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A short primer for sequencing DNA cloned in the single-stranded phage vector M13mp2

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ABSTRACT

In this paper we describe the synthesis and cloning of a short segment of DNA complementary to the region immediately adjacent to the EcoRI insertion site in the single-stranded bacteriophage vector M13mp2. This segment is useful as a "universal" primer for DNA sequencing by the dideoxynucleotide chain termination method; the template can be any DNA species cloned in M13mp2 or its derivatives. The primer has been cloned into the tetracycline resistance gene of plasmid pBR322 as one strand of a 26 bp EcoRI/BamHI fragment. This fragment may be readily prepared from an EcoRI + BamHI restriction digest of the parent plasmid (designated pSP14) by a simple size fractionation.

INTRODUCTION

DNA sequencing by the dideoxynucleotide chain termination method [1] generally requires the availability of single-stranded template and the preparation of a different primer for every 200-300 nucleotides of sequence generated. The requirement for template may be conveniently satisfied by cloning the DNA species of interest in a single-stranded phage vector such as M13mp2 [2-4]. Cloning can also reduce or eliminate the need for multiple distinct primer preparations since a single "universal" primer, prepared from the vector DNA flanking the insertion site, can be used to sequence up to several hundred nucleotides of any inserted DNA. This allows the sequence of a relatively large piece of DNA to be quickly obtained by fragmenting it into smaller pieces, cloning these fragments, sequencing the clones using exclusively a flanking universal primer, and then building up the final sequence from overlaps[1].

The 96 bp EcoRI fragment of M13mp2962 RFI DNA [5] has been used extensively in this laboratory as a source of primer for sequencing M13mp2 clones [4][1]. The only disadvantage of this primer is its excessive length, roughly three

[1] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A., submitted for publication.

to four times longer than is necessary for a stable, specific interaction with the template. Because the amount of sequence data that can be obtained from a single DNA sequencing run is limited mainly by the size resolution of the polyacrylamide gels, it is advantageous to use a shorter primer. In this paper we describe the synthesis and cloning of a 30-nucleotide-long primer for sequencing M13mp2 clones. Because of its small size this primer easily allows sequences greater than 300 nucleotides in length to be obtained from a single priming.

MATERIALS AND METHODS

Synthesis of nonanucleotides

Oligodeoxynucleotides pA-C-G-T-T-G-T-A-A (I) and pA-A-C-G-A-C-G-G-C (II) were chemically synthesised by the solid phase phosphodiester method previously described [6,7]. Final purification was by reversed-phase HPLC on μ-Bondapak C-18 (Waters Associates) [8]. Nonanucleotide I was dephosphorylated before use with bacterial alkaline phosphatase (Worthington) [9]. Nonanucleotide I is complementary to nucleotides 34-42 and nonanucleotide II to nucleotides 25-33 of the *E. coli* β-galactosidase gene [2-4].

Construction of primer fragment

Single-stranded DNA complementary to the two nonanucleotides was obtained by digesting 0.02 μg of the M13mp2 962 bp *Eco*RI fragment [5] with two units of exonuclease III (New England Biolabs) for 90 min at 15°C in 70 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 1 mM DTT, then heating for 3 min at 100°C. This was annealed with 30 ng of dephosphorylated nonanucleotide I and 15 ng of nonanucleotide II for 5 min at 37°C, then incubated for 12 hr at 4°C with 0.6 units of T4 DNA ligase in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM rATP (5 μl final volume) to join the two nonanucleotides. The ligase was inactivated, after the addition of 1 μl of 1.0 M NaCl and 0.5 μl of 1.0 M Tris-HCl (pH 7.8), by heating for 3 min at 100°C; the mixture was then slowly cooled to 4° to allow reannealing of the joined nonanucleotides to the complementary strand. "Filling in" of the primer strand and "trimming back" of the 3' end of the complementary strand was accomplished by adding 3 μl of a solution containing 4 μM [α-^{32}P]dGTP (Amersham; specific radioactivity 400 Ci/mmole) and 167 μM each of dATP, dCTP and dTTP (P-L Biochemicals), followed by 0.5 μl (0.1 units) of *E. coli* DNA polymerase I Klenow fragment (Boehringer-Mannheim). The mixture was incubated for 5 min at 18°C, "chased" for 5 min at 18°C with 1 μl of 0.5 mM dGTP, then the reaction was terminated with 4.5 μl of 1.5% Sarkosyl, 30 mM EDTA. The terminated reaction mixture

was extracted with 0.5 vol of phenol plus 0.5 vol of 24:1 (v/v) chloroform: isoamyl alcohol, and with 10 vol of diethyl ether, then the DNA was precipitated with ethanol and redissolved in 20 µl of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA.

Cloning of the primer fragment

In order to phosphorylate the 5' end of the primer strand (contributed by nonanucleotide I) a 2 µl aliquot of the DNA was incubated with five units of T4 polynucleotide kinase (P-L Biochemicals) in 66 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 16.6 mM DTT, 1 mM spermidine, 350 µM rATP (final volume 10 µl) for 30 min at 37°C [10]; 0.05 µg of BamHI "linkers" (sequence C-C-G-G-A-T-C-C-G-G; Collaborative Research) were similarly treated. The phosphorylated DNA and BamHI linkers were then combined, mixed with 2 µl of fresh 10 mM rATP, and incubated with six units of T4 DNA ligase for 3 hr at 15°C [10]. The ligation reaction was terminated by the addition of 5 µl of 100 mM EDTA, extracted with phenol plus chloroform:isoamyl alcohol and with ether, as described above, then precipitated with ethanol and redissolved in 6 µl of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA. The DNA and linkers were initially digested with 2.5 units of EcoRI (New England Biolabs) in 100 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$ (final volume 10 µl) for 30 min at 37°C, then the digestion was completed by adding 2 µl of 30 mM $MgCl_2$, 2 µl of 1.0 M NaCl and 20 units of BamHI (New England Biolabs) and incubating for an additional 2 hr at 37°C. After adding excess EDTA to chelate the Mg^{++} the digestion mixture was extracted with phenol plus chloroform:isoamyl alcohol, then electrophoresed on a 16% polyacrylamide "thin" gel [11] for 12 hr at 400 V (4 mA) to separate the primer DNA fragment from the digested linkers. The region of the gel containing the primer fragment was cut out and the DNA eluted by soaking the gel piece for 16 hr at 37°C in 5 vol of 10 mM Tris (pH 7.8), 1 mM EDTA, 100 mM NaCl; E. coli tRNA (20 µg) was added as carrier then the eluted DNA was concentrated by two ethanol precipitations and redissolved in 10 µl of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA.

Closed circular DNA from plasmid pBR322 [12] was digested with EcoRI plus BamHI, and the large fragment (3980 bp) was isolated by neutral sucrose gradient sedimentation. A 70 ng sample of this was combined with 3 µl of the concentrated gel eluate and the mixture heated for 2 min at 45°C to denature any pre-existing base-paired termini, then the DNA was cooled and incubated with one unit of T4 DNA ligase in a buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM rATP (20 µl final volume) for 20 hr at 4°C. The ligation reaction was stopped by the addition of 4 µl of 100 mM EDTA, and

half of the resulting mixture was used to transform strain HB101 (F^-, r_k^-, m_k^-, recA) of E. coli [13]. Transformants were selected on YT plates [14] containing ampicillin (15 μg/ml), then replica-plated onto plates containing ampicillin and tetracycline (10 μg/ml). Of the 52 clones tested 13 were found to be tetracycline-sensitive; single-colony lysates of these were analysed by agarose gel electrophoresis [15], revealing three clones that harboured plasmids approximately 90% of the size of pBR322. Deproteinised cleared lysates were prepared from 10 ml cultures of these three clones [16], aliquots of the DNA digested with EcoRI and BamHI, and the resulting restriction fragments end-labelled by filling-in using [α-^{32}P]dATP and E. coli DNA polymerase I Klenow fragment (see above). Subsequent analysis of the DNA by electrophoresis on a 12% polyacrylamide gel showed that two of the plasmids contained a small EcoRI/BamHI fragment of the expected size (26 bp) for the primer fragment. One of these (pSP14) was chosen for further characterisation.

Preparation of pSP14 DNA

An inoculum was prepared by plating E. coli carrying pSP14 onto a plate of solid YT medium containing 25 μg/ml ampicillin, then resuspending the resultant colonies in 2 ml of 2 x YT medium. This was added to one litre of 2 x YT medium in a two litre flask and the cells were shaken at 37°C until they had grown to a density of approximately 0.8 A_{620} units; 150 mg of chloramphenicol was then added and the culture was incubated for an additional 16 hr at 37°C. Cells were harvested and a cleared lysate prepared as described by Clewell and Helinski [16], except 0.1% Triton X-100 was substituted for the 1.0% Brij 58, 0.4% sodium deoxycholate. The cleared lysate (volume approximately 40 ml) was adjusted to 0.1 M NaCl and 0.2% Sarkosyl then incubated for 60 min at 37°C with 4 mg of RNAaseA (Worthington; from a 5 mg/ml stock solution in 0.1 M sodium acetate, pH 5.5, previously heat-treated for 10 min at 100°C). Then 1.0 ml of 1 mg/ml proteinase K (BDH Biochemicals) was added and the incubation continued for 90 min at 55°C. After two extractions with one-half volume phenol (equilibrated with 0.2 M Tris-HCl, pH 7.8) plus one-half volume chloroform:isoamyl alcohol (24:1) the DNA was precipitated with ethanol and redissolved in 15 ml of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA. Then 15.4 gm of CsCl and 0.4 ml of 5 mg/ml ethidium bromide were added to the DNA solution and it was centrifuged at 38,000 rpm for 44 hr at 18°C in a Beckman 50Ti rotor using two polyallomer tubes. The prominent lower band (clearly visible when illuminated by a longwave UV light source) was collected by opening each tube, puncturing the side with a 19 gauge needle, and drawing

out approximately 1 ml of solution with a syringe. The ethidium bromide was removed by extracting the DNA four times with an equal volume of isopropanol, then the DNA was freed of residual oligoribonucleotides and CsCl by passing the sample over a 30 ml column of Sephacryl S-300 (Pharmacia) or Ultrogel AcA34 (LKB) equilibrated with 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 300 mM NaCl. Finally, the purified DNA was concentrated by ethanol precipitation and redissolved to a final concentration of 1 mg/ml in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 10 mM NaCl. The final yield from a one litre culture was typically 0.5-1.0 mg of covalently-closed circular pSP14 DNA.

Sequence analysis of pSP14

The portion of pSP14 representing the inserted synthetic DNA fragment was sequenced by the dideoxynucleotide chain termination method [1]. Template was prepared by cutting the pSP14 DNA at the unique SalI site present in the pBR322 moiety [12] then digesting the resulting linear molecule with exonuclease III [17]; an 85 bp pBR322 HhaI/HaeIII restriction fragment, mapping immediately to the left of the EcoRI site [12,18] was used as a source of primer.

Preparation of primer

A 200 μg sample of purified pSP14 DNA in 2 ml of 10 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 50 mM NaCl, was treated with 50 units of EcoRI or BamHI (New England Biolabs) for 2 hr at 37°C. Each set of products was checked by electrophoresis on a 1% agarose gel to verify that at least 95% of the circular DNA molecules had been linearised, then EcoRI was added to the BamHI digest, and BamHI to the EcoRI digest, followed by an additional incubation for 2 hr at 37°C to completely release the primer fragment. The two digests were pooled, mixed with six volumes of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.4 M NaCl, and loaded onto a 15 x 0.4 cm (2.0 ml bed volume) RPC-5 column equilibrated with the same buffer. The DNA was eluted from the column with a 40 ml gradient in which the NaCl concentration of the buffer increased linearly from 0.4 to 0.8 M. A flow rate of approximately 0.5 ml per minute was maintained by a Milton Roy "miniPump" operating at a pressure of 200-400 psi; fractions were monitored for absorbance at 260 nm using a flow cell. Under these conditions an excellent separation of the small primer fragment from the bulk of the plasmid DNA was achieved. Fractions containing the primer fragment were pooled, mixed with 3 vol of ethanol, and stored overnight at -20°C. The DNA was pelleted by centrifugation in a Beckman SW60 rotor at 45,000 rpm for 1 hr at 0°C, then redissolved in 0.4 ml of 10 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 5 mM NaCl. The primer fragment solution was divided into

aliquots which were stored frozen at -20°C in siliconised glass tubes.

Sequencing M13mp2 clones using the primer fragment

A 2 µl portion of the primer fragment solution, prepared as described above, was sufficient to prime a set of four dideoxynucleotide sequencing reactions using 2 µg of purified DNA from an M13mp2 clone as template [1,4]. Sequencing reactions were carried out as previously described [1,4] except for a modification in the annealing procedure. The primer fragment and the M13mp2 template, in 10 µl of 7 mM Tris-HCl (pH 7.8), 6.6 mM $MgCl_2$, 50 mM NaCl, were denatured in a sealed capillary by heating for 2 min at 100°C, then the capillary was immersed in approximately 5 ml of hot (85-90°C) water in a small test tube. The primer was annealed by allowing the test tube to cool slowly (15-30 min) to room temperature on the bench.

Materials

Dideoxynucleoside triphosphates were purchased from P-L Biochemicals or Collaborative Research. T4 DNA ligase and RPC-5 were gifts from Dr. A.R. MacLeod and Dr. B.A. Roe, respectively. M13mp2962 was a gift from Dr. P.H. Schreier. An amber derivative of M13mWJ22 and pBR322 DNA were gifts from Dr. G.P. Winter.

RESULTS AND DISCUSSION

Synthesis of a short primer

The ideal universal primer for sequencing DNA cloned into the EcoRI site of the single-stranded phage vector M13mp2 should prime very close to the insertion site and have the minimum size required for stable and specific duplex formation with the template (approximately 20-30 nucleotides). Since no restriction fragments meeting these criteria could be easily prepared from M13mp2 RFI DNA we decided to construct and clone a fragment of DNA with the appropriate properties. As described in detail below, this task was simplified by taking advantage of the ability of E. coli DNA polymerase I Klenow fragment to process a partially duplex DNA fragment, by simultaneously "filling-in" and "trimming-back" 5'- and 3'-terminal overhangs, respectively, to produce a fully duplex, flush-ended fragment.

The EcoRI site in M13mp2 RFI DNA occurs 15 nucleotides from the beginning of the β-galactosidase (lacZ) gene [2-4]. The sense strand of the gene is in the plus strand of the vector, so primed DNA synthesis across the EcoRI site requires that the primer (minus strand sense) be complementary to that portion of the lacZ gene distal to the EcoRI site. Accordingly, an oligodeoxynucleotide nine residues long (nonanucleotide I), complementary to

nucleotides 34-42 of the lacZ gene, was made by the solid phase phosphodiester method [6,7]. This oligonucleotide proved to have limited stability and poor specificity in its interaction with the template, possibly due to its high A+T content, so it was lengthened by the attachment of a second nonanucleotide (II) complementary to positions 25-33 of the lacZ gene. Using the exonuclease III-treated 96 bp EcoRI fragment from M13mp2962 [5] as a source of plus strand DNA (Fig. 1), the two nonanucleotides were annealed to the two contiguous complementary regions and joined by T4 DNA ligase. This 18 bp partially duplex structure was then treated with E. coli DNA polymerase I Klenow fragment in the presence of the four dNTPs. The polymerase extended the joined nonanucleotides (minus strand) to produce a filled-in EcoRI site and simultaneously trimmed back the overhanging 3'-terminus (plus strand) to produce a flush-end at the 5'-terminus of nonanucleotide I (Fig.1).

BamHI "linker" oligomers (sequence C-C-G-G-A-T-C-C-G-G) were attached to the resultant 27 bp fully duplex DNA fragment via blunt-end ligation with T4 DNA ligase. Addition of the BamHI linkers regenerated the EcoRI site at the right-hand end of the fragment (Fig. 1). Thus, sequential EcoRI and BamHI digestions removed the linkers and left a 26 bp BamHI/EcoRI fragment containing a primer complementary to nucleotides 20-43 of the lacZ gene. This fragment was cloned into the tetracycline resistance region of plasmid pBR322 [12] (see "Materials and Methods"), yielding a plasmid, designated pSP14 (Fig. 2A), that conferred ampicillin resistance on the host strain and could be amplified by chloramphenicol treatment.

The predicted sequence of the primer was verified by the dideoxynucleotide chain termination method [1]. Purified pSP14 DNA was cut with SalI then digested with exonuclease III to provide template [17], and a HhaI/HaeIII restriction fragment from pBR322 was used as a source of primer. The sequence of the region of pSP14 containing the primer fragment is shown in Figure 2B.

Preparation of the primer

An EcoRI + BamHI double digestion is required to release the primer fragment from the plasmid[1]. In order to avoid wasting enzyme during large scale primer preparations, it is advisable to do a series of small scale digestions to find the minimum amount of EcoRI or BamHI required to linearise 95% or

[1] A 27 bp derivative of the primer fragment has recently been constructed (S. Anderson, unpublished) and inserted into the chloramphenicol resistance gene of plasmid pACYC184 [19]. Release of this primer fragment from the parent plasmid requires an EcoRI digestion only.

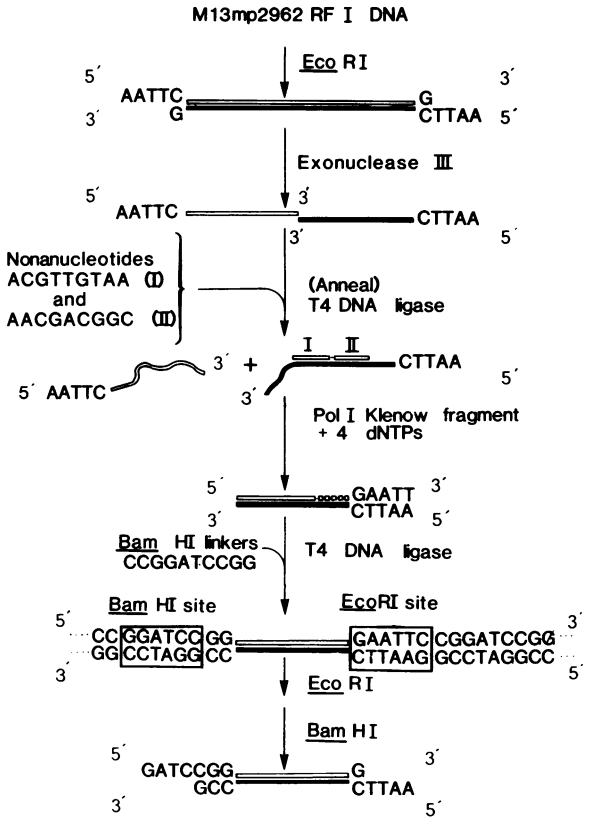

Figure 1. Construction of the primer fragment. A schematic pathway shows the construction of the primer fragment starting with the 96 bp EcoRI fragment from M13mp2962 and two synthetic nonanucleotides. The solid bars represent DNA from the sense strand of the lacZ gene (plus strand of vector); the open bars represent DNA from the anti-sense strand. The "right-hand" EcoRI site represents the normal insertion site for the DNA cloned in the vector M13mp2.

more of the plasmid. The primer fragment may be conveniently labelled after the digestion by treating an aliquot of the DNA with [γ-^{32}P]rATP and polynucleotide kinase [20,21]. If enough DNA is present in the preparation the primer can be followed by absorbance at 260 nm or ethidium bromide staining. However some label in the primer makes it easier to monitor recoveries during the ethanol precipitation steps.

The small size of the primer fragment relative to the large EcoRI/BamHI vector fragment contributes greatly to the ease of its purification. For

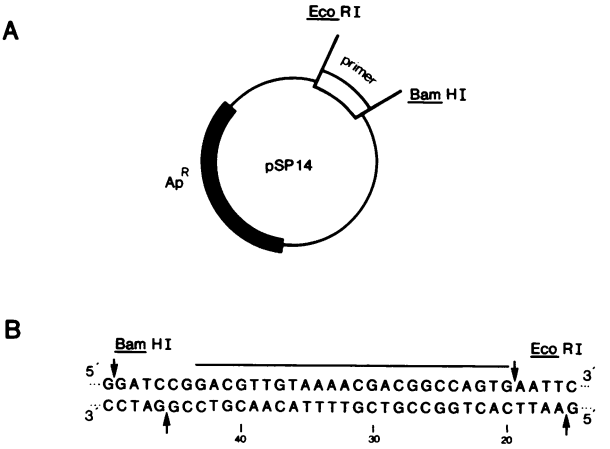

Figure 2. Structure of the cloned primer fragment. A: Plasmid pSP14 consists of the 3980 bp EcoRI/BamHI fragment from pBP322 (carrying the ampicillin resistance gene) joined to the primer fragment. B: The sequence of the 26 bp primer fragment is shown. The overlined portion represents that part which is complementary to the M13mp2 template; the numbers indicate base positions in the lacZ gene. Some of the 5'-terminal nucleotides of the primer (contributed by the BamHI linker) will be mismatched when it is annealed to the template, but this does not affect its performance as a primer for DNA sequencing.

large scale preparations we have routinely used RPC-5 HPLC to separate the primer fragment from the bulk of the plasmid DNA (Fig. 3) [22,23]. Digests containing 0.5 mg of DNA have been chromatographed with no loss of resolution, therefore it is likely that under the conditions given in "Materials and Methods" several mg of DNA could be successfully fractionated in a single run. For smaller scale preparations we have also used 10% polyacrylamide "thin" gels [11] to purify the primer fragment; approximately 5 μg of digested plasmid per cm of slot width can be run. Lower resolution size fractionations using gel filtration or neutral sucrose gradient sedimentation would probably also yield primer fragment preparations of sufficient purity, although these methods have not been tested.

Sequencing M13mp2 clones

The utility of the primer described in this paper derives mainly from its small size. Compared to the previously described primer from the 96 bp M13mp2962 EcoRI fragment [4,5] it allows an additional 70 residues of useful sequence information to be obtained from a single set of sequencing reactions. The T_m of the primer has not been measured, but the annealing

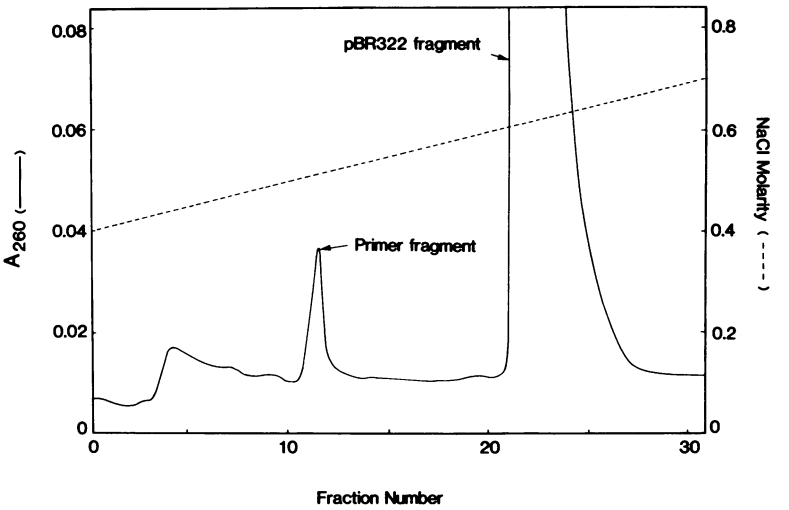

Figure 3. Purification of the primer fragment by RPC-5 chromatography. Purified pSP14 DNA (250 μg) was digested with EcoRI plus BamHI then chromatographed on an RPC-5 column as described in "Materials and Methods". UV absorbance of the eluate, with the baseline arbitrarily set at 0.01 A_{260} units, was continually monitored using a flow cell; fraction volume was approximately 1 ml. The trough and peak at the beginning of the gradient are characteristics of the system and are not due to UV-absorbing impurities in the sample.

procedure, in which the primer and template are allowed to cool slowly to room temperature after denaturation at 100°C ("Materials and Methods"), works well. Because the renaturation reaction is relatively fast under these conditions, slow cooling of the DNA subjects it to highly stringent annealing conditions as it passes through its T_m.

A typical dideoxy sequencing run using the short primer is shown in Figure 4. The dark band at the bottom of the gel is due to filling-in of the reannealed primer fragment. We recommend that [α-^{32}P]dCTP be used to label the DNA in sequencing reactions that employ the short primer (Fig. 4); other labelled nucleotides (e.g. [α-^{32}P]dATP) yield excellent sequence, but their use may result in the appearance of a strong artifact band that obscures the first three to four residues above the filled-in primer band (not shown). The origin of this artifact band has not been established, but it is probably due to hairpin formation and self-priming by the unannealed strand complementary to the primer.

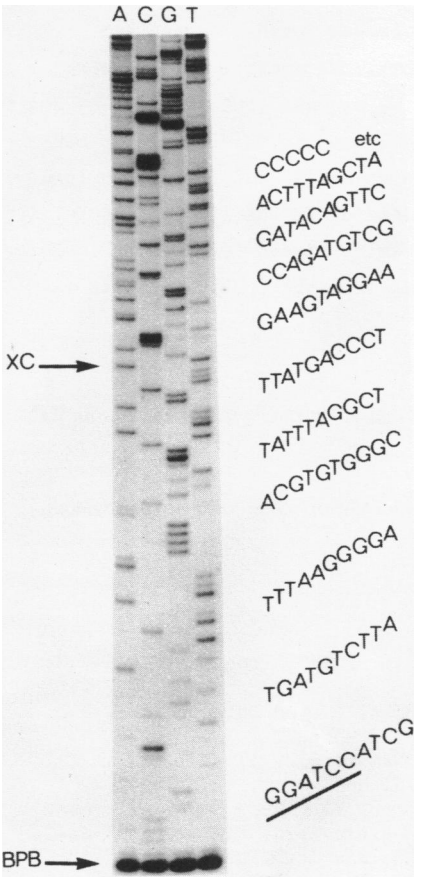

Figure 4. DNA sequencing using the short primer. Shown is an autoradiograph of an 8% polyacrylamide-7 M urea DNA sequencing gel giving the 5'-end H-strand sequence of the MboI fragment 5 from human mitochondrial DNA. This fragment was inserted into an amber derivative of the M13mWJ22 vector [24], and one of the resulting clones provided template for dideoxynucleotide chain termination sequencing using the short primer. All sequencing sections (vol 4 µl) contained [α-^{32}P]dCTP (specific radioactivity 400 Ci/mmole) at a concentration of 0.8 µM. Unlabelled nucleotide concentrations were as follows: A-reaction, 2 µM dATP and 133 µM ddATP; C-reaction, 40 µM ddCTP; G-reaction, 2 µM dGTP and 110 µM ddGTP; T-reaction, 2 µM dTTP and 133 µM ddTTP; except as noted, all other deoxynucleoside triphosphates were present in the reactions at a concentration of 50 µM. Reactions were "chased" by the addition of 1 µl of a solution containing all four dNTPs, each at a concentration of 45 µM. A faint artifact band, the source of which is uncertain, runs just behind the primer band in each track. The BamHI insertion site (underlined) in this vector [24] is separated from the EcoRI site by three C residues. Arrows denote the positions of the xylene cyanol (XC) and bromphenol blue (BPB) tracking dyes.

Other primers

Besides M13mp2 and its derivatives other single-stranded phage vectors exist or are under development [25-32]. Because of the relative ease with which short oligodeoxynucleotides may be made by modern solid phase organic synthesis techniques [6,7,33], the methods described in this paper may be generally applicable to the construction of optimal DNA sequencing primers for these other vectors. There are two advantages of this approach over the totally synthetic route to the production of primers. First, the method allows the construction of a primer having a length in excess of that which is conveniently attainable using existing techniques of organic synthesis. Second, because the primer is cloned it may be readily and inexpensively prepared whenever needed using commonly employed biochemical procedures.

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