Preparation and separation of $d(pT)_{10.n}$ oligonucleotides

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ABSTRACT

A series of oligomers having the general formula $d(pT)_{10.n}$, n varying from 2 to 20, has been prepared by enzymatic joining of $d(pT)_{10}$, annealed on poly dA, employing T_4 polynucleotide ligase. The oligomers could be separated on 8 or 12% polyacrylamide gels. Such oligomers may prove useful as molecular weight markers and as initiators for various polymerases.

INTRODUCTION

Separation of nucleic acids on polyacrylamide gels, both for preparative and analytical purposes, has proven to be an exceedingly useful technique¹⁻³. This laboratory has for some time employed polyacrylamide gel electrophoresis in studying the mechanism of action of various enzymes involved in the nucleic acid metabolism, in particular T_4 polynucleotide kinase and ligase. The present work describes one aspect of this study, namely, the nature and separation of the products of the T_4 polynucleotide ligase catalyzed joining of $d(pT)_{10}$ annealed on poly dA. The method presented here may prove useful in other areas of nucleic acids research as well.

MATERIALS

T_4 polynucleotide kinase and ligase were prepared as previously described⁴. Bacterial alkaline phosphatase was a product of Worthington Biochemical Corporation. Acrylamide and N,N'-methylenebisacrylamide were products of Eastman Kodak

Company. [γ - ^{32}P] ATP with a specific activity of approximately 50-100 Ci/mmol was prepared according to a modified procedure of Glynn and Chappell⁵. Poly dA with average molecular weight of approximately 1×10^5 and $\text{d}(\text{pT})_{10}$ were obtained from P-L Biochemicals. Prior to use $\text{d}(\text{pT})_{10}$ was dephosphorylated by treatment with bacterial alkaline phosphatase⁶ the reaction being carried out at 67° to inactivate possible nucleases. The $\text{dT}(\text{pT})_9$ was then phosphorylated at the 5' hydroxyl terminus by employing T_4 polynucleotide kinase⁷ and the phosphorylated oligonucleotide separated from excess ATP by gel filtration on a column of Sephadex G-50 equilibrated with 0.05M triethylammonium bicarbonate (TEAB). After separation the buffer was removed by lyophilization and the phosphorylated oligonucleotide dissolved in a small volume of 10 mM Tris/HCl pH 8.0, containing 0.1 mM EDTA.

METHODS

Conditions for joining of $\text{d}(\text{pT})_{10}$. A typical reaction mixture for the T_4 polynucleotide ligase catalyzed joining of [$5'$ - ^{32}P] $\text{d}(\text{pT})_{10}$ contained the following components: 50 mM Tris pH 8.0, 6 mM MgCl_2 , 10 mM dithiothreitol (DTT), 0.6 mM ATP, 0.14 mM poly dA (phosphate), 0.14 mM [$5'$ - ^{32}P] $\text{d}(\text{pT})_{10}$ (phosphate) and 400 units of T_4 polynucleotide ligase/ml. Poly dA and [$5'$ - ^{32}P] $\text{d}(\text{pT})_{10}$ were annealed prior to addition of ATP, DTT and enzyme by heating the reaction mixture at 60°C for 5 minutes then letting it slowly cool to room temperature. The reaction temperature was 20°C and aliquots were removed at various times and assayed for resistance to bacterial alkaline phosphatase as described elsewhere⁴. After approximately 1 hour the reaction had usually reached a plateau. The reaction was then

stopped by making the reaction mixture 50% with respect to formic acid and the mixture was then kept for 16 hours at 30°C⁸. The latter treatment causes complete depurination of poly dA and thus serves to denature the poly dA-d(pT)_{10.n} duplexes. Upon completion of this step the reaction mixture was lyophilized, then dissolved in 100 µl of 50% glycerol containing 7M urea and the marker dyes bromphenol blue and xylene cyanole. Aliquots of this mixture were subjected to gel electrophoresis.

Gel electrophoresis. Vertical gels (18x18x0.2 cm) of 8 or 12% polyacrylamide containing 7M urea were employed. The gels (95% acrylamide and 5% N,N'-methylenebisacrylamide) were used with a running buffer of 90 mM Tris, 90 mM boric acid and 0.4 mM EDTA, pH 8.3. The electrophoresis was carried out for 12-18 hours at room temperature and usually at 150 V. Upon completion of electrophoresis the gels were subjected to autoradiography.

RESULTS AND DISCUSSION

The [5'-³²P] d(pT)₁₀ oligonucleotide annealed on poly dA was joined to larger oligonucleotides by T₄ polynucleotide ligase and the oligonucleotides thus formed were then subjected to gel electrophoresis. Prior to electrophoresis the duplexes were denatured by depurination of poly dA. Heat denaturation alone, with or without formamide, proved unsatisfactory for this purpose as the strands rapidly reannealed. A separation pattern on a 12% polyacrylamide gel of the oligonucleotides formed is shown in Figure 1 A. It is clear that the action of T₄ polynucleotide ligase on the duplex poly dA-[5'-³²P] d(pT)₁₀ resulted in formation of a series of new

oligomers having the general formula $d(pT)_{10 \cdot n}$. Oligonucleotides with n varying from 1 to 10 could clearly be separated on such a gel. The separation pattern on a 8% gel is given in Figure 1 B. In this case the lower molecular weight oligomers were run out of the gel to allow better separation of oligomers of higher molecular weight. Oligomers from $d(pT)_{50}$ and up to $d(pT)_{200}$ can be well separated by this technique. For both types of gels a plot of $\log$ (molecular weight) or $\log$ (oligomer size) vs relative mobility produced similar curves as shown in Figure 2. In the lower molecular weight regions these plots were linear.

The various oligomers could be eluted from the gel by cutting out the appropriate regions, crushing the gel to smaller pieces with a glassrod in a 15 ml centrifuge tube, then adding 1 M TEAB equal to twice the volume of the gel. This suspension was left at room temperature for approximately 15 hours, after which time the supernatant was pipetted off and the gel particles washed 3 times with 1 times the volume of 1 M TEAB. The TEAB was then evaporated off by lyophilization and the sample dissolved in a small volume of water. In order to remove the urea and the buffer from the gel the sample was subjected to gelfiltration on a small column of Sephadex G-50 (1x15 cm). Usually 70-80% recovery was achieved.

The mobility on the gel of single stranded nucleic acids will undoubtedly be influenced by such factors as stacking interactions and any tendency to form secondary and tertiary structures. Therefore, the mobility of various single stranded nucleic acids may be slightly different than that of the

Figure 1. A. Separation of $d(pT)_{10.n}$ oligomers on a 12% polyacrylamide gel. Autoradiogram of the gel. B. Separation of $d(pT)_{10.n}$ oligomers on a 8% polyacrylamide gel. The lower molecular weight oligomers were run out of the gel. Marker oligonucleotide not shown.

Figure 2. Semi-logarithmic plots of the separation data shown in Figure 1.

d(pT)_{10·n} of the same size. This was clearly revealed by comparing the mobility of d(pT)₁₀ and d(pA)₁₀ where the ratio of their relative mobilities was 1:1.1. Bearing these restrictions in mind the above oligomers may be useful as molecular weight markers. Furthermore, they are also well suited as initiators for various polymerases⁹⁻¹¹.

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