four bottom panels display the result of the multiple-insertion cloning of the 301-bp fragment (prepared from pRW501; Hardies et al., 1979b) into pVH51. For the sake of this drawing, pVH51 is linearized at the *Kpn*I site. Only the restriction sites needed for the characterization of the plasmids are included in this map. Solid lines designate pVH51 DNA, and hatched boxes represent the inserted DNA. The letter b denotes the single *Hin*dII site in pVH51 which defines the orientation of the vector. The single *Eco*RI site in pVH51 was used for the construction of these plasmids. The letter c designates the *Eco*RI sites which separate the inserts from each other and from the vector DNA. The letter a denotes the *Hpa*II sites in the inserts and the two flanking *Hpa*II sites of the vector. Mapping the *Hpa*II fragments was particularly important in determining the orientation of the inserts. The arrows under the inserts define their orientation with respect to the vector DNA; the arrows point in the direction of transcription from the *lac* promoter which is embodied in the inserted fragments.

plasmids was determined on lysates of the colonies (Barnes, 1977). Plasmid DNA was prepared from clones which contained inserts of the expected sizes, and restriction enzyme analyses were performed (Klein et al., 1980). The conservation of all *Eco*RI sites and the number of inserted fragments were determined by partial and complete *Eco*RI digests as described (Hardies et al., 1979a). Restriction analyses of the clones revealed that all of the candidates retained all *Eco*RI recognition sites (Figure 1).

The orientation of the insertions was determined by *Hpa*II digests. The results of the *Hpa*II digestion for several plasmids containing various numbers of copies of the 95- and 301-bp fragments are shown in Figure 2 in comparison to the *Hpa*II/*Eco*RI digested pVH51 DNA. The 95-bp fragment is cut into 53- and 40-bp segments which add up, with the part of pVH51 ranging from the *Eco*RI site to the next *Hpa*II site (compare Figure 1) in the direction of the *Hin*dII site, to 298 and 285 bp (Maxam & Gilbert, 1977; Hardies et al., 1979a). The pVH51 sequence in the other direction yields either 751 or 738 bp which are not resolved on 5% acrylamide gels. Thus,

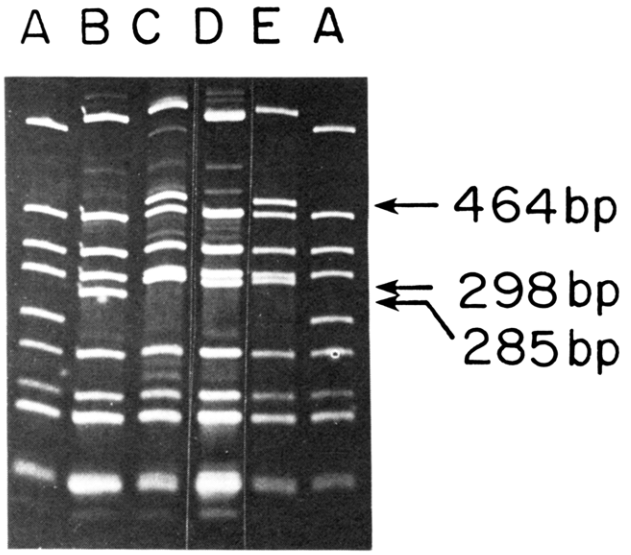

FIGURE 2: *Hpa*II restriction analysis of some multiple insertion clones of the 95- and 301-bp fragments in pVH51. Lanes A on both sides of the gel show the control digest of pVH51 with *Hpa*II and *Eco*RI. Lane B shows the *Hpa*II digest of pRW554 containing four 95-bp inserts. Lane C shows the *Hpa*II digest of pRW574 containing four 301-bp inserts. Lane D shows the *Hpa*II digest of pRW563 containing three inserts of the 95-bp fragment oriented opposite that of pRW554. Lane E shows the *Hpa*II digest of pRW572 containing two copies of the 301-bp fragment in the same orientation as pRW574.

only the orientation of the leftmost insert is unambiguously determined by *Hpa*II digestion. The orientation of the next fragment might be head to head or head to tail. The difference for the 95-bp fragment is either 99 bp including the reconstructed *Eco*RI site if the orientation is head to tail or 84 and 100 bp if they are head to head. None of the plasmids analyzed in this study showed an 84- or 110-bp band in the *Hpa*II digest (Figure 2). Therefore, we conclude that all the 95-bp multicopy plasmids have their inserts in the same orientation. This conclusion is confirmed by *Hpa*II digestion of 95-bp multimers that were isolated from incomplete *Eco*RI digests of pRW554 (data not shown). The orientation of the inserts with respect to the vector was evenly distributed between the two possible configurations, but the orientation of the 95-bp copies within one clone was always the same. Lane B of Figure 2 shows an *Hpa*II digest of pRW554, and lane D shows the same digest of pRW563 as examples.

The conclusions regarding the orientation of the 301-bp multicopy plasmids are more obvious due to the increased size of this fragment. An *Hpa*II digestion of the plasmids containing several copies of the 301-bp sequence defines the complete orientation of the inserts. The 301-bp fragment is cleaved into 80 and 219 bp by an *Hpa*II digestion. This results in either 326 or 464 bp for the *Hpa*II fragment pointing toward the *Hin*dII site of pVH51 (Maxam & Gilbert, 1977; Hardies et al., 1979a). Although both examples shown in Figure 2 have the 219-bp side of the 301-bp sequence pointing toward the *Hin*dII site, the other orientation is found with a similar frequency (data not shown). The size difference between the *Hpa*II fragments of the 301-bp sequence is even large enough to determine the orientation of the last copy pointing away from the *Hin*dII site. The large *Hpa*II fragment is either 782 or 921 bp along. All results suggest parallel orientation of the first and last copy in the multicopy plasmids of the 301-bp fragment as shown for pRW572 in lane E of Figure 2. For the cases of more than two copies, the internal orientation of the 301-bp sequences can also be derived from an *Hpa*II digest. The head to head case would result in 164 and 442 bp including the reconstructed *Eco*RI site whereas the head to tail orientation would result in a 305-bp fragment. The restriction digest of pRW574 in lane C of Figure 2 demonstrates the presence of the 305-bp band only. The same result was found for pRW573 as well.

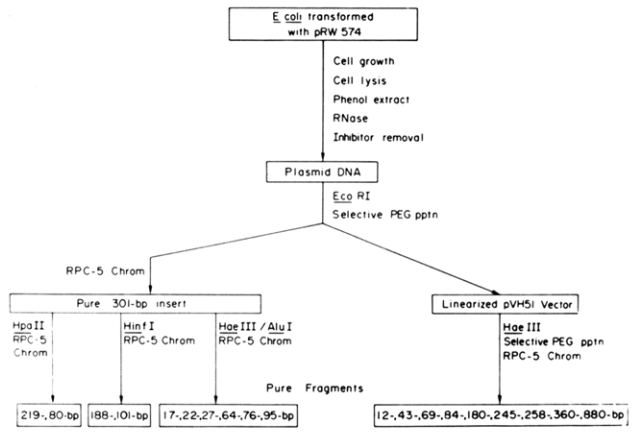

FIGURE 3: Overview of purification schemes of restriction fragments. In addition to isolation of the pure 301-bp insert from pRW574 as shown, the 95-bp insert was purified from pRW554.

Overview of Purification Schemes. Recombinant plasmids were constructed which are amplifiable, can be cleaved with a single restriction enzyme to give the inserted fragment and the vector, and contain the inserted fragment in multiple copies (Figure 1) (Hardies & Wells, 1979; Hardies et al., 1979a). The general approach used for the large-scale purification of small restriction fragments from these plasmids is outlined in Figure 3. The pure plasmids (approximately 0.4–1 g of recombinant DNA from ~1000 g of wet packed cells) were cleaved with *Eco*RI, and the insert fragment (301 or 95 bp in length) was separated from the vector DNA by PEG precipitation. The insert fraction contained a small amount (~10%) of the vector DNA at this stage. The fragment was then purified to homogeneity by RPC-5 column chromatography. The pVH51 fraction, after the first PEG precipitation, contained only a trace (<~5%) of insert DNA.

The lower right portion of Figure 3 outlines our purification scheme for the preparation of nine *Hae*III fragments from the pVH51 vector. After *Hae*III digestion, the mixture of fragments was partially fractionated according to size by selective PEG precipitation. These pools of fragments could then be resolved by RPC-5 column chromatography.

The lower left portion of this figure shows that recleavage of the pure 301-bp insert DNA with another restriction enzyme(s) followed by HPLC on RPC-5 provided a rich source of homogeneous subfragments, in this case from 17 to 219 bp in length.

Lysis of Cells and Removal of Contaminating Proteins and RNA. The lysis procedure as originally described (Clewell & Helinski, 1970) and later modified (Hardies & Wells, 1979) was designed to minimize contaminating chromosomal DNA in the cleared lysate. Other protocols call for removal of contaminating chromosomal DNA from plasmid DNA preparations by using CsCl–ethidium bromide gradients or column chromatography (Hardies & Wells, 1979; Kallai et al., 1980). Following the procedure under Materials and Methods, chromosomal DNA contamination is usually not observed as shown in Figure 4. This figure shows agarose gel electrophoretic analyses of the nucleic acids at four stages during the purification of plasmid DNA. Lane B shows the analysis on the crude lysate; two ethidium-staining bands are observed corresponding to the dimer and tetramer (or form II) recombinant DNAs. If *E. coli* DNA were present, a band would

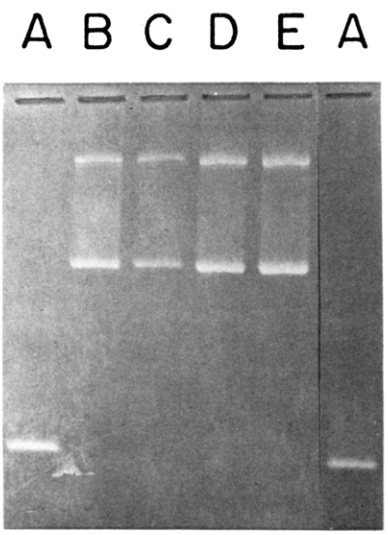

FIGURE 4: Analysis of the dimer plasmid of pRW574 after different steps of the preparation. (Lanes A) pVH51 linearized with *Eco*RI as a control; (lane B) pRW574 in the cleared lysate; (lane C) pRW574 after removal of protein by phenol extraction; (lane D) pRW574 after RNase treatment; (lane E) pRW574 after RPC-5 batch elution. The samples were electrophoresed on 1% agarose. The two bands observed in lanes B–E are the dimer and (probably) the tetramer or nicked circle of pRW574 as indicated by the position of the linear pVH51. The RNA was run off the gels under these conditions.

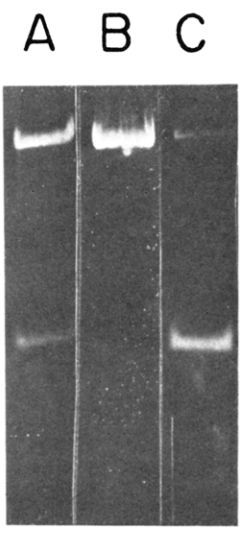

FIGURE 5: Selective PEG precipitation of pVH51 DNA from the 301-bp fragment. (Lane A) Reaction product of the large-scale *Eco*RI digest. (Lane B) Pellet from the 5% PEG treatment. (Lane C) Pellet from the 10% PEG treatment. It is clear that enrichment of the insert occurs without a major loss of the small fragment in the pVH51 pool. The samples were analyzed on 5% acrylamide gels.

be observed on the top of the gel. However, even if trace amounts of chromosomal DNA were present at this stage, the final fragments should not be contaminated since cleavage of the DNA with *Eco*RI would result mainly in large segments from the chromosomal contamination, which would not interfere with the purification of the much smaller inserted fragments. The vector DNA which is cleaved with *Hae*III would contain these contaminations, and they could give rise to small fragments. However, these are removed by high-resolution RPC-5 chromatography unless they coelute with a pVH51 *Hae*III fragment. This event has only a very remote probability.

In working with high cell densities as described (Materials and Methods), nucleolytic degradation of the plasmid DNA was a serious problem throughout all steps of this procedure. We have alleviated this problem by maintaining an EDTA concentration of 0.1 M in the cleared lysate through all steps to the final phenol extraction prior to *Eco*RI cleavage. As an additional precaution, the crude DNA preparation was extracted with phenol as soon as possible after cell lysis.

The pellets from the cell lysis were washed as described under Materials and Methods. Gel analysis revealed that this wash does not contain any detectable chromosomal DNA. The wash was processed separately and the amount of DNA determined after removal of the RNA. Approximately 20% of the final plasmid yield was contained in the cell pellet wash.

RNase treatment (Materials and Methods) cuts the contaminating RNA into small pieces which are easily removed by PEG precipitation. Lane D of Figure 4 shows that the integrity of the plasmid is unchanged by this RNase treatment and the following extraction with phenol and PEG precipitation. This result is significant since RNase is frequently contaminated with DNase.

The next major step in the purification procedure was the *Eco*RI cleavage of the plasmid. Preliminary *Eco*RI reactions on the DNA at this stage revealed that an excessive amount of *Eco*RI was necessary to observe total cleavage. Complete digestion with normal enzyme levels was found after the plasmid DNA was treated batchwise with RPC-5 resin (Materials and Methods). A number of other restriction enzymes (*Hpa*II, *Hin*dII, *Hae*III, *Sal*I, and *Bam*HI) were also inhibited but did cleave the plasmid after RPC-5 treatment. Lane E of Figure 4 shows that the plasmid DNA is unchanged by the batchwise RPC-5 treatment.

In conclusion, it is extremely important that the status of the plasmid DNA remain unchanged throughout all steps of the purification. Figure 4 shows that this is the case for the method described.

Selective Precipitation of Restriction Fragments. Selective precipitation of fragments was used in two different steps (Figure 3) during the purification of the small fragments. This procedure can give a coarse fractionation according to molecular weight (Lis & Schleif, 1975) and, most importantly, is easily scaled up to accommodate gram amounts of DNA. First, the insert was successfully fractionated from the vector DNA following *Eco*RI cleavage of the plasmid in the presence of gelatin (Materials and Methods). Figure 5 shows that 5% PEG precipitated virtually all (>90%) of the 3800-bp vector with little contamination with the insert (lane B). Subsequent treatment of the supernatant fraction with 10% PEG quantitatively precipitated the 301-bp insert with a small amount of contamination by the vector (lane C); total removal of this remaining vector was easily accomplished on HPLC on RPC-5 (see below). This precipitation step was essential in reducing the total amount of DNA to a level that could be accommodated by the RPC-5 column (Wells et al., 1980a); for the example shown in Figure 3, the amount of DNA was reduced from 520 to 110 mg.

Second, the vector fraction, digested with *Hae*III, was selectively precipitated with PEG to give three pools of restriction fragments. The enrichment of classes of fragments is shown in Figure 6. The first pellet from treatment with ~7% PEG contained ~49% of the DNA with the predominant fragments ranging in size from 245 to 880 bp; the second pellet (from ~13% PEG) also contained ~49% of the DNA ranging in size from 69 to 880 bp with the main amount in the 200-bp size class. The final supernatant containing only ~2% of the DNA consisted of the 12–880-bp fragments with the main amount in the size class below 100 bp. This partial fractionation was important since resolution of the small fragments

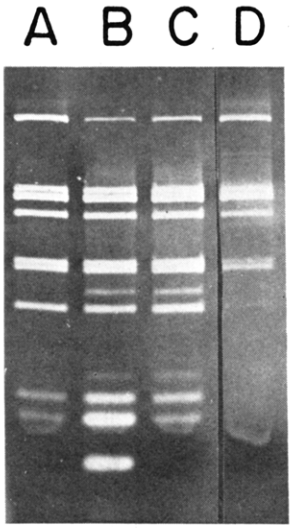

FIGURE 6: Analysis of the selective PEG precipitation of the *Eco*RI/*Hae*III digest of pVH51. Lane A shows the original distribution of fragments after the digest. Lane D shows the analysis of the first pellet which contained 145 mg, lane C shows the analysis of the second pellet which contained 145 mg, and lane B shows the supernatant which contained 8.2 mg of DNA (see Materials and Methods for further details). The samples were analyzed on 5% acrylamide gels.

Table I: Summary of the Fragments Prepared

fragment size[f] (bp)	source	sites at ends	amount (mg)	yield (%)
12	pVH51	*Hae*III	0.6[a]	63
17	301 bp	*Hae*III/*Alu*I	1.8[b]	85
22	301 bp	*Alu*I	2.5[b]	85
27	301 bp	*Alu*I	3.2[b]	89
43	pVH51	*Hae*III	2.3[a]	66
64	301 bp	*Alu*I	6.4[b]	80
69	pVH51	*Hae*III	4.1[a]	75
76	301 bp	*Hae*III/*Alu*I	6.6[b]	70
80	301 bp	*Hpa*II	3.5[c]	85
84	pVH51	*Hae*III	4.6[a]	70
95	301 bp	*Alu*I	7.1[b]	60
95	pRW554	*Eco*RI	50	*d*
101	301 bp	*Hin*fI	4.4[c]	85
180	pVH51	*Hae*III	7.2[a]	50
188	301 bp	*Hin*fI	8.2[c]	85
219	301 bp	*Hpa*II	11.3[c]	85
245	pVH51	*Hae*III	3	*e*
258	pVH51	*Hae*III	3	*e*
301	pRW574	*Eco*RI	90	*d*
360	pVH51	*Eco*RI/*Hae*III	5	*e*
880	pVH51	*Hae*III	5	*e*

[a] From 300 mg of pVH51 DNA. [b] From 37.5 mg of 301-bp DNA. [c] From 15 mg of 301-bp DNA. [d] From plasmids; no meaningful yield can be determined. [e] No attempt to obtain quantitative yield was made. [f] Sticky ends are not counted.

could then be accomplished on the subsequent RPC-5 column due to the application of a smaller amount of DNA.

Fractionation of Restriction Fragments on RPC-5. The high-resolution fractionation of fragments was by large-scale chromatography on RPC-5 (Wells et al., 1980a; Hardies & Wells, 1979; Larson et al., 1979). This procedure was used in each of the pathways in Figure 3 to achieve the final purification of the restriction fragments. Three typical examples of this method are described in detail.

Figure 7 shows the separation of the 301-bp insert from the small amount of contaminating 3800-bp pVH51 vector after the PEG precipitation step (Figure 5, lane C). The pure 301-bp insert eluted in fractions 143–218 (see insert into Figure 7), and the small amount of pVH51 eluted in the following sharp peak (fractions 220–230). Due to the large difference in size of these two fragments, it was possible to successfully fractionate 120 mg of the mixture to homogeneity. The overall yield of 301-bp fragment through the *Eco*RI digest, the selective PEG precipitation, and the RPC-5 separation was 68%. The 95-bp fragment was prepared in comparable yield by the same procedures.

Figure 8 shows the fractionation of 8.2 mg of the lowest molecular weight pool from the selective precipitation with PEG of the *Hae*III digest of linear pVH51 (Figure 6, lane B). This figure shows the purification of the 12-, 43-, 69-, and 84-bp fragments. The larger fragments (180–880 bp) partially overlap under these conditions. Similarly, the intermediate molecular weight fraction displayed in lane C of Figure 6 was fractionated on a 2.5 × 80 cm RPC-5 column. Fractions from the two columns which contained the same fragments were

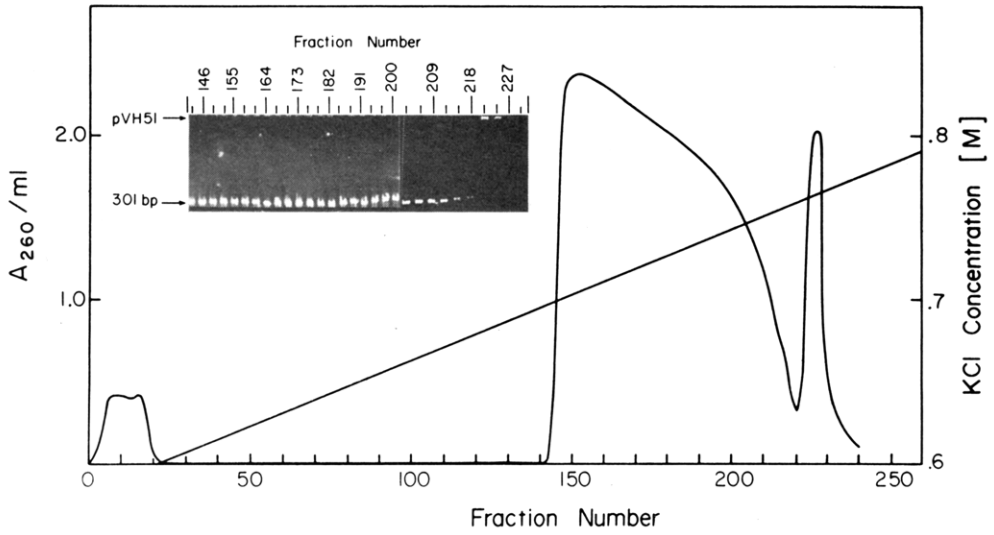

FIGURE 7: Elution profile of the RPC-5 column fractionation of the 301-bp insert from the residual vector DNA. A 2.5 × 85 cm RPC-5 column thermostated at 45 °C was loaded with 120 mg of UV-absorbing material and developed with a linear 3-L gradient from 0.5 to 0.8 M KCl containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Fractions (12 mL) were collected at a flow rate of 1.7 mL/min. The insert shows the 5% polyacrylamide gel analysis of some of the UV-absorbing fractions. No DNA bands were found in the flow-through peak. The 301-bp fragment containing fractions were collected, evaporated until KCl started to precipitate, then dialyzed against 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, further evaporated to a DNA concentration of 2 mg/mL, and redialyzed against the same buffer. The fragment was stored in this state at −20 °C. The total amount of 301-bp fragment purified in this step was 87.5 mg.

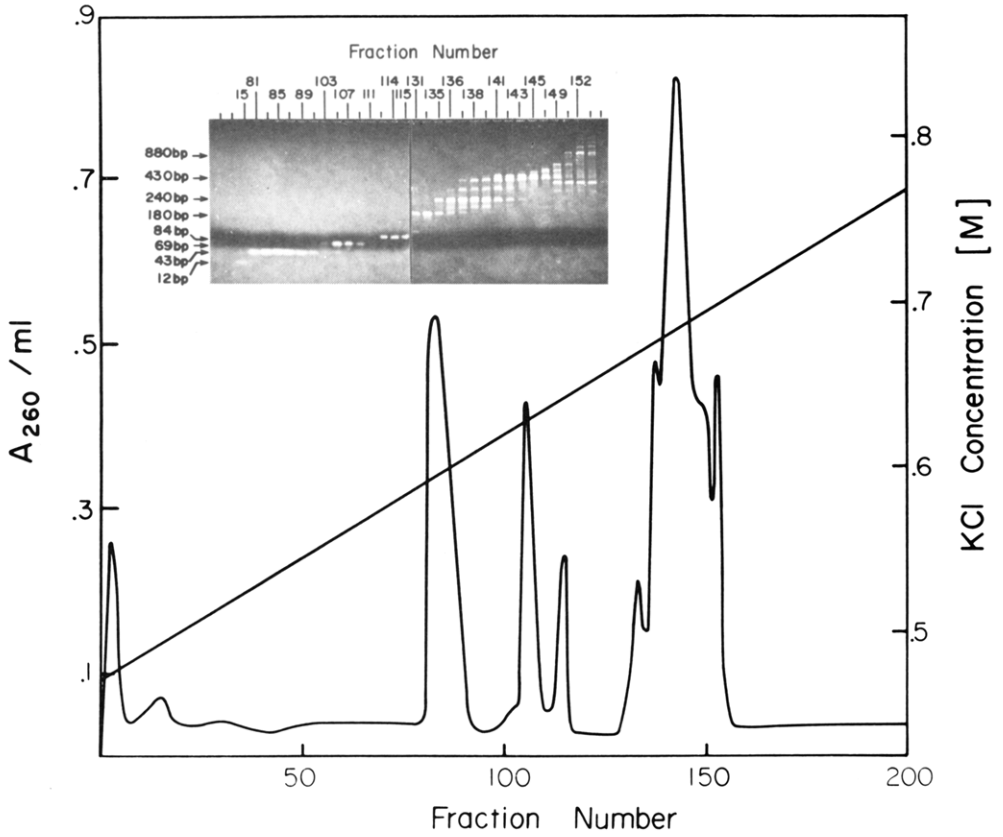

FIGURE 8: RPC-5 elution profile of the low molecular weight pool from the PEG fractionation of the *Eco*RI/*Hae*III digest of pVH51. DNA (8.2 mg) (lane B in Figure 6) was loaded on a 1 × 20 cm RPC-5 column which was thermostated at 45 °C. The column was developed with a linear 2-L gradient from 0.45 to 0.77 M KCl containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. The flow rate was 1 mL/min. The insert shows the analysis of some of the UV-absorbing fractions on 5% acrylamide gels. The broad dark band migrating approximately three-fourths of the distance down the gel is bromophenol blue. Less than 1 mol equiv of the 12-bp fragment was found; it is presumed that a partial loss of this fragment is incurred during the preliminary dialysis step.

pooled and worked up as described in Figure 7 for the 301-bp DNA. The yields of these fragments are summarized in Table I.

As a third source of small DNA restriction fragments, the 301-bp fragment was digested with different restriction enzymes (Figure 3). A *Hae*III/*Alu*I double digest gave 17-, 22-, 27-, 64-, 76-, and 95-bp fragments (Maxam & Gilbert, 1977). The separation of 12 mg of this reaction mixture on an RPC-5 column is shown in Figure 9. The peaks contain the respective DNA fragments in increasing size (gel analysis is not shown); each of these six fragments is pure (>98%).

In addition, an *Hpa*II reaction provided 80- and 219-bp fragments, and a *Hin*fI digest gave 101- and 188-bp fragments; these reaction products were separated on RPC-5 by using similar conditions as described above.

A list of the restriction fragments prepared by these methods together with their respective sources and yields are summarized in Table I. Analytical characterization of the 95- and 301-bp fragments on denaturing gels gave no indication of either depurination or nicking (Hardies & Wells, 1979) that might have occurred during purification.

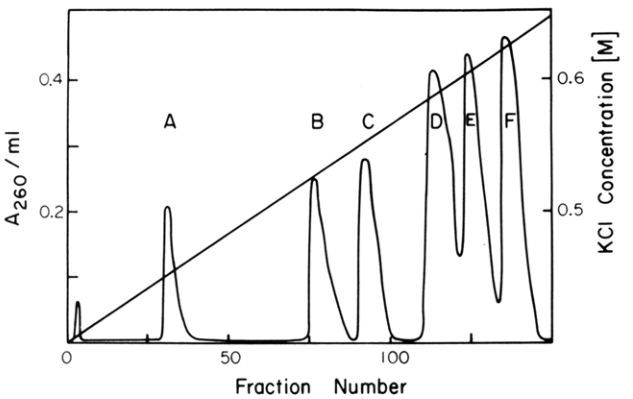

FIGURE 9: RPC-5 elution profile of the *Hae*III/*Alu*I double digest of the 301-bp fragment. DNA (12 mg) consisting of 17-, 22-, 27-, 64-, 76-, and 95-bp fragments was loaded onto a 1 × 25 cm RPC-5 column which was thermostated at 45 °C. The column was developed with a 2-L gradient from 0.4 to 0.75 M KCl containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Fractions of 9 mL were collected at a flow rate of 0.65 mL/min; 6% polyacrylamide gel analysis of the UV-absorbing fractions revealed that peaks A–F contained the pure fragments in the order of increasing size. The first small peak (fraction 3) does not contain DNA.

Discussion

A procedure which involves only methods applicable for large-scale preparations is described for the isolation of large amounts of DNA fragments from plasmids. Procedures that limit the amount of DNA are only used in the final step; all other steps are easily scaled up to multigram amounts. These methods may be applied to all plasmid DNAs from which fragments are to be prepared. However, they are especially powerful when used in conjunction with recombinant methods.

The purification of large amounts of 21 DNA restriction fragments, ranging in size from 12 to 880 bp, is described. All fragments were pure (less than 2% contamination by other fragments). The 95- and the 301-bp fragments which contain the *E. coli* lactose control region were cloned as multimers into pVH51 in up to four copies per plasmid; all inserts were separated by *Eco*RI sites. The presence of tandem copies increases the amount of inserted DNA from 2.5% to 9% for

the 95-bp fragment and from 7.3% to 24% for the 301-bp fragment of total plasmid DNA.

Ten other fragments were obtained by a *Hin*fI, *Hpa*II, or *Hae*III/*Alu*I double digest of the 301-bp fragment. HPLC on RPC-5 provided the necessary resolution to separate the subfragments. Nine other subfragments were prepared by fractionation of a *Hae*III digest of the pVH51 vector. In general, it is easier to prepare large amounts of a fragment by cloning and then separating the inserted fragment from the much larger vector than to achieve the necessary resolution on RPC-5 chromatography for a mixture of fragments of similar size.

Our cloning results confirm that only head to tail orientations are stable for the multiple inserts (Sadler et al., 1979; Hardies et al., 1979a; Hardies & Wells, 1979). In 50 independent multimeric clones that ranged from two to four copies per plasmid for the 95- and 301-bp *lac* fragments, not one case of a head to head or tail to tail configuration was found. In addition, when higher order 95-bp fragment multimers were used for cloning, a smaller number of clones with *lac* insertions were found. The reason why only head to tail orientations were found is unclear, especially since other work (Selsing & Wells, 1979; Gellert et al., 1978; Panayotatos & Wells, 1981) has shown the biological stability of sequences which have the potential to form cruciforms.

The amount of *lac* fragments prepared from 300-L cultures of these plasmids averaged 50 mg for the 95-bp fragment and 90 mg for the 301-bp fragment. Among the preparations carried out so far, the yield of plasmid DNA is higher for pRW554 compared to that for pRW574. The reason for this is not apparent, and the number of preparations may be insufficient to be statistically significant.

PEG precipitation (Lis & Schleif, 1975) was an important step for the large-scale initial separation of the small insert from the much larger vector as well as for the coarse fractionation of a mixture of fragments. The PEG precipitation of the vector from the 95- or 301-bp inserts was possible only in the presence of gelatin. If the gelatin was omitted, the small fragments with *Eco*RI sticky ends behaved as if they were much larger. It is possible that aggregation occurred via the four complementary bases of the single-stranded ends. A similar behavior was also found in several physical measurements requiring a high concentration of these fragments (unpublished observations). On the other hand, the separation of the blunt-ended fragments from the *Hae*III digest of pVH51 succeeded without gelatin (Figure 5). Therefore, it is possible that gelatin interfered with the aggregation of fragments via their sticky ends.

The separation of the *Hae*III digest of *Eco*RI-linearized pVH51 was optimized to prepare the smaller fragments (Hardies et al., 1979a) (up to 180 bp) in homogeneous form and high yield. The purification of the larger fragments (245–880 bp) was only attempted in amounts sufficient for melting and CD studies (Hillen & Wells, 1980).

The homogeneity of the fragments prepared by these procedures was demonstrated by polyacrylamide gel analysis. The analyses were performed so that approximately 2% of a contaminating fragment would have been detected. The UV spectra of the fragments showed A_{260}/A_{280} ratios of greater than 1.8 and A_{260}/A_{230} ratios of greater than 2.5. The absence of nicks or depurinated sites was confirmed by alkaline gel analyses; however, a small percentage of damaged fragments might not be detected. We have used fragments from different preparations for a variety of studies including the following: helix–coil transitions (Hardies et al., 1979b; W. Hillen, T. C. Goodman, A. Benight, R. M. Wartell, and R. D. Wells, unpublished results), circular dichroism (Hillen & Wells, 1980), proton and phosphorus NMR (Early et al., 1981a,b; P. A. Hart, W. Hillen, and R. D. Wells, unpublished results), laser Raman spectroscopy (R. M. Wartell, W. Hillen, and R. D. Wells, unpublished results), mass spectroscopy (J. R. Wiebers, W. Hillen, R. K. Patient, and R. D. Wells, unpublished results), analytical CsCl and Cs_2SO_4 buoyant density (Patient et al., 1979), partial denaturation mapping (Patient et al., 1979; B. Funnell, R. B. Inman, R. D. Klein, W. Hillen, and R. D. Wells, unpublished results), in vitro transcription studies (S. M. Stirdivant, R. D. Klein, and R. D. Wells, unpublished results; Horn & Wells, 1981), catabolite gene activating protein binding studies (S. M. Stirdivant, J. L. Krakow, and R. D. Wells, unpublished results), drug binding studies (G. D. Staffeld and R. D. Wells, unpublished results), and cleavage by "another" restriction endonuclease (Hardies & Wells, 1976; W. Hillen, R. D. Klein, and R. D. Wells, unpublished results). None of the results obtained by these different methods have indicated the presence of impurities or any damage of the DNA.

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A 300- and 600-MHz Proton Nuclear Magnetic Resonance Investigation of a 12 Base Pair Deoxyribonucleic Acid Restriction Fragment: Relaxation Behavior of the Low-Field Resonances in Water†

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ABSTRACT: High-resolution (300- and 600-MHz) proton NMR spectra and 300-MHz relaxation data on the exchangeable imino protons of a 12 base pair (bp) restriction fragment are presented. Analysis of these results permits conclusions on the nature of internal motions in this short DNA helix, the nature of the various interactions which are responsible for the observed relaxation rates, and the mechanism of opening of A·T base pairs. By combining information on the thermal stability and the chemical shifts of the resonances with rudimentary ring current shift calculations, all A·T and G·C resonances in the low-field spectrum can be identified and tentatively assigned to specific base pairs in the molecule. Spin–spin (R_2) and spin–lattice (R_1) relaxation rates of the low-field resonances have been measured at a number of temperatures by using the long-pulse method. The low-temperature relaxation behavior of the low-field resonances can be accounted for theoretically in terms of a model in which the molecule is treated as a rigid rotor and in which the relaxation is entirely attributed to proton–proton and proton–nitrogen dipolar interactions. At 21 °C, we find good quantitative agreement between theory and experiment, and various intercomparisons of the observed and calculated relaxation rates and relaxation rate ratios serve to test different aspects of the theory. Under these conditions, theory predicts that the proton–nitrogen dipolar interaction makes no contribution to the spin–lattice relaxation rates but does contribute significantly to the spin–spin relaxation rates. At higher temperatures, transient opening of the base pairs, with the onset of exchange with the solvent protons, introduces an additional pathway for relaxation. At 38 °C, the highest temperature studied, the exchange mechanism dominates the relaxation of the A·T resonances and is responsible for half of the R_1 observed for the G·C resonances. Both the chemical shift data and relaxation measurements indicate that the opening of the A·T base pairs is faster than that of the interior G·C base pairs. The exchange rates for all of the A·T resonances are faster than for neighboring G·C base pairs, indicating that at 38 °C the T imino proton exchange with solvent occurs *without* opening of the neighboring G·C pairs. This implies that the transient opening of A·T base pairs is independent of the DNA length and sequence for any A·T base pairs not located at or near (within 3 bp) the helix termini.

High-resolution proton NMR has been widely used to investigate the properties of DNA molecules in solution, but most of these studies have been limited to measurement of the standard NMR parameters of chemical shift, intensity, and line width (Patel, 1975, 1978; Patel & Hilbers, 1975; Patel & Canuel, 1976; Kearns, 1977; Selsing et al., 1978; Early et al., 1977; Early & Kearns, 1979; Kan et al., 1975; Rill et al., 1980). Such studies are consequently limited in terms of the detailed structural information they can provide because of uncertainties in the theories used to interpret chemical shifts, the conformational state of DNA in solution, and the role of conformational fluctuations. Proton relaxation measurements, although virtually unused in this area of research, appear to have great promise in providing the detailed information about DNA structure and conformational fluctuations now required (Bolton & James, 1980; Hogan & Jardetzky, 1980; Early et al., 1980a,b; Broido & Kearns, 1980). In those cases where dipole–dipole interactions dominate, the relaxation rates vary

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