A PRIMED-SYNTHESIS METHOD FOR RIBOSUBSTITUTION OF DNA AT A SINGLE SITE

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1. Introduction

A number of techniques are now available for the sequence analysis of DNA. Several of these depend on primed synthesis of DNA using DNA polymerase I to make a radioactive copy of a single-stranded template DNA [1–4]. The rapid 'plus and minus', partial ribosubstitution and chain-termination inhibitor methods for sequencing DNA [1–3] depend on the size analysis of radioactive copy DNA (cDNA) fragments, the 5′-termini of which are identical and the 3′-termini of which are generated in base-specific reactions. DNA fragments generated by a restriction enzyme are commonly used as primers for the synthesis of the cDNA, and the same restriction enzyme is normally used to remove the primer and generate the 5′-termini of the cDNA fragments (the datum restriction enzyme). However, some restriction enzymes are strongly inhibited by the single-stranded DNA present in uncopied regions of the template, and cannot be used to cleave at the priming site (e.g., *Alu* I [5] and *Hph* I, N.L.B. and M. Smith, unpublished observations). Even if the enzyme used is not inhibited by single-stranded DNA, there may be a second cleavage site for the same enzyme within the sequence copied into radioactive DNA. Two sets of fragments, with two different 5′-termini, would be generated by the datum restriction enzyme and a unique DNA sequence would not be obtained.

In this paper a modification of the 'plus and minus' method [1] is described which circumvents the problems arising when the restriction enzyme used to remove the primer DNA fragment is inhibited by single-stranded DNA, or has a second cleavage site in the cDNA sequence. This method involves the introduction of one or more ribonucleotides between the DNA primer and the radioactive cDNA. This site is then susceptible to cleavage with ribonuclease or alkali. This method can be used in conjunction with other rapid methods for DNA sequencing by primed-synthesis [2,3] and may have applications in fields other than DNA sequence analysis.

2. Materials

Ribonucleoside triphosphates and deoxyribonucleoside triphosphates were obtained from P-L Laboratories Ltd. [α-^{32}P]Deoxyribonucleoside triphosphates were obtained from New England Nuclear, Boston, U.S.A. or the Radiochemical Centre, Amersham, U.K. ϕX174*am*3 viral and replicative form (RFI) DNA preparations were gifts of Dr C. A. Hutchison III. Restriction fragment DNA primers were purified electrophoretically as described by Galibert et al. [6]. Pancreatic ribonuclease A and *E. coli* DNA polymerase I (nach Klenow) were obtained from Boehringer Corporation Ltd., U.K. Pancreatic ribonuclease A was dissolved (10 mg/ml) in 5 mM EDTA, 50 mM Tris–HCl, pH 7.4, heated at 80°C for 10 min to inactivate any contaminating

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As this paper is concerned with a technique primarily used for DNA sequence analysis, ribonucleotides are represented by e.g. rC; rCTP etc. Deoxyribonucleotides are not prefixed in the sequences of restriction enzyme sites, which are written 5′ to 3′, the complementary strand sequence being omitted.

deoxyribonucleases, and stored frozen in small aliquots. T4 DNA polymerase was a gift of Dr R. Kamen. Restriction enzymes were prepared by methods similar to those described by Roberts et al. [7].

3. Methods

3.1. *Incorporation of the ribonucleotide and further extension with deoxyribonucleotides*

The principle of the single-site ribosubstitution method is shown in fig.1. In the presence of Mn^{2+} *E. coli* DNA polymerase I will incorporate ribonucleotides into DNA [8]. In the single-site ribosubstitution reaction a ribonucleotide is incorporated at the 3′-end of a DNA primer in the presence of Mn^{2+}, and with no other triphosphates present. Further ribonucleotide incorporation is suppressed during the subsequent extension reaction by the addition of excess deoxyribonucleotide and Mg^{2+}. The procedure described in detail here was used for the incorporation of cytosine ribonucleotide at *Alu* I (AG↓CT) or *Hae* III (GG↓CC) priming sites.

A ϕX174 DNA restriction fragment (as primer; approx. 1.5 pmol in water) and ϕX174 viral DNA (as template; approx. 0.4 pmol in water) were added to annealing buffer (2.5 μl; 500 mM NaCl, 10 mM β-mercaptoethanol, 200 mM Tris–HCl, pH 7.4) and the volume adjusted to 15 μl. The solution was heated at 100°C for 3 min in a sealed capillary tube, then incubated at 67°C for 30 min to anneal the primer to the template DNA. The solution was cooled to 0°C, $MnCl_2$ (2.5 μl; 10 mM), rCTP (0.5 μl; 10 mM) and water (6.5 μl) were added. The reaction was started by the addition of 2 units of DNA polymerase I (nach Klenow), and the mixture incubated at 0°C for 5 min. An equal volume of 'Flood mix' (20 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1 mM dTPP, 0.1 mM dGTP, 0.3 mM dCTP, 0.004 mM [α-^{32}P]dATP, approx 20 μCi; pre-equilibrated to 0°C) was then rapidly added with mixing. One-third of the reaction mixture was taken after 1 min and quenched in EDTA (5 μl; 500 mM). Further aliquots were taken at 3 min and 10 min and quenched in the same solution. This procedure typically generates a series of extension products in which between 10 and 100 nucleotides have been added to the DNA primer.

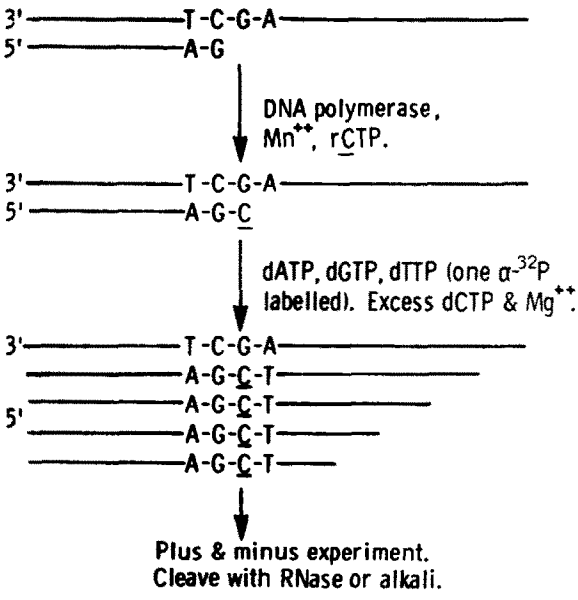


Fig.1. The principle of single-site ribosubstitution, using an *Alu* I fragment as primer.

3.2. *The 'plus and minus' experiment*

DNA polymerase and the unincorporated nucleoside triphosphates were removed from the reaction mixture by phenol extraction and chromatography on Sephadex G-100 [1,9]. The radioactive polynucleotide was lyophilized and taken up in water (25-30 μl). Aliquots (2 μl) of the radioactive polynucleotide were treated with T4 DNA polymerase and one deoxyribonucleoside triphosphate (the 'plus' experiment) or DNA polymerase I (nach Klenow) and three deoxyribonucleoside triphosphates (the 'minus' experiment) as described previously [1,9]. The plus and minus incubations were stopped by heating to 100°C, or with alkali (see below).

3.3. *Cleavage at the ribosubstituted site*

(i) Cleavage with pancreatic ribonuclease: The eight plus and minus samples and an aliquot (6 μl) of the original radioactive extension product (the 'zero' sample: see ref. 9) were sealed in capillary tubes. Each tube was heated at 100°C for 3 min then rapidly cooled in an ice-water bath to denature the DNA. Ribonuclease A (1 μl; 10 mg/ml in 5 mM EDTA, 50 mM Tris–HCl, pH 7.4) was added to each

tube and the samples were incubated at 37°C for 30 min. Formamide loading solution (10 μl; 0.02% bromophenol blue, 0.02% xylene cyanol FF, 0.25 M EDTA in 90% (v/v) deionized formamide was then mixed with each sample. The samples were heated at 90°C for 3 min and immediately loaded onto an analytical acrylamide-urea gel for sequence analysis as described previously [1,9].

(ii) Cleavage with alkali: Sodium hydroxide (1 μl; 2 M) was added to each plus and minus sample and to the 'zero' sample. Each sample was sealed in a capillary tube and incubated at 100°C for 20 min. Samples which were to be analysed immediately by gel electrophoresis were mixed with formamide loading solution (10 μl) and the mixture was applied to the analytical gel. If the samples were not to be analysed immediately, 2 M acetic acid (1 μl) and formamide loading solution (10 μl) were added to each sample. The samples were then heated at 90°C for 3 min immediately before loading the gel.

4. Results and discussion

The result of a 'plus and minus' sequencing experiment using the single-site ribosubstitution technique on φX174 *Alu* I fragment 5 (see ref. 10) annealed to viral strand DNA as template is shown in fig.2. This experiment allowed the sequence of 57 nucleotides starting 12 nucleotides from the priming site to be determined. No detectable radioactivity was found at the top of the gel indicating that all the extended polynucleotide product was susceptible to alkali cleavage, and therefore contained cytosine ribonucleotide at the priming site. In this experiment extended polynucleotide products up to 75 nucleotides from the priming site are detectable. In general the extension reaction goes a shorter distance in the single-site ribosubstitution procedure than in the normal 'plus and minus' procedure. The reason for this is not known, but could be due to an inhibitory effect of rCTP or Mn^{2+} on DNA polymerase I in the presence of Mg^{2+}.

An alternative single-site ribosubstitution procedure for use with *Alu* I fragments as primers is to perform the initial ribosubstitution reaction in the presence of rCTP and [α-^{32}P]dTTP. A single cytosine ribonucleotide and radioactive thymine deoxyribonucleotide are added to the priming fragment. The product can then be purified by phenol extraction and Sephadex G-100 chromatography [1,9] prior to further extension. The incorporated [α-^{32}P]dTTP allows simple detection of the product on Sephadex G-100 chromatography. This modification has proved particularly useful in primed synthesis methods of DNA sequencing using chain-terminating compounds [3]. This modification is not applicable to all priming fragments.

The advantage of single-site ribosubstitution for DNA sequence analysis by primed-synthesis in a region of DNA containing two sites for the restriction enzyme used to generate the primer is shown in fig.3. An intense band is seen in channels *a, b* and *c* which is due to cleavage by *Hae* III both at the priming site (Z3/Z10; ref. 10) and at a second site (Z10/Z9; ref. 10) which is 72 nucleotides away from the priming site. Several bands are seen in channels *b* and *c* which do not correspond to bands of similar mobility in channels *d* and *e*. These additional bands are presumably generated by cleavage of the longer products of the -C and -T reactions at the Z10/Z9 site. A unique set of bands are seen in channels, *d, e* and *f*, which on a full sequencing gel would allow a sequence of approximately 125 nucleotides, starting 12 nucleotides from the priming site, to be determined.

In this and other experiments (not shown) the relative mobilities of bands in the reactions where cleavage was with ribonuclease A, and those where cleavage was with *Hae* III, show that both cytosine nucleotides in the *Hae* III site are fully ribosubstituted during the initial ribosubstitution reaction. It is of interest that *Hae* III can recognise and cleave at this partially ribosubstituted site.

Other experiments (data not shown) have demonstrated that rGTP, rATP and rUTP can also be incorporated at a single site, under the same conditions as

Fig.2. Autoradiograph from a 'plus and minus' DNA sequencing experiment, after single-site ribosubstitution with rCTP using *Alu* I fragment 5 as primer on φX174 viral strand DNA as template.

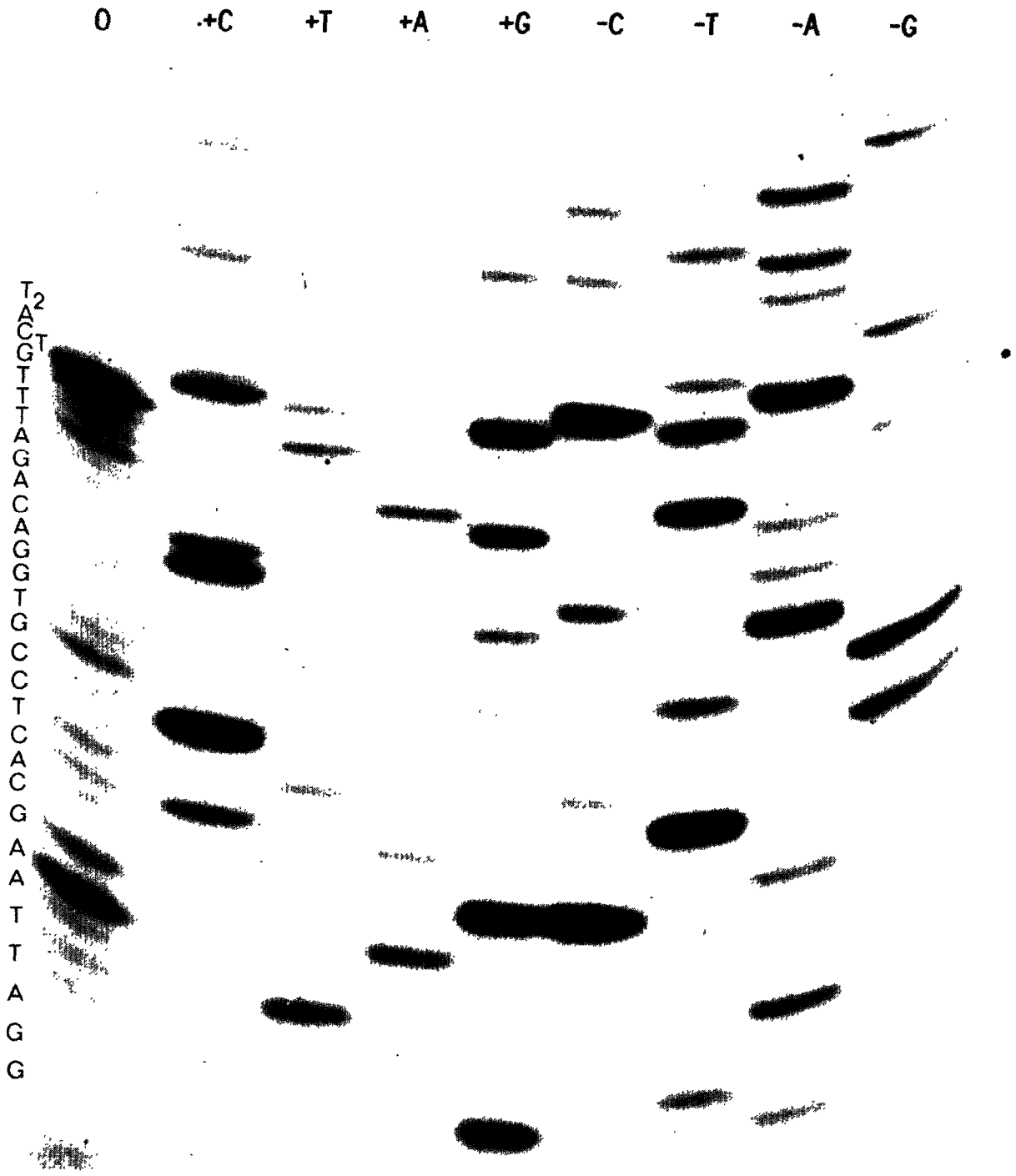
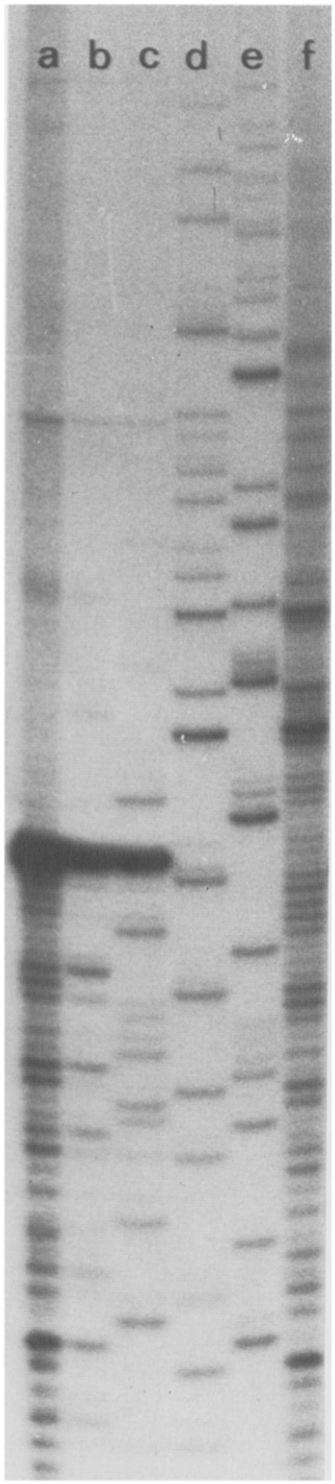


Fig.2



used for rCTP incorporation. rGTP was incorporated in a *Hind* II site (GTPy$^{\downarrow}$PuAC, where Pu was G); rATP was incorporated at a *Hinf* I site (G$^{\downarrow}$ANTC); and rUTP was incorporated at an *Alu* I site (AG$^{\downarrow}$CT) in the presence of dCTP.

Ribonuclease T_1 will cleave at a single rG substitution, pancreatic ribonuclease A will cleave at a single rU or rC substitution. Both rCTP and rGTP gave full ribosubstitution at a single site. rATP gave less than 100% ribosubstitution, but this may have been due to exonuclease activity in the *Hinf* I preparation used to generate the primer fragment. rUTP was the least efficiently incorporated at a single site (approximately 30% incorporation, as estimated from the autoradiographic intensities of bands in cleaved and uncleaved products after alkali treatment and gel electrophoresis). The use of pH 9.1 buffer instead of pH 7.5 buffer [11] did not significantly increase the amount of rUTP incorporation. Due to its limited applicability to DNA sequence analysis in this laboratory no further attempt to optimise rUTP incorporation at a single site was made.

For the restriction enzymes whose cleavage sites have been sequenced, single-site ribosubstitution with rCTP has the widest applicability. Some enzymes do not cleave within their recognition sequences (e.g. *Hph* I [12], *Hga* I [13] and *Mbo* II [14]). The correct ribonucleotide to use in single-site ribosubstitution experiments at priming sites generated by such enzymes must be determined empirically, or the DNA sequence around the cleavage site must be known.

Single-site ribosubstitution may be useful in

Fig.3. Autoradiograph of an experiment to demonstrate the use of single-site ribosubstitution in sequence analysis of a region of DNA containing two sites for the datum restriction enzyme. *Hae* III fragment 3 was used as a primer on ϕX174 viral strand DNA as template, and the Z3/Z10 site (see ref. 10) was ribosubstituted with rCTP as described under Methods. Aliquots (5 μl) of each reaction were analysed on a 8% acrylamide –7 M urea thin gel [15]. (a) An aliquot of the original extension reaction (the 'zero' reaction) cleaved with *Hae* III; (b) a–C reaction cleaved with *Hae* III; (c) a–T reaction cleaved with *Hae* III; (d) a–C reaction cleaved with ribonuclease A; (e) a–T reaction cleaved with ribonuclease A; (f) a 'zero' reaction cleaved with ribonuclease A; The top and bottom 20% of the autoradiograph are not shown.

techniques other than DNA sequence analysis. Ribonuclease or alkali cleavage of a single ribonucleotide incorporated at a restriction enzyme cleavage site cleaves only the non-template strand; the cleavage is at the phosphodiester bond 3′-adjacent to the bond cleaved by the restriction enzyme; the DNA strand synthesised in the extension reaction is left with a 5′-hydroxyl group; the priming fragment has a ribonucleotide-5′,3′-diphosphate moiety added; and cleavage occurs only at the single site in the DNA molecule. Any of these properties of the product may be useful; for example, in the isolation of specific single-stranded fragments of DNA, or for the specific end-labelling of DNA fragments, or for restriction mapping.

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