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Altered mobility of polydeoxyribonucleotides in high resolution polyacrylamide gels due to removal of terminal phosphates

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ABSTRACT

We have compared the electrophoretic mobility of a series of polynucleotides differing solely by the presence or absence of a terminal phosphate. As expected, the effect of removal of a single terminal phosphoryl residue on electrophoretic mobility is dependent on the size of the polynucleotide and therefore is not constant. Removal of a phosphoryl residue from polynucleotides shorter than 30 nucleotides reduces the mobility the equivalent of one nucleotide. Between 30 and 50 nucleotides the reduction in mobility is approximately one-half a nucleotide, while above 50 nucleotides in size the effect of phosphate removal approaches zero.

INTRODUCTION

High resolution polyacrylamide (6-25%) gel electrophoresis in the presence of 7-8 M urea can resolve polynucleotide chains differing in size by a single nucleotide at either the 5' or 3' end (1-3). This has allowed the rapid determination of DNA or RNA sequences by generating a series of polynucleotides with either a common 5' or 3' end and differing in size by increments of one nucleotide (1-3). Recently this technology has been utilized to precisely locate the 5' ends of specific RNA species (4-6), the 3' termination sites of DNA synthesis on specific templates (7, Kaguni, L.S. and Clayton, D.A., submitted), the insertion sites of ribonucleotides in mature mitochondrial DNA (mtDNA) (8), and the locations of 5' ends of nascent DNA chains (Tapper, D.P. and Clayton, D.A., submitted). These analyses have relied on a mobility comparison between polynucleotides of interest and the DNA sequence ladder of a restriction fragment containing a common end. Polyacrylamide gel electrophoresis of the DNA sequence fragments and the polynucleotide of interest in adjacent lanes yields the precise location of the ends of the polynucleotide.

In some instances the polynucleotide to be mapped, although containing a common 5' end, contains a 3' terminus lacking a 3' phosphoryl residue which is generated by the chemical DNA sequencing procedure (3). Thus an exact comparison cannot be made. It has previously been estimated that removal of one of the terminal phosphoryl

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residues will reduce the electrophoretic mobility of a polynucleotide of chain length **n** by the equivalent of one-half the spacing that occurs between a polynucleotide containing **n** residues and polynucleotide **n+1** (4,9). However, one would predict that this estimate of relative mobility would not be a constant value since the difference in the charge to mass ratios of two sets of polynucleotides should be greatest in the case of small oligonucleotides and should approach zero with increasing length of the polynucleotide. We have analyzed the effect of the removal of a terminal phosphate on the electrophoretic mobility of a polynucleotide and report here the chain lengths where this difference in polynucleotide structure significantly alters mobility.

MATERIALS AND METHODS

Growth and isolation of plasmid DNA

E. coli SF-8-C600r^-m^- transformed with pBR322 vector containing either mouse mtDNA sequences from 1751 to 3803 (pHR-D) or from 4014 to 9136 (pHR-C) (10) were grown under P2-EK1 conditions in Bacto-Penassay broth (Difco). Cultures were grown to stationary phase and plasmid DNA was isolated as described (11).

Preparation of DNA fragments

200 μg of pHR-C was digested with 100 units of Taq I. The 1550 nucleotide fragment was isolated from a preparative 1% agarose gel and electroeluted as previously described (12). After 3'-end labeling (below), 1μg of the 1550 nucleotide Taq I fragment was digested with 10 units of Eco RI. The 1207 nucleotide Eco RI/Taq I fragment (mouse mtDNA nucleotides 4014 to 5220) was isolated from a preparative 1% agarose gel and electroeluted (12). 10 μg of pHR-D was digested with 50 units of Bam HI. The 344 nucleotide Bam HI fragment (mouse mtDNA nucleotides 3223 to 3566 (10)) was isolated from a preparative 1.4% agarose gel and electroeluted. After 5'-end labeling (below), the DNA was digested with Ava II and the 85 nucleotide Ava II/Bam HI fragment (mtDNA nucleotides 3481 to 3565 (10)) was isolated from a 5% polyacrylamide gel by electroelution (12). DNA was digested with restriction enzymes under conditions specified by the supplier.

Labeling of 3' ends

The 1550 nucleotide Taq I fragment was labeled with [α-^{32}P]TTP and T4 DNA polymerase as described (13,14). DNA (100μg/ml) was incubated at 11°C for 20 min in 10 μl containing 5 units of T4 DNA polymerase and 10μM [α-^{32}P]TTP (400 Ci/mM) (14). The reaction was terminated and unincorporated label was removed as described (14) prior to restriction endonuclease digestion.

Labeling of 5' ends

The 344 nucleotide Bam HI fragment was dephosphorylated as described (12) and rephosphorylated in the presence of [γ-^{32}P]ATP as described (2).

DNA sequencing and gel electrophoresis

The 3'-end labeled Taq I/Eco RI fragment and the 5'-end labeled Bam HI/Ava II fragment were sequenced by the chemical method (3). Only the pyrimidine and purine specific cleavages were performed. After the removal of piperidine by lyophilization, the samples were resuspended in H_2O and divided into two. These were lyophilized, resuspended in H_2O and lyophilized again. To each was added 4 μl of 10mM Tris-HCl (pH 9.0), 20mM NaCl and 10mM $MgCl_2$. To one aliquot of each sample was added 0.25 units of calf alkaline phosphatase and the samples were incubated at 65°C for 10 min. 10 μl of formamide loading buffer was added to each sample and the samples were stored at -20°C prior to gel electrophoresis. 8% polyacrylamide-7M urea gels were prepared and utilized as described (3).

Materials

All restriction endonucleases were purchased from Bethesda Research Laboratories. T4 polynucleotide kinase was from Boehringer Mannheim. T4 DNA polymerase was from PL Biochemicals. [γ-^{32}P] ATP and [α-^{32}P] TTP were obtained from Amersham-Searle. Calf alkaline phosphatase was purchased from Boehringher Mannheim and additional purification was as described (15).

RESULTS AND DISCUSSION

To produce two series of DNA fragments labeled at a common end but differing solely by a single external phosphoryl residue, a 1550 nucleotide Taq I DNA fragment was labeled at the 3' ends with T4 DNA polymerase. Both 3' ends of this fragment terminate with the sequence 5'-X-T-T-3' and contain single strand 5' extensions, 5'-C-G-3'. Thus reaction of this fragment with T4 DNA polymerase and 10μM [α-^{32}P] TTP will specifically label the 3' terminus (13,14). Digestion of the resulting labeled fragment with Eco RI produces a 1207 nucleotide fragment labeled at the 3' end and containing a 3'-OH group and a 5'-phosphoryl group. This fragment was then subjected to the pyrimidine and purine specific degradations described by Maxam and Gilbert (3), thereby generating a series of fragments containing a labeled 3'-OH terminus with an unlabeled 5'-phosphoryl residue. Half of each of these samples was treated with calf alkaline phosphatase to remove the 5'-phosphoryl residue, resulting in the desired sets of fragments differing solely by a single terminal phosphoryl residue.

Each of the above samples was then subjected to electrophoresis in an 8% polyacrylamide-7M urea sequencing gel (Fig. 1). As expected, comparison of the mobility patterns from autoradiography reveals that the fragments minus the 5' phosphate migrate slower than their cognates containing a 5'-phosphoryl residue. The pair of bands containing the same DNA sequence and differing only at the 3' end can easily be identified since the correct sequence of pyrimidines and purines can be ascertained

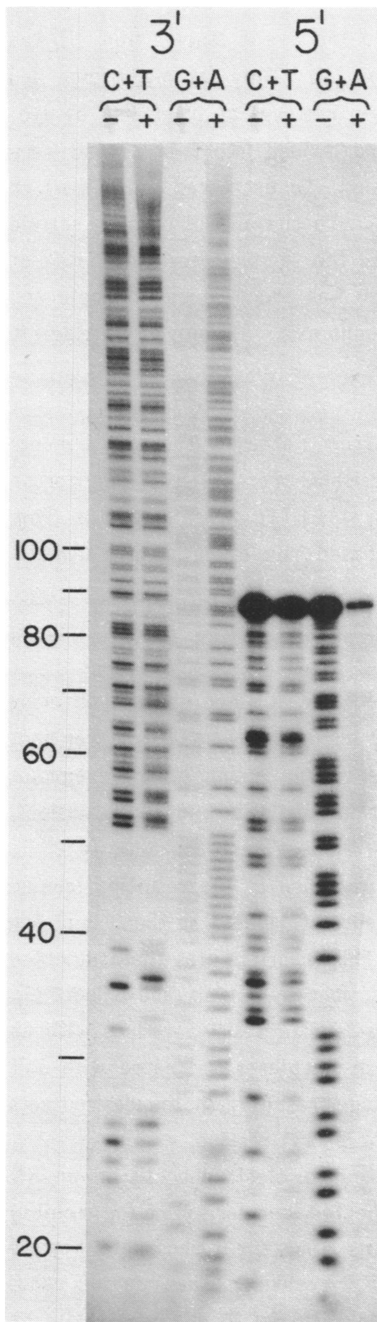

Figure 1. Electrophoresis of 3' and 5'-end labeled DNA before (-) and after (+) phosphatase treatment.

(3') The 1207 nucleotide Eco RI/Taq I fragment from pHR-C labeled with T4 DNA polymerase and [α-^{32}P]TTP at the Taq I site was degraded specifically at either pyrimidines (C+T) or purines (G+A). (5') The 85 nucleotide Bam HI/Ava II fragment from pHR-D labeled with T4 polynucleotide kinase at the Bam HI site was degraded specifically at either pyrimidines (C+T) or purines (G+A). Electrophoresis was in an 8% polyacrylamide-7M urea sequencing gel (3). The numbers on the left are the size in nucleotides of the 3'-end labeled fragments.

from both sets of DNA fragments.

Two independent control experiments demonstrated that the difference in mobility was due solely to the removal of the 5'-phosphoryl group. First, a DNA fragment derived from pHR-D was labeled at the 5' end with polynucleotide kinase and [γ-^{32}P] ATP and was then subjected to the purine and pyrimidine specific degradations as above. Each sample was then divided into two and treated with calf alkaline phosphatase, as with the 3'-end labeled sample. Under the conditions used, it can be seen that the phosphatase removed the vast majority of 5' phosphates (Fig. 1, 5' G+A). Second, when the 5' phosphates are only partially removed (Fig. 1, 5' C+T), not only the expected unmodified fragments are observed, but a second set of fragments can be seen. These are due solely to the removal of the 3'-phosphoryl residue which, as expected, reduces the mobility of the polynucleotides. Thus under the conditions used, 3'-phosphoryl residues are removed and the presence of calf alkaline phosphatase during electrophoresis does not alter the mobility of polynucleotides.

The effect of the removal of the 5'-phosphoryl residue on the electrophoretic mobility of polynucleotide **n** can conveniently be expressed by the ratio of the difference in mobility of polynucleotide **n** and polynucleotide **n** minus the 5' phosphate, to the difference in mobility of polynucleotide **n** and polynucleotide **n+1**. From inspection of the DNA sequence gel (Fig. 1) it can be clearly seen that the absolute difference in mobility due to phosphate removal is greatest for the lowest molecular weight species. However, the ratio discussed above for any polynucleotide **n** will be a constant irrespective of the position in the gel. This is obscured for polynucleotides that have not migrated to the bottom of the gel (e.g., 60 nucleotides in Fig. 1). Electrophoresis of the same 3'-end labeled sample such that a species of 75 nucleotides has migrated to the bottom of the gel (Fig. 2) shows that the effect of the removal of the 5' phosphate is still significant between approximately 75 to 110 nucleotides. In the pyrimidine specific degradation lane, where dephosphorylation was incomplete, this effect is obscured (Fig. 2).

When the ratio described above is plotted against polynucleotide chain length (Fig. 3), it is clear that the effect of the removal of the terminal phosphate is greatest on the electrophoretic mobility of smaller polynucleotides ($<$ 40 nucleotides). Polynucleotides of approximately 40 nucleotides move at least one-half of a nucleotide slower in the sequencing gel. Below 30 nucleotides mobility is reduced by at least one nucleotide. A significant decrease in electrophoretic mobility resulting from the removal of the 5'-phosphoryl residue can be observed in polynucleotides of up to 110 nucleotides. As shown in Fig. 3, the effect of phosphate removal on electrophoretic mobility gradually decreases such that polynucleotides $>$ 50 nucleotides migrate only 0.2 nucleotide equivalents slower than the corresponding fragment in the sequencing

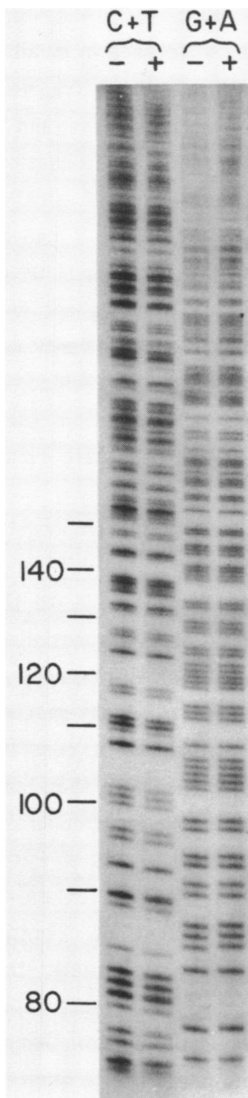

Figure 2. Electrophoresis of 3'-end labeled DNA before (-) and after (+) phosphatase treatment.

The 1207 nucleotide Eco RI/Taq I fragment from pHR-C labeled with T4 DNA polymerase and [α-^{32}P] TTP at the Taq I site was degraded specifically at either pyrimidines (C+T) or purines (G+A). Electrophoresis was in an 8% polyacrylamide-7M urea sequencing gel (3). The time of electrophoresis was twice as long as that shown in Fig. 1. The numbers on the left are the sizes in nucleotides of the fragments from the Taq I site.

ladder. Although removal of the 5' phosphate alters the mobility of polynucleotides containing up to at least 110 residues, the lack of resolution, even when those molecules are allowed to migrate a greater distance into the gel, renders the difference insignificant. Above this size range, the difference in electrophoretic mobility with phosphate removal approaches zero.

In conclusion, these data extend and clarify earlier observations presented in

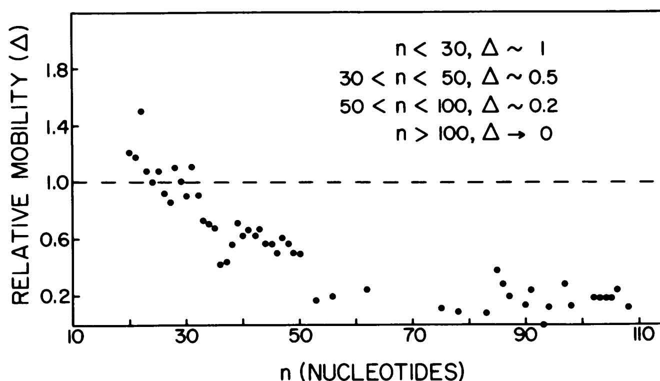

Figure 3. Change in electrophoretic mobility of polynucleotides due to phosphate removal.

The relative decrease in mobility (Δ) due to phosphate removal was determined by electrophoresis of the 3'-end labeled DNA (Figs. 1 and 2) before and after phosphatase treatment. Δ was calculated by measuring the difference in electrophoretic mobility of polynucleotide **n** and polynucleotide **n** after phosphatase treatment and dividing this by the difference in electrophoretic mobility of polynucleotide **n** and polynucleotide **n+1**. When this ratio is one (dashed line), the mobility decrease due to phosphate removal is the equivalent of a shift of one nucleotide in the sequencing ladder. The relative mobility (Δ) is plotted against the size of the polynucleotides (**n**). Between 75 and 110 nucleotides the mobility shift was determined for the G+A reaction only.

the literature on the effect of the removal of terminal phosphates on polynucleotide mobility in DNA sequencing gels. The previous reported correction value of 0.5 nucleotides (4,6) is valid only within a limited range of DNA chain lengths (Fig. 3). The data here show that a significant correction must be made when comparing DNA fragments less than 50 nucleotides in length lacking at least one terminal phosphate with a DNA sequence ladder generated by the chemical degradation procedure. The effect of dephosphorylation is insignificant on DNA chains greater than approximately 100 nucleotides in length.

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REFERENCES

1. Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. **94**, 441-448
2. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA **74**, 560-564
3. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. **65**, 499-560
4. Sollner-Webb, B. and Reeder, R.H. (1979) Cell **18**, 485-499
5. Weaver, R.F. and Weissman, C. (1979) Nucleic Acids Res. **7**, 1175-1193
6. McKeown, M. and Firtel, R.A. (1981) Cell **24**, 799-807
7. Doda, J.N., Wright, C.T. and Clayton, D.A. (1981) Proc. Natl. Acad. Sci. USA **78**, in press
8. Brennicke, A. and Clayton, D.A. (1981) J. Biol. Chem. **256**, in press
9. Tomizawa, J-I., Ohmori, H. and Bird, R.E. (1977) Proc. Natl. Acad. Sci. USA **74**, 1865-1869
10. Bibb, M.J., Van Etten, R.A., Wright, C.T., Walberg, M.W. and Clayton, D.A. (1981) Cell **26**, in press
11. Battey, J. and Clayton, D.A. (1978) Cell **14**, 143-156
12. Gillum, A.M. and Clayton, D.A. (1978) Proc. Natl. Acad. Sci. USA **75**, 677-681
13. Challberg, M.D. and Englund, P.T. (1980) Methods Enzymol. **65**, 39-43
14. Tapper, D.P. and DePamphilis, M.L. (1980) Cell **22**, 97-108
15. Efstradiadis, A., Vournakis, J.N., Donis-Keller, H., Chaconas, G., Dougall, D.K. and Kafatos, F.C. (1977) Nucleic Acids Res. **4**, 4165-4174