
Polyethylene glycol derivatives of base and sequence specific DNA ligands: DNA interaction and application for base specific separation of DNA fragments by gel electrophoresis

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ABSTRACT

Various base pair specific DNA ligands comprising a phenyl phenazinium dye, a triphenylmethan dye and Hoechst 33258 were covalently bound to polyethylene glycol (PEG) via ester or ether bonds. The DNA interactions of the PEG derivatives formed were shown to exhibit the same base pair specificity as the parent compounds. Since the PEG chains thus bound to the DNA could be expected to increase drastically the frictional coefficient of the DNA, the PEG derivatives were used for base specific DNA separations in agarose and polyacrylamide gel electrophoresis. The procedures, which do not require any special techniques, are described in detail. The resolution observed in agarose gels allows one to separate equally sized DNA fragments differing as little as 1% in base composition at mean travel distances of about 10 cm. Examples of gels showing the base compositional heterogeneity of restriction fragments obtained from λ DNA, *E. coli* DNA and calf thymus DNA are given.

INTRODUCTION

Several years ago we could show that a fair number of organic cationic molecules exert pronounced base and sequence specificities on interacting with DNA (1,2,3). It seemed rather obvious to us to make use of these compounds for fractionation of DNA according to base composition in gel electrophoresis and column chromatography. It turned out, however, that the unmodified compounds only exert minor effects. In gel electrophoresis, for instance, base composition dependent mobility changes of DNA fragments due to the presence of such compounds were found to be too small for practical use (11). In hydroxyapatite chromatography intercalating GC specific compounds yielded no better resolution than that obtained in neutral cesium chloride gradients (4).

Improvements were obtained when base specific ligands were incorporated into linear polyacrylamide chains grafted on solid poly-bisacrylamide particles and used as absorbents for DNA affinity chromatography (5,6,7). A more general way of applying these ligands for electrophoretic and

chromatographic procedures was found by linking them chemically to polyethylene glycols (PEG). In agarose and polyacrylamide gels these PEG derivatives exert substantial base composition dependent mobility changes. In liquid-liquid chromatography they act by shifting partition coefficients to an unexpected extent (Manuscript in preparation). The present paper outlines the principles of synthesis of these PEG derivatives and gives some basic results on their DNA interactions and their application in the gel electrophoresis of DNA fragments.

With respect to the latter it should be noted that three base composition dependent electrophoresis procedures have been reported in the literature within the last decade (8,10,12). The first uses the fact that equal sized DNA fragments of substantially different base composition show appreciable mobility differences in agarose-polyacrylamide composite gels (8,9). The second approach uses DNA binding drugs such as antibiotics or "Hoechst 33258" in agarose gels to produce base composition dependent mobility shifts (10), which we found to be too small for general applications, however (11). The third procedure, published by Fischer and Lerman (12) takes advantage of the fact that drastic structural changes of DNA fragments due to loop formation caused by partial denaturation of DNA duplexes practically immobilize the fragments in a polyacrylamide gel.

The technique we describe in the present paper relies on the base composition dependent increase of the frictional coefficient of DNA fragments when complexed with one of the PEG derivatives and is simple enough not to require any special techniques except thermostating the gels during the run. The resolution is mainly governed by the base composition unless strong sequence anomalies as sometimes observed in eucaryotic DNA are present. For such cases the availability of PEG esters of different sequence specificities may provide an advantage.

MATERIALS AND METHODS

Buffers used: TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.8 Tris-NaOAc buffer: 40 mM Tris, 20 mM NaOAc, 2 mM EDTA, pH 7.8 adjusted with acetic acid 40 mM phosphate buffer: 30 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 2 mM EDTA, pH 6.1;

DNAs: a. λ DNA was purchased from New England Biolabs. Digests of this DNA were prepared with restriction enzymes from the same source or from Boehringer, Mannheim, using standard techniques.

b. Hpa I digest of ϕ X 174 RF DNA was obtained from New England Biolabs.

c. DNA from calf thymus was purchased from Boehringer, Mannheim.

d. Bacterial DNAs (M. lysodeicticus-, E. coli- and C. perfringens DNA) were obtained from Sigma Chemical Co. These DNAs were deproteinized by several phenol extractions and stored at 4°C in TE buffer. For preparation of a test mixture consisting of equimolar amounts of these DNAs at narrow size distributions (10^6 and 2×10^6 d) a mixture of the crude DNAs was sheared at 0°C in TE buffer containing 0.2 M NaCl for 4 hours in a Virtis homogenizer at 12,000 rpm at a total DNA concentration of 150 µg/ml. The DNA solution was extracted 3 times with phenol and precipitated with two volumes of ethanol. The precipitate was dissolved in 5 ml of TE buffer and dialyzed. 0.8 mg was loaded on a horizontal slab gel (15 x 12.5 x 1.0 cm) of 1% agarose and electrophoresed at 22°C for 10 hrs. at 1.5 V/cm in the presence of 0.5 µg/ml ethidiumbromide. Strips of 2-3 mm thickness were cut perpendicular to the migration direction at different positions within the broad fluorescent band. The gel strips were dissolved in the seven-fold amount (w/v) of 7 M NaClO₄ by gentle stirring at $\leq 25^\circ\text{C}$. 200 mg of glass-powder (Eagle Ceramics, Bethesda, MD) were added to each solution stirred for 2-3 min, and spun at 2,000-3,000 x g for 10 min. The sediments were washed twice with 2 ml of 7 M NaClO₄ and the sediments extracted with 2 portions of 3-4 ml of TE buffer. The extracts were concentrated by repetitive extractions with n-butanol until a volume of the aqueous phase of 0.5-1 ml was reached. After two additional extractions with chloroform, the DNA was precipitated by two volumes of ethanol chilled to -20°C for 2 hours and centrifuged for 10 min at 3,000 x g. The sedimented DNA was dried for 10 min in a vacuum desiccator over CaSO₄, and dissolved in 0.5 ml of 0.1 x TE buffer. Yield: about 35 µg DNA from each strip. The fractions used for the experiments described had a mean size of 1.9×10^6 d and 0.9×10^6 d (2.9 kb and 1.5 kb respectively).

Polyethylene glycol derivatives: The PEG derivatives used in the experiments described have the structures I, II and III. Their synthesis is shown in principle in the following schemes.

The procedures shown are classical procedures of organic chemistry optimized for the present purposes. (See Appendix) The basic processes follow procedures reported in Beilstein (13) using starting materials of highest purities available. PEG was purchased from Serva, Heidelberg, of a mean mol. weight of 6,000-7,500. The PEG derivatives obtained are dialysed against a slightly acid buffer (10 mM cacodylate, pH 6.0) and stored at 4°C in the dark in solution of 50 to 150 OD/ml. The molar extinction

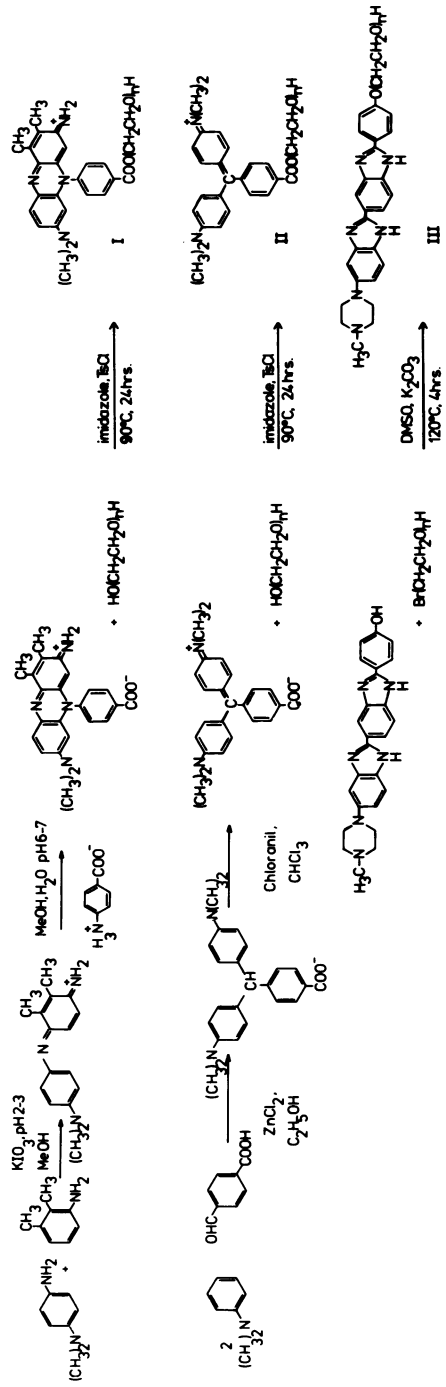
coefficients used for determining concentrations are listed in Table 1. It should be noted that the PEG ester I aggregates appreciably in aqueous media. The value given holds for concentrations $\leq 0.5 OD_{555}/ml$.

The green PEG ester II forms a colorless carbinol form at higher pH. Since the pK of this equilibrium is between 6.1 and 6.6 the photometric measurement of the total ester concentration requires the adjustment of the solution to pH ~ 3 by adding dilute acetic acid and equilibrating for at least 30 min. Adding SDS also shifts the equilibrium towards the carbonium form, therefore yielding total concentrations. Unless otherwise stated, the dye concentration used in gels, etc. indicate the equilibrium concentrations at pH 6.

Binding isotherms for the interaction of the PEG esters with bacterial DNAs: The interaction of the PEG esters with DNAs of various base compositions were studied using the gel filtration method by passing PEG ester-DNA complexes through columns of Sepharose C1 4B. The columns were previously equilibrated with PEG esters at various concentrations and samples of about 0.15 mg of DNA loaded in excess with PEG ester in 0.5 ml passed through the columns at 25 ml/hr under careful thermostating of the column bed. Samples from the fast running complexes were analyzed optically for DNA content and PEG ester content after dissociating the complexes using SDS (2% final concentration).

Gel electrophoresis was performed in Studier-type (14) devices made of plexiglass. The horizontal gels were thermostated by percolating a flat space underneath the plate carrying the gel with water cooled to 2-3°C below the desired gel temperature. The setting was checked by measuring the temperature in the gel using a thermistor. When agarose gels containing PEG esters were prepared, the esters were added to the gel solutions at the end of the annealing period (45-60°C) and the gels poured in the usual way. Two-dimensional gels were set up by pouring the annealed gel around the gel strip containing the DNA-fragments from the first dimensional sizing run. Since the esters migrate slowly in opposite directions to the DNA due to their positive charge, the gel foot on the positive side also contained the PEG ester. The addition of PEG ester to the buffer is useless since it is destroyed at the electrodes unless the space around the electrodes is separated from the residual buffer volume by an extra diaphragm.

Recycling of the buffer in the electrode compartments turned out to be necessary in order to avoid the formation of pH gradients in the gel. Either peristaltic or rubber fitted membrane pumps were used for this



PEG derivative	$\lambda_{\max}(\text{vis})(\text{nm})$		$\epsilon (\text{M}^{-1}\text{cm}^{-1})$
	40mM phosphate, pH 6.1; in 40 mM phosphate, pH 6.1 +2.5%w/v SDS		
PEG ester I	555	44 700	50 000
PEG ester II	637	77 000	89 000
PEG ether III	338	38 000	43 000

Table 1: Absorption maxima and molar extinction coefficients used of the PEG derivatives I-III.

purpose, the back flow in the opposite direction being established by connecting the electrode compartments by 4-8 mm wide tygon tubing.

When vertical polyacrylamide gels were run, the PEG esters were introduced into the gels by soaking the preformed gels (containing 5% bisacrylamide in the monomers) in a PEG ester solution in electrophoresis buffer for 4-5 hours at 60°C in a tape-sealed box. The dilution caused by the gel volume was taken into account when preparing the soaking solution. The gel was cooled down for at least three hours at 4°C in order to restore the original gel size before remounting it into the gel apparatus. For two-dimensional polyacrylamide gels the cylindrical gel from the first dimension run is sealed on top of the second dimension slab gel using a 1.2% agarose solution in electrophoresis buffer. It was found crucial to use an agarose of low electroendosmosis for this purpose; Agarose L from LKB gave perfect results.

For visualizing the fragments, the gels were stained in the usual way by soaking in ethidium bromide (0.5-1.5 $\mu\text{g}/\text{ml}$) solution for 40-100 min. When the gel contains the green PEG ester (II) the ethidium solution should be adjusted to pH 8.5-9 by addition of some sodium bicarbonate. This transfers the green ester into its colorless carbinol form yielding a brighter fluorescence when irradiating the gel with UV light. When the gel contains the red PEG ester (I) the weak background fluorescence of the phenazinium chromophore may be diminished either by prolonged soaking of the gel or by careful reduction of the dye by soaking the gel in 0.04% sodium dithionite solution after staining until the red color has completely disappeared. Since the dye moiety is easily reoxidized by air oxygen, the gel should be covered immediately by polyethylene foil after removing from the dithionite solution. Photography is done with aid of the usual red filter.

RESULTS

Binding studies of PEG derivatives with various DNAs: Parts of the binding isotherms covering the range required for the subsequent studies were measured by the gel filtration technique of Hummel & Dreyer (15) using the same buffer as in the gel electrophoresis experiments at the same temperatures.

In Fig. 1 the partial isotherms of the PEG-derivatives I-III interacting with DNA from M. lysodeicticus (72.5% (G+C)), E. coli (52.5% (G+C)) and C. perfringens (32% (G+C)) are shown as Scatchard plots. The broken lines in these plots connect the binding ratios r obtained with the various DNAs for equal ligand concentrations m , which may be calculated from the reciprocal slopes of these lines. Comparing the r -values obtained for the same values of m reveals higher than first order dependences on the base compositions in all cases. Definite numbers characterizing the specificities are obtained by plotting the logarithms of the r -values against the logarithms of the molar GC or AT content of the DNAs used (not shown). The slopes n of the resultant lines indicate how many base pairs of the same kind form the binding sites occupied at a given value of m (1,2,3). The numbers obtained from the slopes of such plots are listed in Table 2.

Extrapolation of the n -values to $m = 0$ yields $n = 1.64$ for PEG ester I, $n = 1.85$ for PEG ester II and a n -value between 3 and 4 for PEG ether III. These values are in satisfactory agreement with the corresponding figures for the parent compounds: 1.58 for I, (2), 2.0 for II (3), and 3.8 for III (3). In addition, we found only quantitative changes in the sequence specificity as a result of attachment of the PEG side chain: PEG ester II shows no detectable affinity for poly dA · poly dT, while PEG ester III has a comparable affinity for poly (d(A-T)) ($r=0.06$ at $m = 6.5 \times 10^{-7}M$) and poly dA poly dT ($r=0.09$ at $m = 6.5 \times 10^{-7}M$). In the case of both PEG derivatives the specificities agree roughly with those of the parent compounds (3,16).

Applications of the PEG derivatives in gel electrophoresis

Conditions: In optimizing the conditions for the electrophoretic resolution of DNA fragments according to their base composition we had to find an acceptable compromise between the ionic strength of the buffer, the concentration of the PEG derivatives in the gels and the temperature of the runs.

With respect to the ionic strength of the buffer it was found that 40 mM sodium phosphate containing 2 mM EDTA, pH 6, is a suitable medium for all three PEG derivatives. For two dimensional gels, 25 mM EDTA ad-

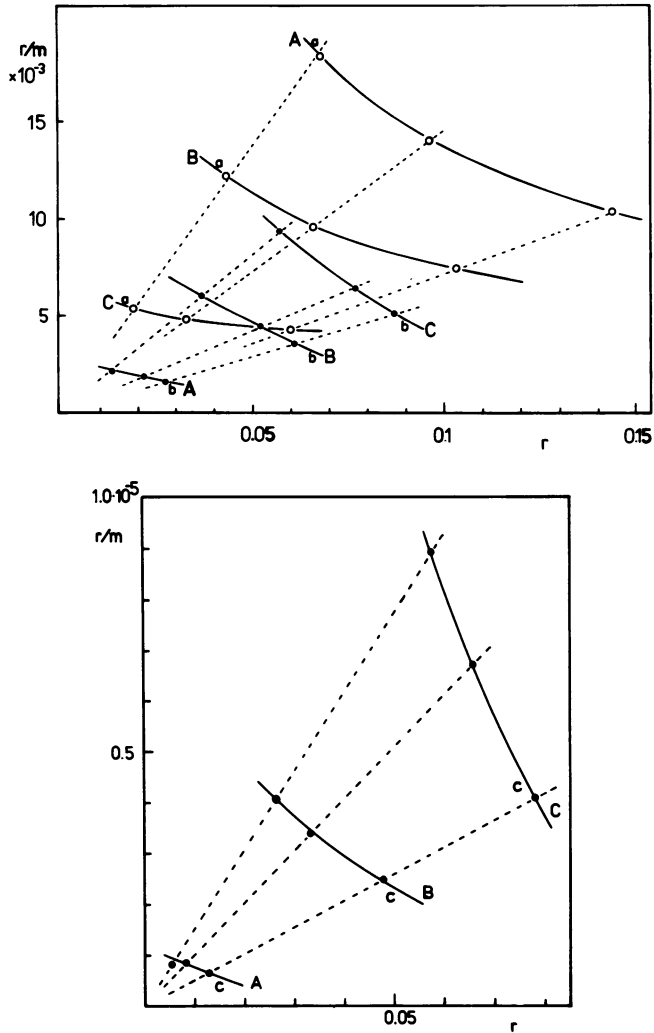


Fig. 1: Part of the binding isothermes of (A) *M.lysodeicticus*-, (B) *E.coli*- and (C) *C1.perfringens* DNA with a) PEG ester I (17°C), b) PEG ester II (10.5°C) and c) PEG ether III (25°C) in 40 mM phosphate buffer 2 mM EDTA, pH 6.0.

justed to pH 5.9-6.0 with sodium hydroxide turned out to be a better medium for the second dimension, since the DNA fragments tend to form circular spots rather than stripes in this medium. Its total ionic strength is similar to the 40 mM phosphate buffer. The following temperatures were adopted for

PEG ester I		PEG ester II		PEG ether III	
m ($\times 10^{-5}M$)	n (GC-pairs)	m ($\times 10^{-5}M$)	n (AT pairs)	m ($\times 10^{-7}M$)	n (AT pairs)
0.349	1.48 \pm 0.05	0.615	1.62 \pm 0.05	6.48	3.1 \pm 0.1
0.698	1.31 "	1.20	1.44 "	9.84	2.4 "
1.40	1.04 "	1.70	1.30 "	19.4	2.02 "

Table 2: Number of adjacent base pairs forming the binding sites for the PEG derivatives I-III in 40 mM phosphate, 2 mM EDTA, pH 6.0 at 17°C for PEG ester I, 10.5°C for PEG ester II and 25°C for PEG ether III.

electrophoretic runs with the different PEG derivatives in order to keep their concentrations in the gels low enough to counteract unfavorable side effects PEG ester I: 16-18°C, PEG ester II: 10-11°C, PEG ester III: 25°C.

The effect of the concentrations of the PEG derivatives in the gels on the resolution is shown in Fig. 2 in which the mobility differences of the fragments C and D from λ DNA-EcoRI digests is plotted against the concentration of the retarding agents. The data were obtained by running

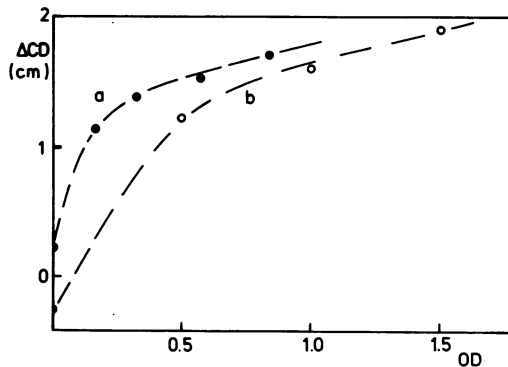


Fig. 2: Differences in relative mobilities between the λ -EcoRI fragments C and D caused by increasing concentrations of a) PEG ester I and b) PEG ester II in agarose gels. The distances between the two fragments observed after a travel distance of 10 cm of the faster one is plotted against the PEG derivative concentration in the gels. (40 mM phosphate, 2 mM EDTA, pH 6, at a: 10°C and b: 10.5°C, 1.5 V/cm).

digests in one-dimensional gels at various concentrations of PEG derivatives I and II and identifying the fragments by comparing the gel patterns with two dimensional gels as shown in Fig. 3. The plots in Fig. 2 show that the optimal resolution is achieved at PEG derivative concentrations which yield r -values between 0.03 and 0.05. Higher r -values produce minor

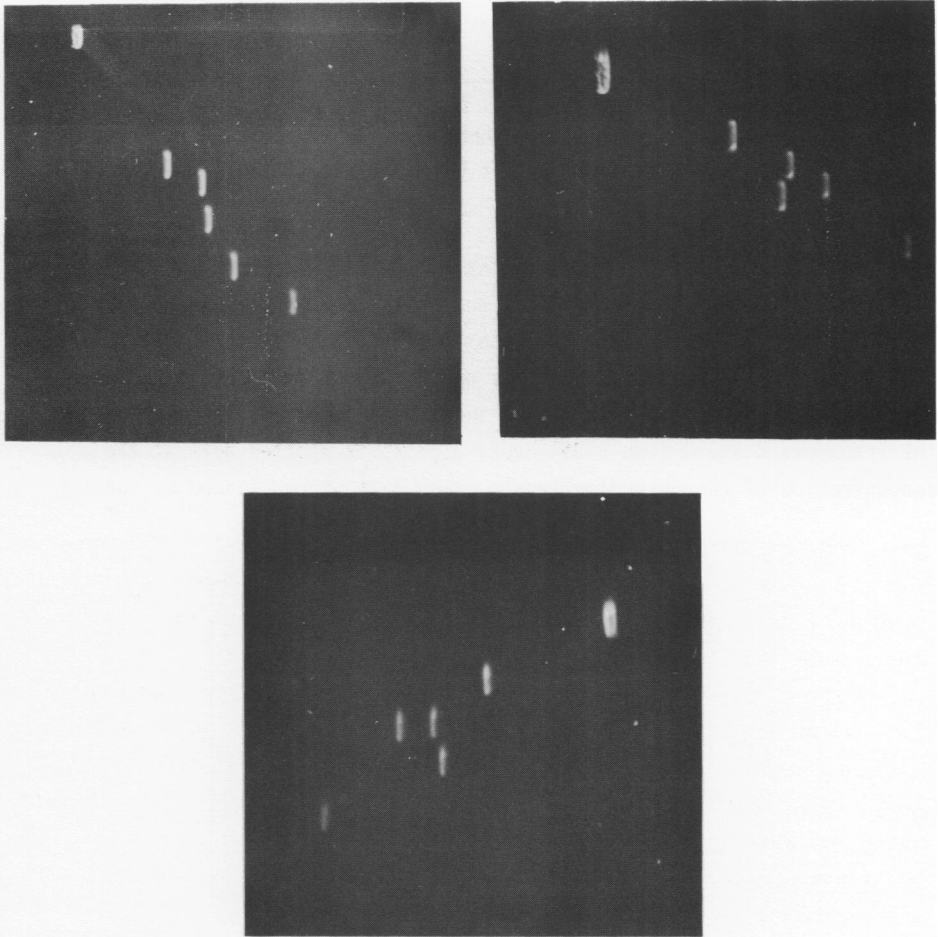


Fig. 3: Diagonal agarose gels of λ -EcoRI fragments in the presence of equivalent concentrations of a) PEG ester I, b) PEG ester II and c) PEG ether III yielding mean binding ratios r of 0.05 in each case. 40 mM phosphate, 2 mM EDTA, pH 6, at 17°C (a), 10.5°C (b) and 25°C (c); 1.5 V/cm, 20 hrs. The first dimension was a sizing gel from left to right (a,b) or right to left (c), followed by a base specific gel from top to bottom.

additional effects only.

Examples: Two dimensional gels of λ DNA fragments formed by EcoRI were run in the presence of the three PEG derivatives at temperatures and concentrations of the PEG derivatives to yield the same binding ratio r of about 0.05 in each case. The patterns shown in Fig. 3 reveal the complementary behavior of the three PEG derivatives, I retarding GC-rich fragments, II and III retarding the AT-rich ones. The stronger retardation of the E fragment (5'th from the origin) by derivative III as compared to II reflects the different sequence specificities of the two agents, implying that the E fragment is relatively rich in non-alternating AT runs.

A similar comparative test with Hae III digests of λ DNA is shown in Fig. 4 and 5 using agarose and polyacrylamide gels, respectively. The better resolution of the smaller fragments in polyacrylamide is evident. Further examples deal with the base compositional heterogeneity of EcoRI and Hind III fragments of *E. coli* DNA. The "diagonal" gels (Fig. 6) run with the PEG derivatives I and III clearly demonstrate the increasing heterogeneity with decreasing fragment size. Hae III restriction nuclease produces fragments smaller in their mean size than EcoRI. The diagonal gel of a Hae III digest run in the presence of PEG derivative III in polyacrylamide is shown in Fig. 7.

Eucaryotic DNAs degraded with restriction enzymes yield distinct bands from repetitive sequences. The diagonal gels of calf thymus DNA digests obtained with EcoRI and Hae III clearly reveal base or sequence inhomogeneities for some of these bands (Fig. 8,9). This also holds for the prominent 1400 b.p. fragment produced by EcoRI from satellite I. A more convincing way to demonstrate the inhomogeneity of this fragment is shown in Fig. 10, in which the bands formed in the sizing gel were cut out and the thin agarose blocks embedded separately side by side in a gel containing the PEG derivative II. Running this gel, the bands were allowed to proceed in the same direction as in the sizing gel, thus increasing the resolution. The pattern clearly indicates the existence of two species of the 1400 b.p. fragment (prominent band, second lane from left) well separated from the broad band containing relative AT-rich background material formed by non-repetitive DNA. It should be noted that this heterogeneity is not observed when the GC specific PEG ester I is used instead of the PEG ester II, suggesting differences in the kind of AT runs in the two subfragments rather than differences in GC content.

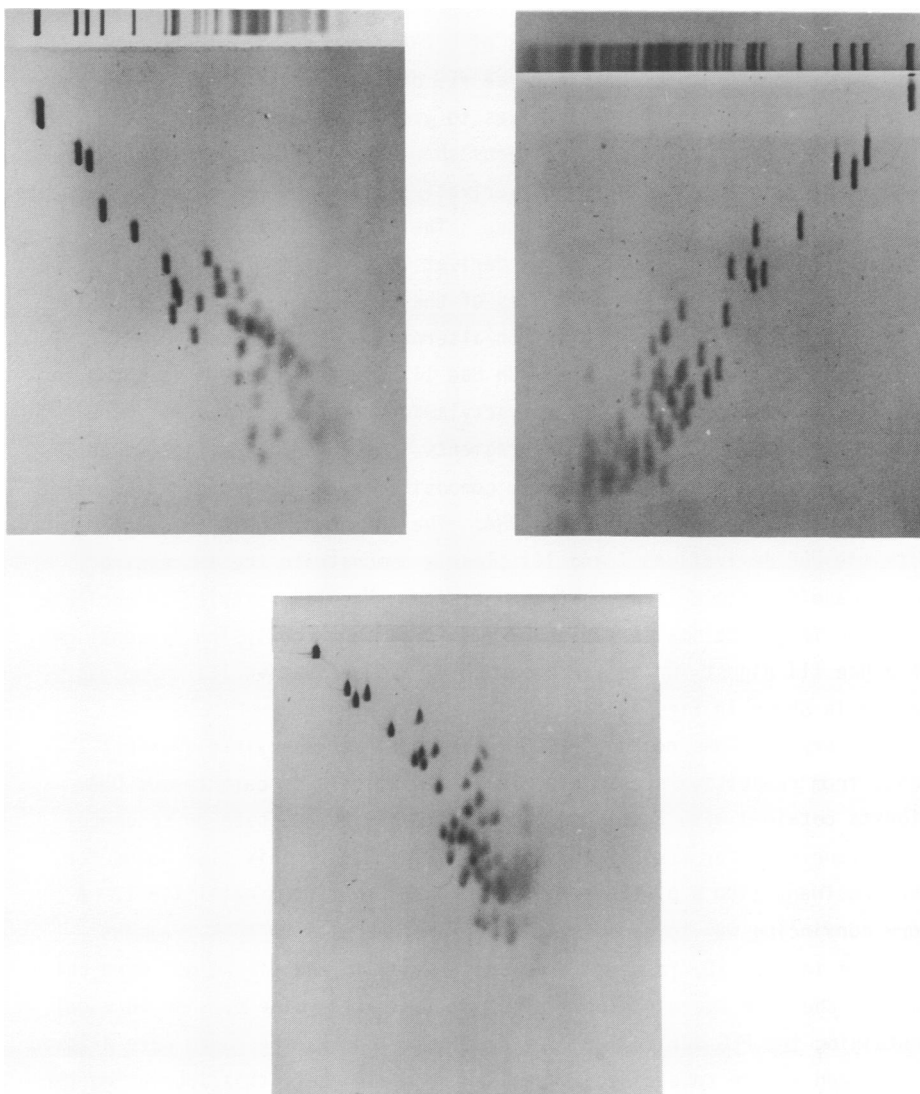


Fig. 4: Diagonal gels of Hae III digests of 5 μ g of λ DNA in 1.8% agarose in the presence of
 a) PEG ester I (8×10^{-6} M, 18°C)
 b) PEG ester II (1.2×10^{-5} M, 10°C)
 c) PEG ether III (6.5×10^{-7} M, 25°C)
 and a and b were run in 40 mM phosphate, 2 mM EDTA, pH 6.0, c was run in 25 mM EDTA, pH 5.9 in the second dimension at 1.5-2V/cm. The patterns from the first dimensional sizing gels are shown on top for a and b. Negative prints of the fluorescence emission are shown for better visualization.

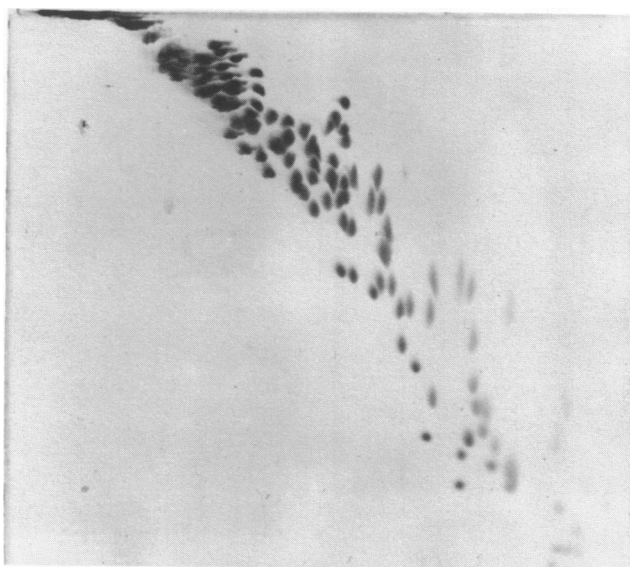


Fig. 5: Diagonal gel of a Hae III digest of 5 μ g of λ -DNA in 5% polyacrylamide (20+1) in the presence of PEG ether III (6.5×10^{-7} M), 25°C, 4V/cm. Buffer: tris-sod. acetate-EDTA, pH 7.8. A negative print of the fluorescence emission is shown.

Application of PEG derivatives for electrophoretic determination of base composition of DNA fragments.

In order to calibrate separations by base composition, we prepared a set of calibration fragments by shearing a mixture of *M. lysodeicticus*, *E. coli* and *Cl. perfringens* DNA and fractionating this mixture by size in a preparative gel as described in Materials and Methods. Samples of 1.9 and 0.9×10^6 d were run simultaneously in the absence and in the presence of one of the PEG derivatives in a horizontal gel apparatus (40 cm in length) divided lengthwise into two compartments. In Fig. 11 such a gel stained with ethidium is shown. PEG ether III was used as retarding agent at 9.8×10^{-7} M concentration in the left half of the gel. The large mobility differences between the three equally sized bacterial DNAs result from about 20% difference in AT content between the three species. For evaluation of the gel, the relative mobilities of the fragments in the absence (v_0) and in the presence (v) of the PEG derivative were measured.

An attempt to correlate the retardation observed with the base compo-

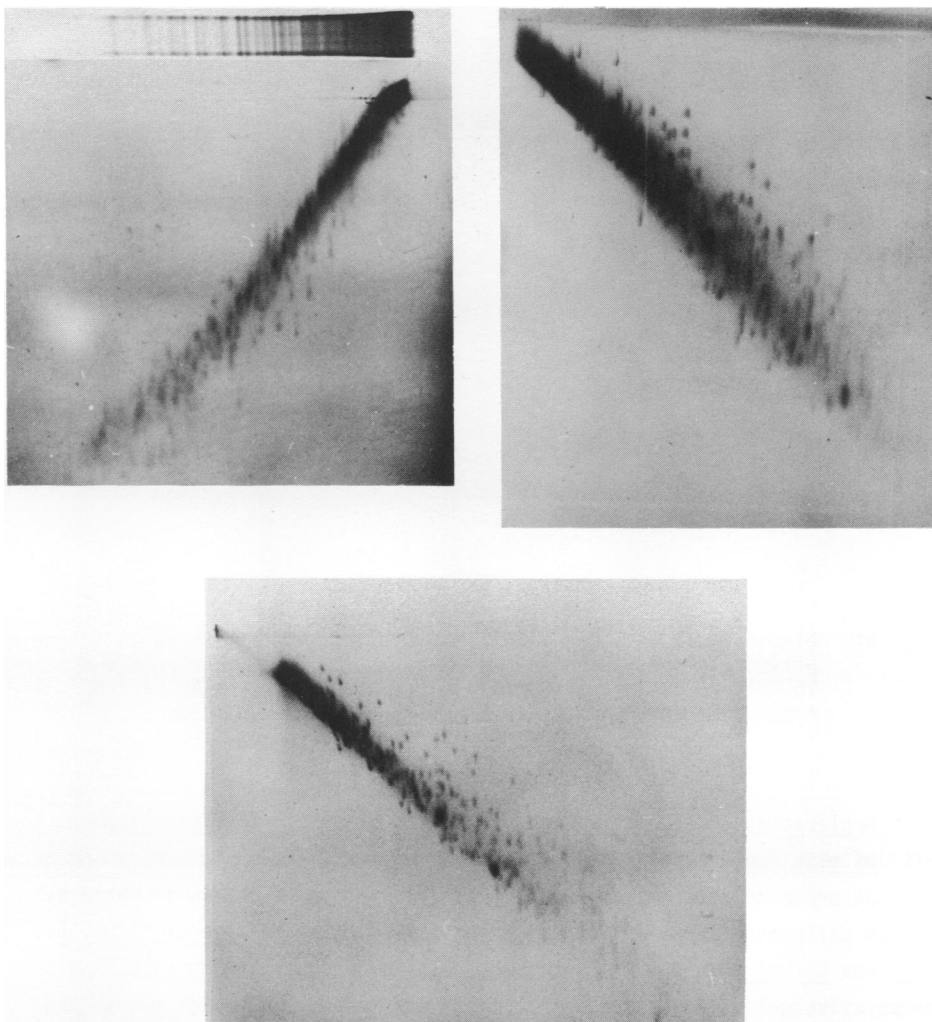


Fig. 6: Diagonal gels of *E. coli* DNA fragments (10 μ g) produced by EcoRI (a and b) and Hind III (c). a in the presence of PEG ester I (4×10^{-5} M) b and c in the presence of PEG ether III (2×10^{-6} M) a was run in 1% agarose at 22°C using 40 mM phosphate, 2 mM EDTA, pH 6 as buffer, b and c were run in 1% agarose at 25°C using 25 mM EDTA, pH 5.9, as buffer. In a the PEG ester I was reduced after ethidium staining using 0.04% sod. dithionite before the UV fluorescence photography. For better visualization reproductions of the negatives are shown.



Fig. 7: Diagonal gel of a Hae III digest of E.coli DNA (10 μg of the smaller and 5 μg of the larger fragments) obtained in 5% polyacrylamide (20+1) in the presence of PEG ether III ($2 \times 10^{-6} \text{M}$) at 4V/cm, 25°C. Buffer: 25 mM EDTA pH 5.9. Reproduction of the negative.

sitions of the fragments was based on the well-known fact, that the product of mobility and frictional coefficient (f_0) of a charged particle (as a DNA molecule) in an electrical field is constant:

$$v_0 f_0 = A \quad (1)$$

Assuming that the introduction of side chains into the particle (as binding PEG derivatives to DNA) acts at low ratios mainly by increasing the frictional coefficient, one might tentatively write for the latter:

$$f = f_0 + kr^\beta \quad (2)$$

in which k is a constant, r the binding ratio indicating the number of PEG side chains introduced per base pair, and β allows for a non-linear power dependence of the frictional coefficient on the number of PEG chains.

Writing the analogous expression to (1) for the complexed DNA fragment using relation (2), forming the ratio of v_0/v and rearranging results in:

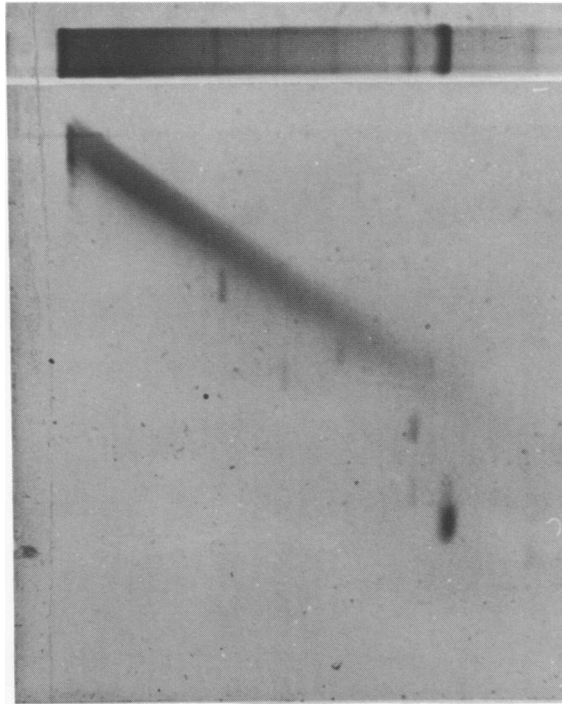


Fig. 8: Diagonal gels of EcoRI digests of calf thymus DNA (10 μ g) in 1.2% agarose using 40 mM phosphate, 2 mM EDTA, pH 6.0 as buffer in the presence of PEG ester II (1.1×10^{-5} M) at 10.5°C. The pattern of the first dimensional sizing gel is shown on top, and a negative print is presented.

$$\frac{v_0}{v} - 1 = \frac{k}{f_0} r^\beta \quad (3)$$

which predicts a simple relation between the retardation ($v_0/v - 1$) and the binding ratio r . If this relation is sufficient to account for the mobility changes observed, a double logarithmic plot of the retardation against r should yield a straight line with a slope of β . In Fig. 12 such plots for DNA fragments from M. lysodeicticus E. coli and C. perfringens DNA obtained with the PEG derivatives I-III are shown.

They indicate that the expected linear relations exist within the limits of error if the PEG derivatives I or III are used. The source of the deviation observed for derivative II is not clear to us; it might be



Fig. 9: Diagonal gel of a Hae III digest of calf thymus DNA in 5% polyacrylamide (20+1) using 25 mM EDTA, pH 5.9 as buffer, in the presence of PEG ether III ($2 \times 10^{-6}M$) 4V/cm, 25°C.

due to technical problems. The values obtained from the slopes are clearly above unity: for PEG ester I a value of $\beta = 1.5 \pm 0.1$ is found while PEG ether III exhibits a value of 1.9 ± 0.1 . The plots in Fig. 12 also reveal a slight increase of the retardation effects with the size of the DNA fragments used. This implies that calibration mixtures of comparable size to the DNA fragments to be studied have to be used for precise determinations of their base compositions.

Obtaining the base composition from the retardation is straight forward. Since $\ln X_{GC}$ is proportional to $\ln r$, so $\ln (v_0/v-1)$ should also vary linearly with $\ln X_{GC}$ or $\ln X_{AT}$. The corresponding scales are shown on the right hand axes of Figure 12a and b. These calibration curves can then be used to determine base composition by measurement of v/v_0 .

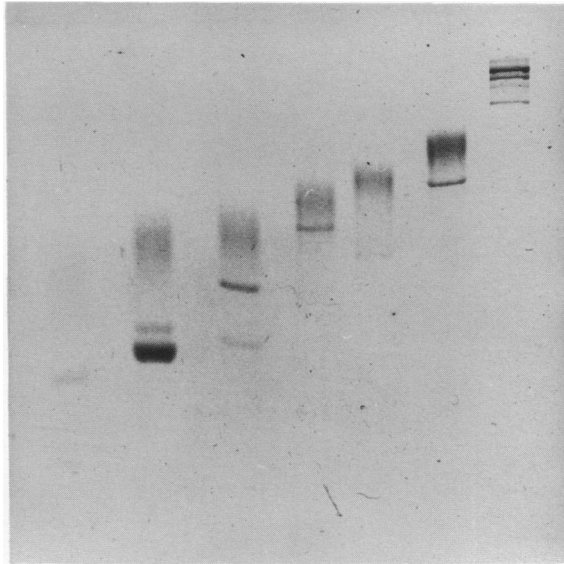


Fig. 10: Electrophoretic separation of the stronger bands formed in a sizing gel (1.2% agarose) of an EcoRI digest of calf thymus DNA. The bands were cut out and embedded into a 1.2% agarose gel containing PEG ester II ($1.2 \times 10^{-3}M$) thus allowing the bands to continue moving in the same direction as in the sizing gel. 2V/cm, 12°C. The slots from left to right, are the prominent restriction bands visible (from right to left) in Figure 9b. The slot on the far right contains the highest molecular weight material (at the far left in the gel at the top of 9b).

From a set of test experiments the base compositions listed in Table 3 were obtained for the two Hpa I fragments of ϕ X 174 RF-DNA as well as for the EcoRI fragments of λ DNA. The figures in Table 3 allow one to estimate that the precision of the method is not better than $\pm 1\%$, mainly limited by the sequence specificities of the PEG derivatives used. The importance of using suitably sized markers is shown drastically by the large difference obtained for the two values for the A fragment of the EcoRI digest of λ DNA (values in brackets). The markers used (0.9 and 1.9×10^6 d, see Materials and Methods) may yield reliable data for the Hpa I fragments of ϕ X 174-RF DNA and the smaller EcoRI fragments of λ DNA (D-F) only.

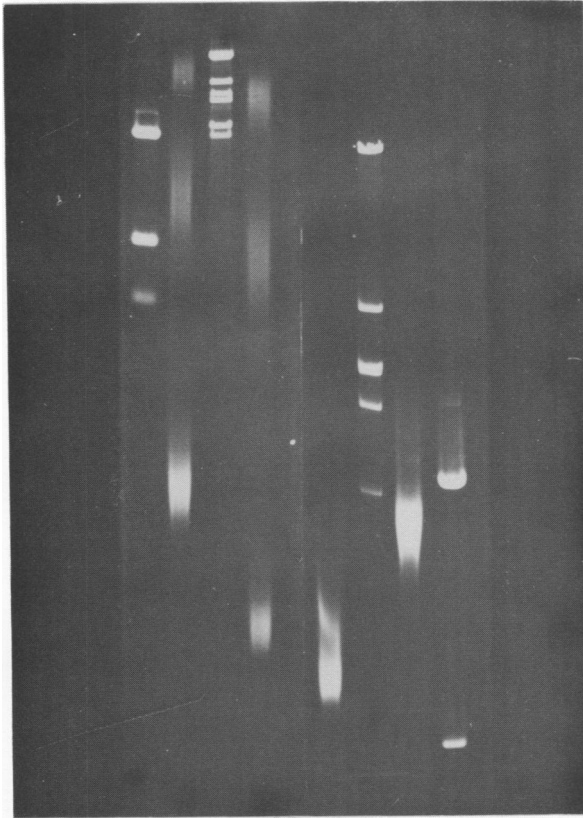


Fig. 11: Lengthwise divided horizontal agarose gel (0.5%) showing the retardation of various DNA fragments by PEG ether III ($1.5 \times 10^{-3} M$) which was added to the left half only. The gel was run at $25^{\circ}C$ in $40 mM$ phosphate, $1 mM$ EDTA, pH 6.0 for 20 hours at $1.5 V/cm$. The different samples consist of (counting from the center to left (1) and right (1')):

- 1 and 1': equimolar mixtures of *M.luteus*-, *E.coli*- and *Cl.perfringens* DNA, $0.9 \times 10^6 d$;
- 2 and 2': *EcoRI* digest of λ DNA;
- 3 and 3': as 1 and 1', but $1.9 \times 10^6 d$;
- 4 and 4': *HpaI* digests of $\phi X 174$ -RF DNA

DISCUSSION

The results shown in the previous sections raise the question of what difference in base composition of two equally sized DNA fragments may still be detected, within reasonable times of electrophoresis under the

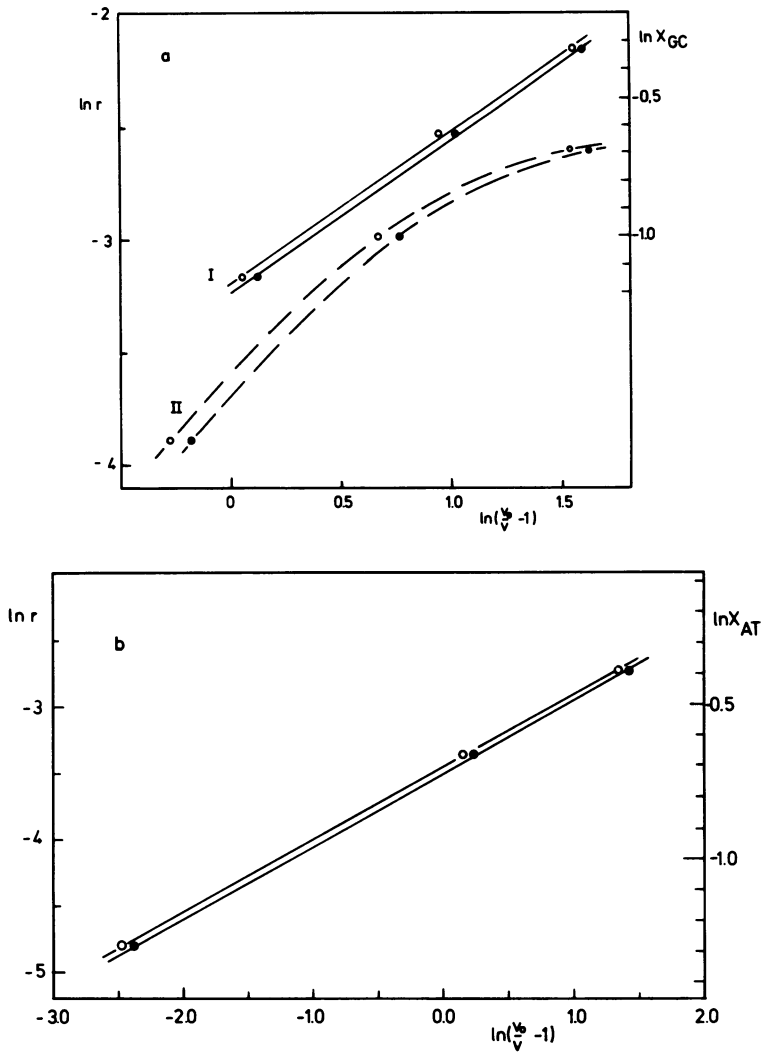


Fig. 12: Logarithmic dependence of the retardations ($v/v_0 - 1$) on the binding ratios r as observed for the bacterial DNAs used in gels as shown in Fig. 11. The full circles correspond to the DNAs of 1.9×10^6 d, the open circles to the DNAs of 0.9×10^6 d. In (a) the effects caused by the PEG esters I (straight lines) and II (curved lines) are shown, in (b) the dependence observed for the PEG ether III is shown. The right hand axis shows the scale corresponding to $\ln X_{GC}$ (a, I) or $\ln X_{AT}$ (b) for the base composition of the three bacterial DNA's. This constitutes a calibration curve, from which the base composition of a fragment of roughly the same molecular weight can be determined by measurement of its v/v_0 .

Source	DNA fragment		% AT content			
	Size kb	M(x10 ⁶)	obtained with PEG deriv I.	from PEG deriv.III	sequence (17)	
ØX174 RF-HpA	I	3.73(18)	2.47	55.1	56.0	55.6
"	B	1.26(18)	0.834	54.4	55.1	55.3
DNA-EcoRI	A	21.8	13.7	(16.5)	(48.7)	---
	B	7.55	4.73	54.8	55.4	---
	C	5.93	3.72	50.0	52.2	---
	D	5.54	3.47	55.5	56.3	---
	E	4.80	3.00	58.3	57.9	---
	F	3.38	2.12	54.4	55.6	---

Table 3: Base composition of various DNA fragments obtained from retardation measurements in agarose gels using PEG derivative I and III and mixtures of M. lysodeicticus, E.coli- and C1. perf. DNA of 0.9×10^6 and 1.9×10^6 d as calibration markers.

conditions given. This may be answered using an expression which relates the normalized fractional change in mobility to the fractional change in base composition. Such an expression may be formed departing from equation (3) which relates the retardation ($v_0/v - 1$) to the binding ratio r . At constant free ligand concentration the binding ratio r may be substituted by the product Kf_{AT}^n in which K is a constant, f_{AT} the molar AT fraction of the DNA fragment in the binding equilibrium and n the number of adjacent AT pairs used by the ligand as binding site. Differentiation and rearrangement yields:

$$\frac{1}{v} \frac{dv}{df_{AT}} = - \frac{n \beta Y}{f_{AT} (1+Y)}, \text{ with } Y = \frac{v_0}{v} - 1 \quad (4)$$

This expression shows that the normalized change of mobility with base composition increases linearly with increasing n and β and decreases with increasing AT content of the fragment.

Relation (8) may be used to calculate the mobility difference between two equally sized DNA fragments differing by 1% in AT at a mean AT content close to 60% which is typical for many eucaryotic DNAs. Using PEG derivative III at 0.9×10^{-6} M concentration in the gel with $n=2.5$ $\beta=1.8$ and $Y=2.0$ the distances travelled by the two fragments differ after 10 cm of mean distance by

$$v = - \frac{2.5 \times 1.9 \times 2.0 \times 10 \times 0.01}{0.6 \times 3.0} = -0.53 \text{ cm}$$

which is easy to detect.

With PEG ester I at $0.7 \times 10^{-5} \text{ M}$ concentration, relation (8) yields with $n=1.3$, $\beta=1.5$ and $Y=2$ a difference of 0.22 cm after the same mean migration distance of the fragments.

Thus the answer to the original question is that for fragments with AT contents in the range from 35 to 65% a difference in base compositions of 0.5% should be detectable, especially if the technique shown in Fig. 11 is applied. This resolution should allow one to locate point mutations in phages or bacterial genomes if the mutational event happened between two restriction sites not more than 200-300 base pairs apart from each other.

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APPENDIX

Preparation of Polyethylene Glycol Derivatives of Base and Sequence Specific DNA Ligands

- I. Polyethyleneglycol ester of 1,2-dimethyl-3-amino-5-(4-carboxyphenyl)-7-dimethyl-phenazinium salt. (Peg derivative I)

- a) Synthesis of the chromophore:

To 4.2 g N,N-dimethyl-p-phenyldiammoniumchloride (anal. grade) dissolved in a mixture of 100 ml of methanol and 8 ml of 2 N HCl, 2.5 ml of 2,3-dimethylaniline (highest purity available, freshly distilled) is added. The excess acid is buffered by addition of 20 ml of acetate buffer, pH 4.6 (1 M in sodium acetate), and the formation of the indamine intermediate triggered by addition of 80 ml of 1/6 M KIO₃ solution. After 10 min. at room temperature the dark green suspension formed is diluted to 800 ml with dist. water, the indamine precipitated by addition of 96 g KBr and collected after chilling in an ice bath for 45 min. by filtration. The crystals are washed with about 100 ml of ice cooled 1 M KBr and dissolved in 800 ml of methanol. 55 g of p-aminobenzoic acid are added under stirring and when dissolved the mixture is diluted to 1300 ml with dist. water and heated gently (500 W) on a magnetic stirrer to 55°C. 40 ml of 1/6 M KIO₃ sol. are added and the blue solution turning to deep red is concentrated under gentle boiling and stirring to a volume of 750 to 800 ml. The solution is cooled to 4°C for 20 hrs, filtered through a Buchner funnel and the black crystalline solid formed rinsed with some ice cold water. The filtrate is concentrated to 750 ml again, kept at 4°C for 15 hrs and filtered. The combined solids are redissolved in 600 ml of 0.2 M Na₂CO₃ by gentle heating and the fine brown precipitation formed after cooling is collected after rinsing with 0.1 M NaHCO₃ and ice water to yield 2.3 g (~27%) after drying over KOH in vacuum. Identified by mass spectroscopy and UV spectroscopy.

- b) Esterification of the chromophore with PEG:

A mixture of 15 g PEG (6000-7500 MW, Serva or MCB) and 10 g of imidazol are melted in a stoppered tube (20 x 3 cm) at 90°C in an oilbath under stirring 120 mg of the dry chromophore obtained under a) are dissolved in the melt (20-30 min.). After addition of 1.2 g tosylchloride the mixture is stirred magnetically at 90°C for 20-24 hrs. A small sample filtered through a column of Sephadex G 25 (0.9 x 15 cm) in 10mM acetate buffer, pH 4.6-5, should indicate from absorbance measurements at 555 nm that more than 95% of the colored material is contained in the fast running exclusion peak. Otherwise further 0.3-0.5 g of tosylchloride are added and the heating continued until the conversion has reached this percentage. The melt is cooled to about 30°

C and dissolved in 300 ml of acetone. After slow addition of 150 ml of ether the mixture is kept at 0°C for 90 min. The solid is filtered through a Buchner funnel, washed with 500 ml of acetone-ether (1:2, v/v) and dried for about 10-15 min. in a gentle air stream. The red solid is dissolved in 700 ml of dist. water and passed through a column (65 x 5 cm) of CM Sephadex C 50 prewashed with dist. water. The absorbed PEG derivative is washed with 2.5-3 L of dist. water and desorbed with several 50 ml portions of 2 M KCl. The eluate of the PEG derivative is concentrated in vacuum at 40°C bath temperature to about 25 ml and dialysed against 10 mM cacodylate buffer containing 1 mM EDTA, pH 5.9-6.0. Yield: 10300 OD₅₅₅ (~90%). (Stable in the dark at 4°C.) Identified by UV spectroscopy.

II. Polyethyleneglycol ester of 4"-carboxy-malachite green (PEG derivative II).

a) leuco-4" -carboxy-malachite green:

A solution of 300 mg of p-carboxy benzaldehyde, 800 mg of anhydrous ZnCl₂ and 0.8 ml of N,N-dimethylaniline (freshly distilled) in 20 ml of absol. ethanol is refluxed under N₂ for 24 hrs. The viscous mixture is suspended in 10 ml of methanol and is adjusted to pH 5 by dil. HCl after adding of 10 ml of dist. water. The slightly greenish crystals formed are removed by filtration, washed with dist. water and dried over KOH in vacuum. Yield 658 mg (88%)

b) 4" -carboxy-malachite green (Chromgrün, C.I. Mordant Green 13):

250 mg of the leucocompound obtained are dissolved in 30 ml of CHCl₃ and 0.5 ml of glacial acetic acid, 200 mg of chloranil are added and the mixture gently stirred at 36°C for 60-80 min. (Best results were obtained by slow rotation of the flask in a water bath). The oxidation product forming a crystalline layer on the glass wall is removed mechanically and the suspension filtered 15 min. after the addition of 10 ml of CCl₄. The crystals are washed with CHCl₃/CCl₄ (1:1) and dried over KOH in vacuum. Yield: 330 mg (The product contains bound CHCl₃).

c) Esterification of 4"-carboxy-malachite green with polyethyleneglycol (PEG derivative II):

120 mg of 4"-carboxy-malachite green are dissolved in a melt of 12 g PEG 6000-7500 (Serva or MCB) at 90°C under magnetic stirring as described for PEG derivative I. 1.2 g of tosylchloride are added and the reaction mixture kept for 26 hrs at 90°C. The test column run in acetate buffer pH 4.5 should indicate complete esterification. The melt is dissolved in 150 ml of acetone and 300 ml of ether are added in small portions at 10°C. The mixture is kept at 0°C for 2 hrs, filtered and the solid washed with 500 ml of acetone-ether (1:2). The air dried product is dissolved in 700 ml of dist. water, 3 ml of acetic acid are added and the solution is filtered in two portions through a column of SP Sephadex 50 (the column is prepared from 10 g of SP Sephadex swollen in 2 L of dist. water, the gel is activated by rinsing with 1 L of 0.05 N HCl and washed with 2 L of dist. water).

The bound PEG derivative is washed with 2.5 L of 0.01 M acetic acid and desorbed with 25 ml portions of 2 M KCl. Part of the product is eluted in a double protonated pale yellow form. After adjusting the pH to 4.6 by sodium acetate, the eluate is concentrated to about 20 ml by vac. distillation (40°C bath temp.) and dialysed extensively against 10 mM cacodylate - 1 mM EDTA, pH 5.5-6. Yield 10 000 OD₆₃₅ in total, measured at pH 5.9. The solution is stable at 4°C in the dark. The product is identified by UV spectroscopy.

III. Polyethylene glycol ether of "Hoechst 33258" (PEG derivative III):

104 mg of Hoechst 33258 are dissolved in a solution of 40 g of PEG bromide⁺ in 120 ml of dry DMSO (0.05% H₂O, Merck, Darmstadt) at 85°C and and kept at 80-85°C after addition of 4 g of dry K₂CO₃ for 2.5 hrs. A sample run on a Sephadex G 25 test column should indicate that more than 95% of the fluorescent material is bound to PEG. The solution is filtered, diluted to 500 ml with dist. water, adjusted to pH 5 with acetic acid and extracted 3 times with 200 ml portions of methylene chloride. The extracts are filtered through siliconized paper (Whatman PA) and the methylene chloride removed by vac. distillation (40°C bath temp.). The viscous residue is diluted to 700 ml with dist. water, residual methylene chloride removed by short term. vac. distillation, and the clear solution passed in two portions through SP Sephadex 50 (60 x 5 cm, see sect. IIc). The bound PEG ether is washed extensively with 0.05 M acetic acid (2-3 L) and desorbed with 2 M KCl in 0.5 M acetic acid applying 25-50 ml portions. The eluates are concentrated to 30 ml by vac. distillation and dialysed against 10 mM cacodylate buffer - 1 mM in EDTA, pH 5.5-6. The solution is filtered (if necessary) through a nitrocellulose filter (0.45). Yield: 6000-7000 OD₃₄₀ (80%) The product was identified by UV and fluorescence spectroscopy.

⁺) prepared according to the procedure of G. Johansson (Biochim. Biophys. Acta 222, 381-389 (1970)) by applying half the amount of thionyl bromide indicated.