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DNA chain length markers and the influence of base composition on electrophoretic mobility of oligodeoxyribonucleotides in polyacrylamide-gels

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ABSTRACT

The specific influence of the four nucleobases on electrophoretic mobility of oligodeoxyribonucleotides in polyacrylamide-gels under denaturing and nondenaturing conditions has been investigated using homooligomers from the four deoxyribonucleotides as chain length standards. Homooligomers of same chain lengths exhibit remarkable differences in mobility. Specific retardation of any other oligonucleotide investigated was found to be mainly dependent on base composition but not on sequence. A simple procedure is presented for calculating mobilities relative to the standards on denaturing gels. This allows a reliable identification of oligonucleotides on acrylamide-gels by exact chain length determination with respect to base composition and furthermore a detailed interpretation of complex reaction mixtures. The homooligomers also show the same differences in mobility on nondenaturing gels. The significance of this effect for strand separation is discussed.

INTRODUCTION

Polyacrylamide-gel electrophoresis under denaturing conditions has been described by many authors[1-4] to be an accurate method for determination of chain length and molecular weight of small DNA or RNA molecules. During our work on the synthesis of a structural gene for the peptide hormone angiotensin II (AII-DNA)[5] we performed the enzymatic ligation of chemically synthesized oligodeoxyribonucleotides without any intermediate purification in a multistep reaction[6]. Polyacrylamide-gel electrophoresis was used to analyse successive steps of ligation. A mixture of thymidine oligomers $p(dT)_n$ with $n \geq 3$ was coelectrophoresed to determine the chain length of synthesized products. These chain length standards were prepared by polymerization of dTTP with terminal deoxynucleotidyl transferase using the chemically synthesized trinucleotide $p(dT)_3$ as primer. But none of the well characterized segments of the AII-DNA showed a mobility as the corresponding thymidine oligomer of same chain length. Thus direct correlation between joining products and chain length standard proved not to be reliable. We

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concluded that under conditions used oligodeoxyribonucleotides are not only separated according to their chain length.

To study the influence of nucleobases on electrophoretic mobility homo-oligomer mixtures of the other three nucleotides were also prepared. As a result of this work we report in this communication a reliable method to calculate the electrophoretic mobility of any oligodeoxyribonucleotide relative to one of the chain length standards on denaturing gels. Furthermore some estimations are made for electrophoretic mobilities of oligonucleotides under strand separation conditions.

MATERIALS AND METHODS

All chemicals were of p.a. quality and obtained from the following sources: acrylamide and N,N'-methylenebisacrylamide (twice crystallized), N,N,N',N'-tetramethylethylenediamine from Serva Feinbiochemica (Heidelberg); urea, ammonium persulfate, buffer salts and other chemicals from E. Merck (Darmstadt) or Riedel-deHaën (Hannover); deoxynucleosidetriphosphates from Boehringer (Mannheim) and ATP from Papierwerke Waldhof (Aschaffenburg); ^{14}C-labelled dATP, dTTP and dCTP from Amersham and carrier free [^{32}P]orthophosphoric acid from New England Nuclear.

[γ-^{32}P]ATP (specific activity 200-400 Ci/mmol) was prepared according to Szekely[7] and stored in water at -20°C at a concentration less than 5mCi/ml.

Enzymes: Alkaline phosphatase (from calf intestine), phosphodiesterase (from spleen and snake venom), T4 polynucleotide kinase, and restriction endonuclease Eco RI were purchased from Boehringer (Mannheim). T4 DNA-ligase was prepared as described[6] and terminal deoxynucleotidyl transferase isolated in this laboratory by Dr. D. Skroch[8].

Standard enzyme reactions: The oligonucleotides and samples of joining reaction were phosphorylated using T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction mixtures (10 µl) contained 10-1000 pmol of oligonucleotide, a three fold excess of [γ-^{32}P]ATP, 60mM Tris·HCl (pH 7.6), 6mM $MgCl_2$, 10mM dithioerythritol (DTE) and 2 units of polynucleotide kinase. Before adding the enzyme the mixture was heated to 80°C for 1 min and chilled in ice. Incubation was carried out at 37°C for 30 min.

T4 DNA-ligase catalyzed joining was performed in reaction mixtures (10µl) containing equimolar concentrations (10µM) of each oligonucleotide (aliquots taken from phosphorylation mixtures without purification), 60mM Tris·HCl (pH 7.6), 10mM $MgCl_2$, 10mM DTE and 0.2mM ATP. This mixture was

heated to 80°C and cooled to 20°C within 1h. Then 0.2 units of T4 DNA-ligase were added, after cooling to 5°C within 3h again 0.2 units of enzyme were added and incubation at 5°C was continued for another 20h.

Kinase and ligase reactions were stopped either by heating to 80°C for 5 min and storage at -20°C or by addition of an equal volume of dye-mix containing 10M urea, 0.05% bromophenol blue, 0.05% xylene cyanol, and 20mM EDTA followed by storage at 4°C or direct use in gel electrophoresis. Yields of phosphorylation- and ligation reactions were determined using a previously described assay[6].

Oligodeoxyribonucleotides: d(TTAAAAGGGAT), d(GAATATAAACG), d(CGATCCATTTA), d(TAAATG), d(GATCGCGTTT), d(ATATTCATCCC), and d(TTTTAA) are segments of the chemically synthesized angiotensinII-DNA and their syntheses have been described[9]. The trinucleotides d(TTT), d(AAA), d(TAA), d(TTA), d(CCC), d(GGG), d(GAT) and the two hexanucleotides d(GAATAT), d(ATATTC) were intermediates in the synthesis of the AII-DNA segments. They were isolated from the fully protected oligonucleotides by known methods[10], purified by paper chromatography (Schleicher + Schüll No. 2043bMgl) in propanol-(2)/NH_3(25%)/H_2O (7/1/2, v/v) or propanol-(1)/NH_3(25%)/H_2O (55/10/35, v/v), and characterized by digestion with phosphodiesterase from spleen and snake venom[10]. The synthesis of d(TTACAT) is described elsewhere[11]. $p(dT)_7$ was synthesized chemically according to Khorana and Connors[12] and was a gift from Dr. D. Skroch. d(GGAATTCC) was obtained from Collaborative Research Inc. d(pAATTCCGG) was prepared by oligomerization via blunt end ligation of d(pGGAATTCC) followed by EcoRI digestion. 1 nmol of d(GGAATTCC) was phosphorylated as described above. Ligation was performed in a 100 µl reaction volume under conditions described above but incubation was carried out at 20°C for 20h and resulted in 68% alkaline phosphatase resistent radioactive phosphate. Oligomers were separated from unreacted octanucleotide by preparative polyacrylamide-gel electrophoresis as described below. Products were eluted by the crush and soak method[13] and precipitated with ethanol. The pellet was dissolved in 200 µl containing 60mM Tris·HCl(pH 8.0), 6mM $MgCl_2$ and 50mM NaCl, heated to 80°C and slowly cooled down to 37°C. 60 units of EcoRI endonuclease were added and the mixture incubated for 1h at 37°C and for 20h at 10°C. After precipitation with ethanol the digestion product was isolated by preparative gel electrophoresis. The yield was 0.25 nmol of d(pAATTCCGG). This sequence was confirmed by successful oligomerization via sticky end joining with T4 DNA-ligase and successive digestion with EcoRI which produced again the same

octanucleotide. All reactions were followed by analytical polyacrylamide-gel electrophoresis.

Polymerization of deoxynucleosidetriphosphates[14,15]: The reaction mixture (100µl) containing 10 nmol primer, 200 nmol dNTP, 40mM sodium cacodylate (pH 6.8), 1mM $CoCl_2$ and 10 units of terminal deoxynucleotidyl transferase was incubated at 37°C. After 4h and 8h another 10 units of enzyme were added. The reaction was stopped after 16h by addition of 100µl 0.2 M Tris·HCl (pH 9.0) of 0°C. For 5'-dephosphorylation the mixture was incubated with 2µg of alkaline phosphatase at 50°C for 30 min and the enzyme inactivated by addition of 4µl 0.25M EGTA and heating for 5 min on a boiling water bath. This solution was then stored at -20°C.

Polymerization may be followed by analytical paper chromatography on DE81-strips (Whatman) when the dNTP is ^{14}C or ^{32}P labelled. In 0.3M ammonium formiate (pH 7) as solvent polymerization products remain at the origin while the dNTP moves with an R_f of 0.4 to 0.6.

1µl aliquots of the reaction mixture containing 50pmol of 5'-hydroxyl termini were then ^{32}P-labelled by phosphorylation as described above. The reaction was stopped by addition of dye-mix. This solution may be layered onto the gel directly or after suitable dilution with water and dye-mix.

Polyacrylamide-gel electrophoresis: A normal vertical slab gel apparatus was used. The gels (40x13.5x0.1 cm or 40x31x0.1cm) were 20% in acrylamide (3.33% N,N'-methylenebisacrylamide), 7M urea, 50 mM Tris·borate (pH 8.3), 1mM EDTA and 0.07% ammoniumpersulfate[13]. Buffer reservoirs were filled with 50mM Tris·borate (pH 8.3), 1mM EDTA (1xTBE).

Aqueous samples for electrophoresis were made 0.05 N in NaOH and an equal volume of dye-mix was added. The samples were heated to 90°C for 15 sec and immediately layered onto the gel. Electrophoresis was carried out at room temperature at constant voltage (1200V, 30V/cm) and stopped when the bromophenol blue dye had moved about 30 cm (the trinucleotides have reached the end of the gel). Wet gels were covered with plastic wrap and exposed to an X-ray film (Kodak, X-Omat R) at -20°C or below.

The following remarks should be noticed for good reproducibility of relative mobilities and a high resolution. Prior to use the gel should be kept at room temperature for at least 10h. After removing of the slot former, slots should be filled with 1xTBE containing 7M urea. The gel should be pre-electrophoresed at 600-800 V until current at 1200 V has fallen to 15mA. Then all the urea in the slots has to be washed away with 1xTBE and hot samples should be loaded immediately. For each electrophoresis all

samples should have the same volume. Voltage (and temperature of the gel) should not be reduced too much to prevent oligonucleotides from diffusion and renaturing.

RESULTS

The mixture of homooligomers of thymidine-5'phosphate that differs only by one nucleotide in length was prepared by polymerization of dTTP with terminal deoxynucleotidyl transferase and a chemically synthesized short thymidine oligomer as primer. The use of a trinucleotide $p(dT)_3$, although a poor substrate for the enzyme, is advantageous in this case. The reaction with e.g. the heptanucleotide $p(dT)_7$ resulted in a high polymerization rate but relative narrow chain length distribution. The trinucleotide, however, is slowly elongated producing better primer molecules. Throughout the whole reaction time a constant but low level of these more reactive primers is maintained while the nucleoside triphosphate concentration is rapidly decreasing. The result is a small polymerization rate with even distribution of products over a wide range of chain lengths (see Figure 1a). The radioactive label was introduced by successive action of alkaline phosphatase from calf intestine and T4 polynucleotide kinase with $[\gamma\text{-}^{32}P]$ATP directly on small samples of the polymerization reaction mixture.

Electrophoresis on 20% acrylamide /7M urea denaturing gels provides best resolution for the range of chain lengths of our interest ($5 < n < 50$). Figure 1b shows the analysis of three joining reactions with segments of the AII-DNA. For details see legend to Figure 1b. Two samples were withdrawn from the reaction mixture and one treated with T4 polynucleotide kinase and $[\gamma\text{-}^{32}P]$ATP before electrophoresis. This reaction was performed because some of the oligonucleotides in the joining reaction mixtures were not 5'-phosphorylated which was assumed to influence their electrophoretic mobility. Comparison of the two samples after electrophoresis clearly shows the accelerating effect of an additional 5'-phosphate (slot g and h in Figure 1b). Thus for exact determinations only oligonucleotides should be compared that are in the same state with respect to their 5'- and 3'-termini.

During synthesis of AII-DNA we always observed a greater mobility for each oligonucleotide if compared to the corresponding thymidine oligomer of same chain length. This deviation was greater with adenosine and cytidine rich molecules and less with guanosine rich molecules. Thus,we predicted an accelerating effect of A and C and a retarding effect of G on electrophoretic mobilities relative to T. To prove this prediction homooligomers of

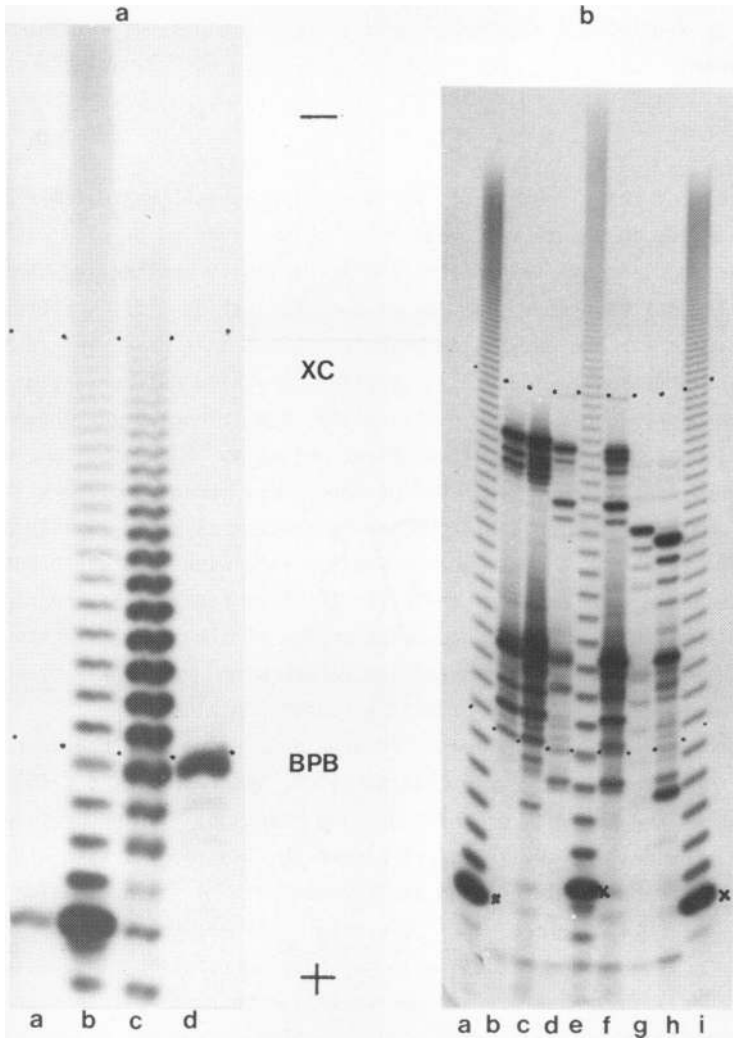

Figure 1a: Electrophoresis of polymerization reaction mixtures of dTTP with terminal deoxynucleotidyl transferase and $p(dT)_3$-primer(b) or $p(dT)_7$ primer (c); a) $p(dT)_3$; d) $p(dT)_7$. XC=Xylene cyanol. BPB=Bromophenol blue.

Figure 1b: Analysis of three joining reactions on a 20% acrylamide/7M urea gel: a and i) A-standard; b) reaction 1 with d(TTAAAAGGGAT) as acceptor, d(pGAATATAAACG) as donor, and d(ATATTCATCCC) as splint; c) reaction 1 after phosphorylation; d) reaction 2 with d(GAATATAAACG) as acceptor, d(pCGATCCATTTA) as donor, and d(GATCGCGTTT) as splint; e) T-standard; f) reaction 2 after phosphorylation; g) reaction 3 with d(TAAATG) as acceptor, d(pGATCGCGTTT) as donor, and d(CGATCCATTTA) as splint; h) reaction 3 after phosphorylation. x=Trinucleotide.

the other three nucleotides were prepared by the same procedure as for $p(dT)_n$. The result of gel electrophoresis is shown in Figure 2a. Direction of base specific shifts had been deduced accurately. The 33 nucleotides long (+)- and (-)-strands of the AII-DNA migrate with $T_{31/32}$, $A_{35/36}$ and $C_{37/38}$. The faster moving weaker band is due to incomplete repair synthesis[16]. Figure 2b shows the same samples but electrophoresis was carried out under nondenaturing conditions. Polymerization of dGTP with $p(dG)_3$ as

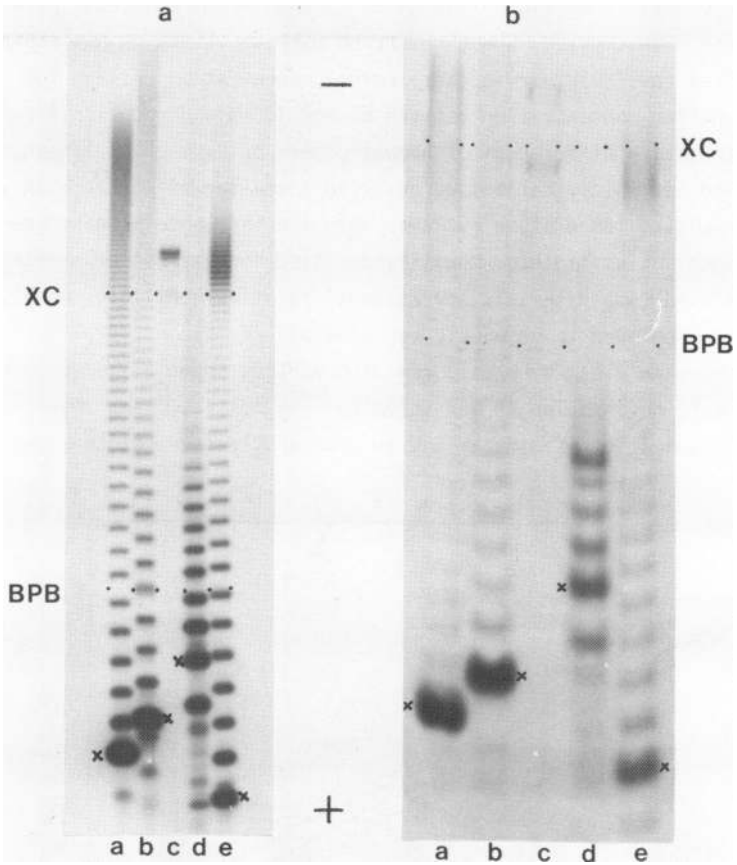

Figure 2a: Electrophoresis of the four homooligomer-standards on a 20% acrylamide /7M urea gel: a) A-standard, b) T-standard, c) AII-DNA, d) G-standard and e) C-standard. x=Trinucleotide.

Figure 2b: Electrophoresis of the same samples as in Figure 2a but on a nondenaturing 20% acrylamide-gel under strand separation conditions (Ref. 13).

primer has also been successful but phosphorylation was very poor and a distinct banding during electrophoresis is only detectable up to the dodecamer. This might be due to the extreme strong tendency of guanosine to selfassociation.

The curves relating mobility to log of chain length (Figure 3) have a relative similar form for all four series of homooligomers and follow sigmoidal functions. This has also been observed for various gel systems and other molecular weight standards that have been used to date[3,4,17].

Dependence of base specific influence on various electrophoretic parameters has been investigated. Changing the pH without effecting net charge of the molecules (pH 7-9), varying acrylamide concentration between 10% and 20%, and buffer concentration between 50 and 100mM did not significantly alter the relative differences of homooligomers of same chain length. For established gel specifications we observed a smaller effect at high voltage and elevated temperature but even then the influence of base composition becomes not negligible. When standardized conditions for electrophoresis were used (see Materials and Methods) relative mobilities of oligonucleotides were found to be highly reproducible.

Figure 4 shows an electrophoretic run with various short oligodeoxynucleotides of well defined length and sequence. The most striking results are identical mobilities of a deca- and an undecanucleotide (slots 1 and m),

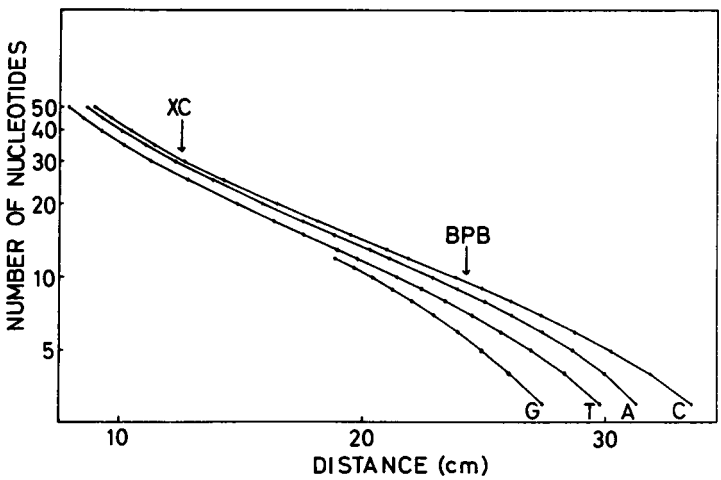

Figure 3: Plot of electrophoretic mobility as log of chain length for the 5'-phosphorylated homooligomers.

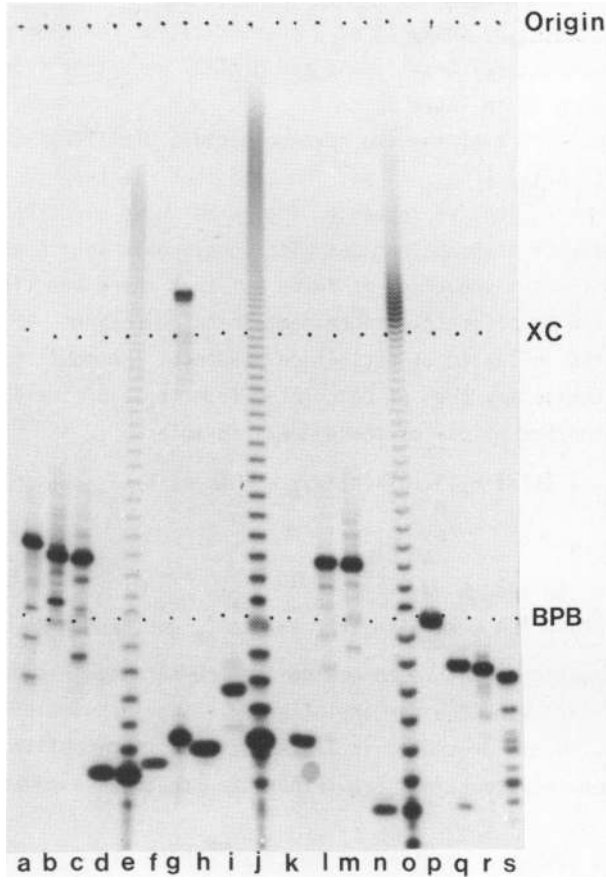

Figure 4: Electrophoresis of various oligodeoxyribonucleotides on a 20% acrylamide/7M urea gel: a) d(pTTAAAAGGGAT), b) d(pGAATATAAACG), c) d(pCGATCCATTTA), d) d(pAAA), e) A-standard, f) d(pTAA), g) d(pGAT) and AII-DNA, h) d(pTTA), i) d(pGGG), j) T-standard, k) d(pTTT), l) d(pGATCGCG-TTT), m) d(pATATTCATCCC), n) d(pCCC), o) C-standard, p) d(pGGAATTCC), q) d(pTAAATG), r) d(pTTTTAA), s) d(pTTACAT).

and the different mobilities of the hexanucleotides (slots q, r and s) and the undecanucleotides (slots a, b and c). Comparison of four trinucleotides d(pAAA) (slots d, e), d(pTAA) (slot f), d(pTTA) (slot h) and d(pTTT) (slots j, k) strongly indicate that base specific retardation of oligonucleotides is an additive effect. So the mobilities for d(pTAA) and d(pTTA) can easily be calculated by linear interpolation between those of $p(dA)_3$ and $p(dT)_3$.

The behavior of T/A-oligomers of longer chain length has been investigated under the same aspect by analysis of a polymerization reaction of dTTP with terminal deoxynucleotidyl transferase and d(pTAA) as primer (Figure 5a). Results are presented in Table 1.

Identical mobilities of the two hexanucleotides d(pTTTTAA) (Fig. 4, slot r) and d(pTAATTT) (Fig. 5a) may indicate that electrophoretic mobility is independent on nucleotide sequence. Figure 5b shows electrophoresis of three other pairs of oligonucleotides with equal chain length and base composition but different sequence. It turns out that there are little differences at least with oligonucleotides containing guanosine.

Neglecting the influence of nucleotide sequence, the mobility (x) of any oligodeoxynucleotide may then be calculated from those of the four corresponding homooligomers by use of the general formula:

$$x_{(T_tA_aC_cG_g)} = [t/n]\cdot x_{(T_n)} + [a/n]\cdot x_{(A_n)} + [c/n]\cdot x_{(C_n)} + [g/n]\cdot x_{(G_n)}$$

x= mobility [cm]

t+a+c+g = chain length n

e.g. d(pGGAATTCC) = $T_2A_2C_2G_2$ with t=2, a=2, c=2, g=2, and n=8

The exact position relative to one of the four standards can now be determined. This has been done for the oligonucleotides shown in Figure 4 and 5b and results are presented in Table 2. Values for mobilities (x) of the homooligomers were obtained from Figure 2a that was used as a standard nomogram.

This method, however, was limited to chain length $n \leq 12$ because of the short G-standard available (Figure 2a). Therefore efforts were made to deduce theoretical values for mobilities of higher G-oligomers from electrophoretic behavior of the T, A and C standards. Good results were obtained by the graphic method shown in Figure 6. Files of autoradiographic spots from Figure 2a can be arranged in a way that all lines connecting spots of same chain length cross in one point. Only spots of very short oligonucleotides ($n < 5$) do not coincide very well. The short file of G-oligomers was then adjusted with maximum accuracy and the G-standard extended to n=50. Reliability of this method was tested with well defined joining products of oligonucleotides shown in Figure 4. Data are presented in Table 3. The nomogram used to determine mobility values (x) of homooligonucleotides for calculation of theoretical chain lengths is shown in Figure 7. It is a graphic representation of Figure 2a including the extended G-standard from Figure 6.

Table 1. Observed and calculated mobilities of dT/dA-oligomers from Figure 4 and 5a in denaturing polyacrylamide-gel electrophoresis expressed in theoretical chain length of a coelectrophoresed homooligomer standard.

Nucleotide Sequence	Theoretical Chain Length of a Corresponding Homooligomer[a]			
	observed[b]		calculated	
	A	T	A	T
d(pAAA)	3.0	-	3.00	-
d(pTAA)	3.4	-	3.38	-
d(pTTA)	3.8	-	3.75	-
d(pTTT)	4.2	3.00	4.13	3.00
d(pTAAT)		3.43		3.45
d(pTAATT)		4.46		4.50
d(pTAATTT)		5.52		5.55
d(pTTTTAA)		5.54		5.55
d(pTAATTTT)		6.58		6.60
d(pTAATTTTT)		7.65		7.64
d(pTAATTTTTT)		8.70		8.68
d(pTAATTTTTTT)		9.72		9.70

a) Values are obtained by linear interpolation between mobilities of the two neighboring homooligomers of a chain length standard.
b) Average values of about three electrophoretic runs under exact the same conditions.

Table 2. Observed and calculated mobilities of various oligodeoxyribonucleotides in denaturing polyacrylamide-gel electrophoresis expressed in theoretical chain length of a coelectrophoresed homooligomer standard.

Nucleotide Sequence	Theoretical Chain Length of a Corresponding Homooligomer[a]							
	observed[b]				calculated			
	T	A	C	G	T	A	C	G
1 d(pGAT)	3.2	4.5	5.4	-	3.21	4.37	5.50	-
2 d(pTAAATG)	5.6	7.0	7.9	3.9	5.57	6.94	7.90	3.86
3 d(pGAATAT)	5.5	6.9			5.57	6.94	7.90	3.86
4 d(pTTTTAA)	5.5	6.9	7.8	3.8	5.55	6.93	7.87	3.83
5 d(pTTACAT)	5.2	6.6	7.5	3.5	5.14	6.52	7.47	3.46
6 d(pATATTC)	5.2	6.6			5.14	6.52	7.47	3.46
7 d(pGGAATTCC)	7.2	8.6	9.6	5.7	7.34	8.76	9.71	5.80
8 d(pAATTCCGG)	7.6	9.0			7.34	8.76	9.71	5.80
9 d(pGATCGCGTTT)	9.9	11.4	12.3	8.6	9.67	11.23	12.13	8.33
10 d(pTTAAAAGGGAT)	10.8	12.3	13.2	9.4	10.67	12.20	13.08	9.33
11 d(pGAATATAAACG)	10.2	11.8	12.7	8.9	10.11	11.71	12.60	8.82
12 d(pCGATCCATTTA)	10.2	11.7	12.6	8.7	10.02	11.57	12.46	8.67
13 d(pATATTCATCCC)	9.8	11.3	12.2	8.5	9.67	11.23	12.13	8.33

a,b) see footnotes of Table 1.

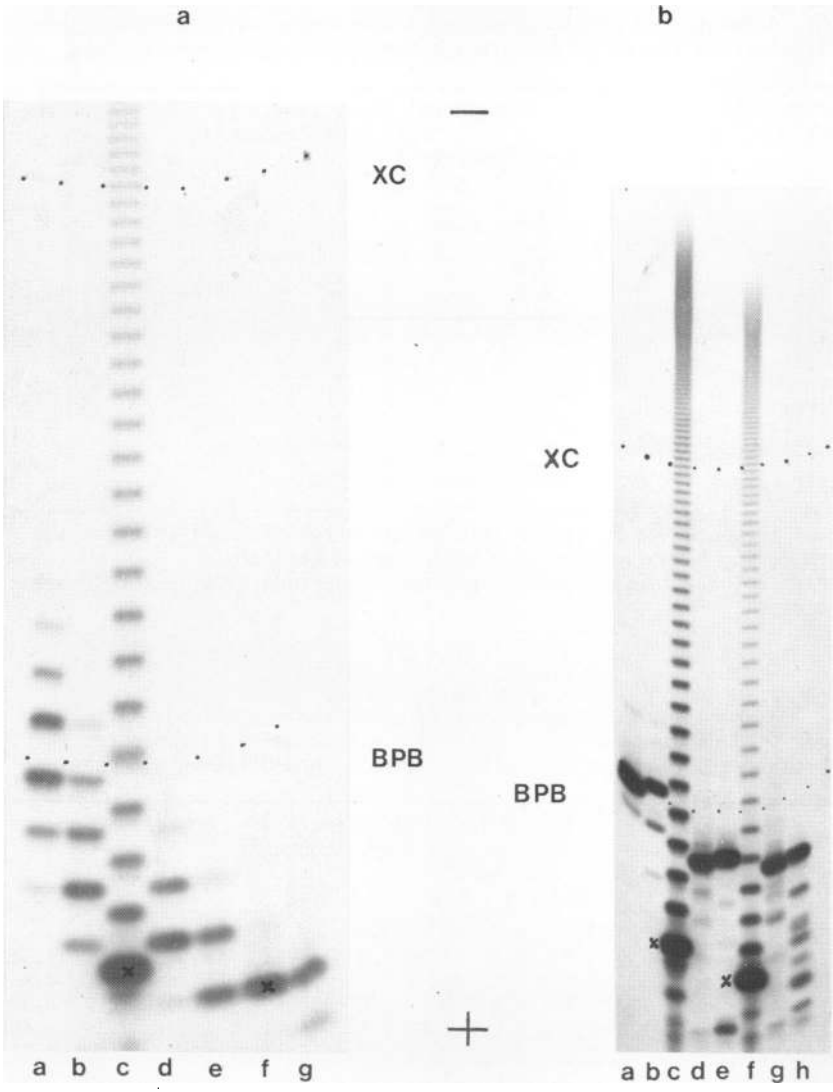

Figure 5a: Electrophoresis of T-standard (slot c) and fractions from Sephadex G-50 filtration of a polymerization mixture of dTTP with terminal deoxynucleotidyl transferase and d(pTAA) as primer (slots a,b,d,e,f,g).

Figure 5b: Electrophoresis of three oligonucleotide pairs of same chain length and base composition but different nucleotide sequence: a) d(pGGAATTCC), b) d(pAATTCCGG), c) T-standard, d) d(pGAATAT), e) d(pTAAATG), f) A-standard, g) d(pATATTC), and h) d(pTTACAT). x=Trinucleotide.

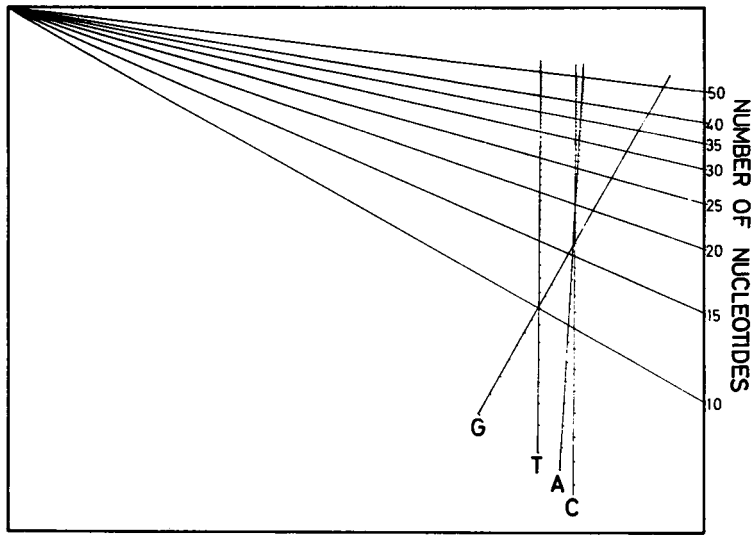

Figure 6: Graphic determination of electrophoretic mobilities of higher guanosine oligomers.

DISCUSSION

The advantageous use of homooligonucleotides of known chain length as an analytical marker system in polyacrylamide-gel electrophoresis has been pointed out before[18]. As the log molecular weight/mobility plot deviates

Table 3. Observed and calculated mobilities of some joining products of oligodeoxyribonucleotides from Table 2 in denaturing polyacrylamide-gel electrophoresis expressed in theoretical chain length of a coelectrophoresed homooligomer standard.

Joined Segments	Number of Nucleotides	Theoretical Chain Length of a Corresponding Homooligomer[a]					
		observed[b]			calculated		
		T	A	C	T	A	C
2 + 9	16	15.5	17.4	18.5	15.41	17.41	18.45
7 + 7	16	15.0	16.9	18.0	15.05	17.00	18.04
9 + 13	21	19.8	22.2	23.4	19.79	22.20	23.39
10 + 11	22	21.1	23.7	24.9	20.96	23.59	24.82
11 + 12	22	20.8	23.2	24.5	20.60	23.10	24.32
2 + 9 + 13	27	25.6	29.0	30.4	25.68	29.04	30.41
10 + 11 + 12 (AII-DNA (-)strand)	33	31.5	35.6	37.5	31.63	35.68	37.50
2 + 9 + 13 + 4 (AII-DNA (+)strand)	33	31.5	35.6	37.5	31.54	35.59	37.40

a,b) see footnotes of Table 1

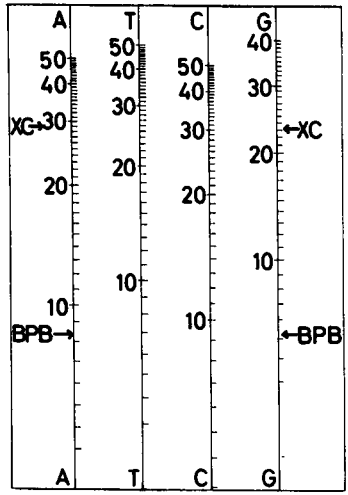

Figure 7: Nomogram for determination of mobility values for homooligomers. Xylene cyanol = 12.5cm, bromophenol blue = 24.5cm

from linearity especially for tighter gels (e.g. for 20% polyacrylamide-gels, Figure 3) oligonucleotide markers allow a far more reliable molecular weight determination than dye markers. In analysing joining reactions with short synthetic oligodeoxyribonucleotides our problem was to determine the exact chain length of joining products and their reliable idenfication in order to detect undesirable side reactions. Coelectrophoresis of thymidine oligomers led to the conclusion that under conditions used electrophoretic mobility depends on base composition.

Investigations on electrophoretic behavior of various oligonucleotides of well defined nucleotide sequence presented in this communication allow the following statements for totally denatured oligonucleotides: a) homo-oligomers of same chain length show a different mobility in the order C>A>T>G; this holds as well for very short as for long molecules; b) oligonucleotides of any other sequence but same chain length also show different mobilities in the order C>A>T>G with respect to their base composition; c) the effect does not depend on pH (7 to 9), concentrations of acrylamide and urea and ionic strength; d) the effect is dependent on field strength and temperature; e) the effect is additive with respect to base composition; f) the effect is almost independent on base sequence.

It is one important conclusion from the work of Flint and Harrington[19] on gel electrophoresis of DNA, that field strength dependence of relative mobilities might be due to DNA-gel interaction. If this model also holds for the electrophoretic behavior of single stranded linear polynucleotides, interaction with the gel matrix will take place at least partially via nucleobases. The observed order of interaction strength (G>T>A>C) is similar to that described for polystyrene anionexchangers at pH 9.0[20,21] and differs from hydrogen bonding tendency to e.g. cellulose[22]. We therefore suggest nonionic interaction of nucleobases with the polyacrylamide-gel to be responsible for base specific retardation of oligodeoxyribonucleotides during electrophoresis. The minor influence of nucleotide sequence on mobilities (Fig. 5b) is not yet understood by us. Stacking of bases may be of importance. But to confirm this further studies are needed.

If the chain length standards are used for calibration of the gel, electrophoresis should be performed at high voltage and temperature to keep base specific influence at a minimum. In the range of chain lengths up to 50 nucleotides the T-standard fits best with oligonucleotides of not too unbalanced base composition. But in any case each standard allows a more accurate chain length determination than the normally used dye markers.

Without much effort the conditions for electrophoresis can be standardized to obtain a high reproducibility of relative mobilities on denaturing gels. Only temperature control is somewhat critical as specific conductance and temperature of the gel are decreasing during the run. After an appropriate prerun temperature is sufficiently constant and a thermostatically controlled apparatus is not necessary.

Reproducibility and additivity of base specific retardation are the basis of the described calculation procedure for relative mobilities of oligodeoxyribonucleotides. Electrophoresis of more than twenty oligonucleotides of known chain length and base composition gave an excellent correspondance of observed and calculated values (see Tables 1, 2 and 3) and justify to neglect the influence of nucleotide sequence. This method now opens up a new feature of polyacrylamide-gel electrophoresis: the identification of and discrimination between oligonucleotides by their mobility shift relative to homooligomer standards. This may be helpful in various applications of gel electrophoresis but is restricted to oligonucleotides of known or at least evaluable base composition.

One example is the analysis of the three joining reactions shown in Figure 1b. To achieve selective ligation only the 5'-phosphate-"donor" is

labelled and the "acceptor" and the "splint" are employed with free 5'-hydroxyl termini. Thus, also joining procucts are expected to have free 5'-hydroxyl termini. Therefore a second sample of the reaction mixtures is treated with [γ-^{32}P] ATP and T4-kinase prior to electrophoresis. The untreated samples show the labelled donor molecules with their contaminations and the joining products bearing an internal label. In the phosphorylated samples appear in addition the acceptor molecules and splints with their contaminations (see also Figure 4) and the slightly faster moving 5'-phosphorylated joining products. Their mobility can now be compared with calculated values of theoretically possible molecules. The results from analysis of Figure 1b are given in Table 4 and may demonstrate the superiority of acrylamide-gel electrophoresis with a homooligomer marker system in analysing complex reaction mixtures.

A further interesting application is to calculate the mobility differences between oligonucleotides and to estimate the distance of electrophoresis necessary for their separation. This may be of importance for molecules that are expected to have very similar mobilities (e.g. oligonu-

Table 4. Identification of joining products from Figure 1b.

Nucleotide Sequence[a]	Mobility Relative to the T-standard calc.	obsv.	NNA[b]	Yield and Rel.Amount[c] %
Reaction 1			Tp	73
d(TTAAAAGGGATGAATATAAACG)	21.0	21.1		48
d(TTAAAAGGGATGAATATAAAC)	20.0	19.9		26
d(TTAAAAGGGATGAATATAAA)	19.1	19.1		26
Reaction 2			Gp	35
d(GAATATAAACGCGATCCATTTA)	20.6	20.8		45
d(GAATATAAACGCGATCCATTT)	19.7	19.8		15
d(pTATAAACGCGATCCATTTA) [d]	17.7	17.7		30
d(pTATAAACGCGATCCATTT) [d]	16.8	16.7		10
Reaction 3			Gp	65
d(TAAATGGATCGCGTTT)	15.4	15.5		70
d(TAAATGGATCGCGTT)	14.4	14.5		18
d(TAAATGGATCGCGT)	13.4	13.4		7
d(TAAATGGATCGCG)	12.4	12.4		5

a) Sequences were selected under consideration of given data and known contaminations of oligonucleotides.
b) NNA=Nearest Neighbor Analysis.
c) Determined by scanning X-ray films with an ORTEC densitometer and integration of peaks.
d) These products have an unlabelled 5'-phosphate as detected by alkaline phosphatase treatment of a sample resulting in a shift of these spots to slower mobility without the loss of radioactivity.

cleotides of same chain length but different base composition, the two complementary strands of a duplex DNA etc.). The minimal difference between two discrete spots on an autoradiogram depends on their width. This may easily be estimated by comparing the resolution of the standard homooligomer mixture under electrophoretic conditions used. For example the two strands of the chemically synthesized 26 nucleotides long lac operator DNA (Caruthers 1977[23,24]) have the following calculated mobilities

5'TGTGGAATTGTGAGCGGATAACAATT (G-rich)= $T_{25.7}$,A_{29},$C_{30.4}$

3'ACACCTTAACACTCGCCTATTGTTAA (C-rich)= T_{24},A_{27},$C_{28.4}$.

Their mobility difference is equal to that of two nucleotide units at the homooligomer level and is due to the extreme difference in guanosine and cytidine content. The cytidine rich strand is that of higher mobility. This corresponds to the experimental data[25]; the influence of one uridine introduced at position 13 was considered equal to thymidine.

From the nomogram (Fig.7) one can determine the ratio between a calculated mobility difference [cm] and the mobility [cm] of a dye marker. For a minimal difference necessary for separation the corresponding distance of the dye marker is given by the same ratio. In the case of lac operator DNA with a difference of 0.6 cm (12.5 cm XC) and electrophoretic conditions allowing 0.2 cm resolution the separation of the two strands is achieved when xylene cyanol has travelled about 4 cm. The two strands of the AII-DNA, however, have nearly identical mobilities (see Table 3) and accordingly separation on a denaturing acrylamide-gel has been unsuccessful[16].

Electrophoretic strand separation on acrylamide-gels normally is performed under conditions that neither promotes the denatured nor the native form of complementary oligonucleotides: DNA samples are denatured and electrophoresis is carried out at low temperature and voltage on gels that do not contain urea or magnesium chloride. The basis of strand separation is not clear at present. Several authors suggest that secondary structure of the single strands[26,27] or base composition[28] may play an important role. We tried to confirm these results by analysis of the four homooligomer standards on a 20% nondenaturing acrylamide-gel. Autoradiography shown in Figure 2b was compared with electrophoresis of the same samples under denaturing conditions (Figure 2a) and reveals the same relative mobility shift. This shows that urea does not interfere in base specific interaction. We therefore conclude that specific retardation by nucleobases must be one parameter for strand separation. Szalay et al.[28]

reported that mainly strands of higher thymidine contents migrate slower than the corresponding complementary strand. The same was observed with the two strands of AII-DNA[16]. These results correspond to the behaviour of the T- and A-standards on Figure 2b, but the influence of guanosine and cytidine should be much stronger. As this seems not to be the case we suggest that guanosine and cytidine are predominantly involved in formation of specific secondary structure. Thus, mobility of a polynucleotide under nondenaturing conditions may be the result of both effects.

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