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Electrophoresis of DNA in Agarose Gels. Optimizing Separations of Conformational Isomers of Double- and Single-Stranded DNAs†

Paul H. Johnson* and Lawrence I. Grossman

ABSTRACT: The electrophoretic behavior of closed circular, nicked circular, and linear duplex forms of bacteriophages ϕX174 and PM2, plasmid ColEl, and rat mitochondrial DNAs has been analyzed by agarose gel electrophoresis as a function of gel concentration, electric-field strength, and ionic conditions. The logarithm of electrophoretic mobility (μ) is a linear function of gel concentration (T) at all agarose concentrations and field strengths tested. The retardation coefficient ($d\mu/dT \equiv K_R$) is characteristic of DNA conformation under controlled conditions. Alterations in electric-field strength and ionic conditions lead to predictable changes in the relative migration rates of the various DNA forms, thus defining conditions under which their separation can be optimized. The alkaline titration of a mixture of duplex conformational isomers was carried out on gels at neutral pH. Under conditions where closed circular DNA is stable to denaturation, separation of the linear and circular complementary strands of ϕX174, ColEl, and PM2 was observed. Electrophoresis of closed circular DNA in the presence of the intercalating dye ethidium bromide provided an estimate of superhelix density for PM2 DNA in good agreement with values determined by other methods. In addition, the use of ethidium bromide offers additional control over conditions for optimizing separations of DNA conformational forms. At ethidium bromide concentrations above that necessary to remove all negative superhelical turns, the relative change in the electrophoretic mobility with increasing dye concentration appears to be distinguishably different for the closed circular, nicked circular, and linear duplex forms of PM2 DNA. The combined results of this investigation define several experimental conditions and strategies for the application of agarose gel electrophoresis to studies of DNA structure and function.

Agarose gel electrophoresis is a technique of high resolving power for the analysis of DNA structure and function. The electrophoretic migration rate of a macromolecule moving through a gel matrix under the influence of an applied electric field is dependent on molecular size, conformation, and net charge. This technique has found important application in nucleic acid research for the separation and molecular weight determination of specific double- and single-stranded DNA fragments and in the analysis of DNA conformation (Thorne, 1966; Hayward, 1972; Hayward and Smith, 1972; Aaij and Borst, 1972; Helling et al., 1974; Tibbets et al., 1974; Flint et al., 1975; Keller, 1975; Pulleyblank et al., 1975; Depew and Wang, 1975; Bailey and Davidson, 1976; DeLeys and Jackson, 1976; Espejo and Lebowitz, 1976; Meyers et al., 1976; Shure and Vinograd, 1976).

In the present investigation, we have examined the effects of electric-field strength, gel concentration, and ionic conditions on the electrophoretic mobility of various molecular weight and conformational forms of DNA. The results define a set of generalizations which may be used to improve conditions for (a) separation of the major conformational isomers of duplex DNA molecules in the molecular weight range of

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approximately 3–11 × 10^6, (b) separation of the linear and circular complementary strands produced by alkali denaturation of several DNA species, and (c) the use of ethidium bromide in enhancing the separation of closed circular, nicked circular, and linear DNA, and in measuring superhelix density.

These results provide a systematic approach for the use of agarose gel electrophoresis in the study of DNA structure and replication.

Materials and Methods

DNA Preparations

Bacteriophage φX174 replicative form DNA was isolated from *Escherichia coli* C cells infected by φX174 *am*3, an amber mutant of cistron E with a lysis defective phenotype (Hutchison and Sinsheimer, 1966). The phage titer was determined by assay on the amber suppressor strain *E. coli* Hf4714. Wild-type revertants were detected by parallel plating on *E. coli* C; their frequency was less than 10^{-5}. Cells were grown to a concentration of 4 × 10^8/mL in the minimal media described by Knippers et al. (1969) but with potassium phosphate added to 13 μM and phage added at a multiplicity of infection of 10. Ten minutes later, 50 μCi of $H_3{}^{32}PO_4$ (Amersham-Searle) and 30 μg of chloramphenicol (Calbiochem) were added per mL. Cells were collected by centrifugation after 2 h of incubation in a gyrotory shaker at 37 °C and lysed with egg-white lysozyme (Worthington) and Brij 58 (Atlas Chemical) (Komano and Sinsheimer, 1968). The supernatant solution from the high-speed centrifugation was extracted twice with phenol (saturated with 0.5 M Tris-HCl (pH 8.0),[1] 1 mM EDTA, containing 0.1% hydroxyquinoline) followed by ethanol precipitation. φX174 replicative forms were purified by two neutral sucrose gradient centrifugations as described by Johnson and Sinsheimer (1974). Forms I (21 S) and II (16 S) DNA were pooled separately, ethanol precipitated, and dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl (TEN buffer). Form III DNA was prepared as the limit product of the circular forms upon digestion with the restriction enzyme *Pst*I (New England Biolabs, Boston, Mass.).

φX174 viral [^{32}P]DNA was isolated from *am*3-infected cells prepared as described above, except that the chloramphenicol treatment was omitted. Cells were lysed with lysozyme and three cycles of freeze–thawing. The supernatant solution was treated with micrococcal nuclease (500 units/mL, Worthington) in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM $CaCl_2$ at 37 °C; after 30 min, EDTA was added to a concentration of 50 mM, the DNA solution applied to a 1 × 90 cm column of porous glass beads (G-240-50, Sigma), and eluted with 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.2 M NaCl. The peak of radioactivity emerging in the void volume was ^{32}P-labeled phage, which proved to be greater than 90% pure as judged by sucrose gradient centrifugation. The phage DNA was extracted with phenol, ethanol precipitated, and dialyzed against TEN buffer.

ColEl DNA was isolated from the *E. coli* K12 strain JC411 *thy*$^-$, kindly provided by Dr. David Jackson, University of Michigan. The cleared lysate, prepared as described by Clewell and Helinski (1970), was phenol extracted, ethanol precipitated, and purified by sucrose gradient centrifugation and CsCl–EthBr equilibrium centrifugation. ColEl form III DNA was prepared by digestion with the restriction enzyme *Eco*RI.

Bacteriophage PM2 DNA was the kind gift of E. Gay Vasaris of this laboratory. Phage were purified through the poly(ethylene glycol) precipitation step described by Hinnen et al. (1974). The precipitate was suspended in their buffer B and centrifuged to remove cellular debris. The phage were then pelleted at 33 000 rpm in a Spinco Ti60 rotor for 1 h at 0 °C and suspended overnight in buffer B. Phage were further purified by centrifugation through a 5–20% sucrose gradient in 50 mM Tris-HCl (pH 8.0), 2 M NaCl, 5 mM EDTA for 90 min at 23 000 rpm at 10 °C in a Spinco SW27 rotor. Phage banding near the center of the gradient were pooled, diluted with an equal volume of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and pelleted as above. The phage were lysed by resuspending the pellet in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA containing 1% $NaDodSO_4$ and DNA purified by sucrose gradient centrifugation (Espejo et al., 1971). PM2 form III DNA was prepared by digestion with the restriction enzyme *Hpa*II (Johnson et al., 1973; Sharp et al., 1973).

Mitochondrial DNA (mtDNA), extracted from purified mitochondria isolated from adult rat liver, was the kind gift of Brian Nickoloff of this laboratory. Livers were removed and immersed in 0.21 M mannitol, 0.07 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and then disrupted in a motor-driven Potter-Elvehjem homogenizer with a fitted Teflon pestle. Mitochondria were purified by sucrose gradient centrifugation as described by Clayton and Vinograd (1969). DNA was phenol extracted from $NaDodSO_4$-lysed mitochondria, ethanol precipitated, and purified by CsCl–EthBr equilibrium centrifugation. A mixture of forms I, II, and III DNAs was prepared by brief treatment of purified form I DNA with either *Eco*RI or *Hin*$_d$III restriction endonucleases (Moore et al., 1977).

Molecular Weights. We take φX174 RF DNA to have a molecular weight of 3.51 × 10^6, based on 5372 base pairs. From electron microscopic measurements relative to φX174, we take the molecular weights of other DNAs used in this study to be: PM2, 6.68 × 10^6 (PM2/φX174 = 1.904; D. L. Robberson, personal communication); rat mtDNA, 10.6 × 10^6, in analogy with the value for mouse mtDNA (mtDNA/φX174 = 3.03; Brown and Vinograd, 1974). ColEl DNA is taken to be 4.2 × 10^6 based on sedimentation analysis (Bazaral and Helinski, 1968).

Agarose Gel Electrophoresis

Preparation of Gels. The electrophoresis equipment described by Studier (1973) was purchased from Aquebogue Machine Shops, Aquebogue, N.Y. A thin coat of silicone vacuum grease (Dow Corning) was used to seal the spacers in the glass-plate molds. The notched plate was sandblasted so that the gel would preferentially adhere to it when the mold was disassembled. This helped to eliminate gel distortion and to stabilize low-percentage gels in the mold. Gels containing 0.4% agarose could be adequately supported in this way.

A modified gel mold was also used in which three gels of different concentrations could be formed. Three separate compartments were made by the use of two additional spacers placed 4.5 cm in from each side. The lucite comb was cut into three equal pieces, each containing four sample well-formers.

The gel solution was prepared by dissolving agarose (0.6–2.0%, w/w) (SeaKem, Marine Colloids, Inc., Rockland, Maine) in a boiling water bath in buffer (TAE) containing 40

[1] Abbreviations used are: form I, covalently closed circular duplex DNA; form II, nicked circular duplex DNA; form III, linear duplex DNA; EthBr, ethidium bromide; $NaDodSO_4$, sodium dodecyl sulfate; mtDNA, mitochondrial DNA; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

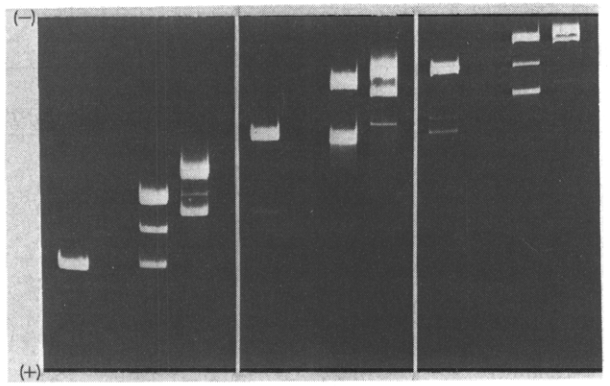

FIGURE 1: Electrophoretic migration of ϕX174 RF, PM2, and mtDNA forms at three agarose concentrations. Electrophoresis was performed simultaneously in a partitioned slab gel apparatus containing, from left to right, 0.6%, 1.0%, and 1.4% agarose. Electrophoresis was for 4.0 h at 4.93 V/cm. The identification of all species and conformational forms for this gel is presented schematically in Figure 3d.

mM Tris base, 5 mM sodium acetate, and 1 mM EDTA (pH 8.2). The solution was then readjusted with deionized water to the original weight and cooled to 55 °C. The gel solution was poured into a mold which had also been equilibrated to 55 °C, and allowed to solidify at room temperature for several hours before use. The gel dimensions were approximately 15.5 × 13.5 × 0.25 cm.

Running of Gels. Sample solutions were adjusted to 5% Ficoll 70 (Pharmacia) and 0.025% bromophenol blue before being applied to each well in 20–50 μL. Electrophoresis was carried out at a constant potential of 25–100 V using a modified Heathkit power supply (Model IP-17). The electrophoresis tank was equipped with an overflow device on the cathode chamber which allowed continuous recirculation of the running buffer between the chambers. This eliminated the significant pH changes which developed during long runs and maintained a constant ion concentration in both chambers. During a sample experiment, electrophoresis of a 2% gel for 16 h at 50 V using 250 mL of TAE buffer containing 25 mM NaCl (pH 8.4) in each chamber yielded a final pH of 8.6 and 8.2 in the cathode and anode chambers, respectively, when recirculation was used. In the absence of recirculation, the cathode buffer was pH 11.6 and the anode buffer was pH 3.5.

Temperature changes within the gels were monitored by an iron–constantan thermocouple (10 Ω external resistance) sealed in a polyethylene capillary and set in the center of the gel. Measurements of gel temperature at 25 and 100 V, using TAE buffer, indicated a small effect of voltage gradient on the temperature during the experiment. For a gel initially at 24 °C, a 25-V potential produced a temperature rise of 2.4 °C in 35 min; at 100 V, the rise was 4 °C in 80 min. No further changes in temperature took place.

Visualization of DNA. After electrophoresis, unlabeled DNA bands were stained with ethidium bromide and ^{32}P-labeled DNA was visualized by autoradiography, both as described (Moore et al, 1977).

Measurement of Mobilities. The electrophoretic mobility was defined as $\mu = d/Et$, where d is the distance migrated in cm, E is the potential gradient in V/cm, and t is the time in seconds.

Films of stained or autoradiographed bands were projected with a photographic enlarger at a total magnification of 5×. Band distances were measured from the origin to the zone center using a Numonics Corp. (North Wales, Pa.) Model 224 electronic digitizer (resolution 0.25 mm) interfaced with a Hewlett-Packard Model 9820A calculator. Mobilities were

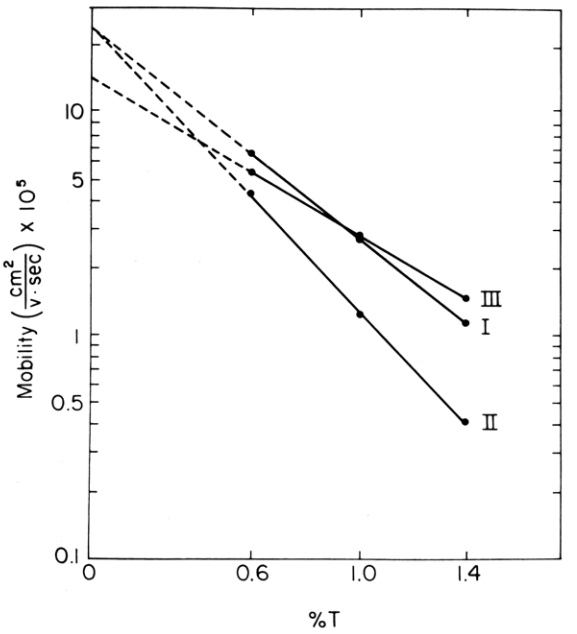

FIGURE 2: Electrophoretic mobility of PM2 DNA forms as a function of agarose gel concentration. The mobilities of PM2 forms I, II, and III were calculated with the band velocity determined by an analysis of distance migrated vs. time. Electrophoresis was performed at 3.29 V/cm as described in the legend to Figure 1. The computed least-squares line (r = 0.99) and the experimental points are shown. The calculated values for K_R are presented in the text.

obtained from the slope of the computed least-squares line of the logarithm of distance migrated vs. time. Retardation coefficients were similarly calculated from the slope of log μ vs. %T.

Results

(*a*) *The Logarithm of Electrophoretic Mobility Is a Linear Function of Gel Concentration*

Ferguson (1964) first demonstrated for proteins that a linear relationship exists between the logarithm of electrophoretic mobility (μ) and gel concentration (T):

$$\log \mu = \log \mu_0 - K_R T$$

where μ_0 is the free electrophoretic mobility and K_R is the retardation coefficient, a quantity which is related to the properties of the gel and the size and shape of the migrating molecule (Rodbard and Chrambach, 1970).

We have measured the electrophoretic mobility of several duplex DNA species in the molecular weight range of 3–11 × 10^6 at gel concentrations between 0.6 and 2.0%. Forms I, II and III were examined in each case. The migration of the various conformational isomers of three of the DNAs used is shown in Figure 1. Each section of a partitioned slab gel (see Materials and Methods) contained a different agarose concentration. Figure 2 shows a graph of log μ vs. %T for the three duplex forms of PM2 DNA, determined from gels similar to that shown in Figure 1. The migration of these DNA forms in agarose gels is demonstrated to obey the Ferguson relationship. The retardation coefficients (K_R) calculated for PM2 were 1.28 ± 0.05 (form II), 0.96 ± 0.02 (form I), and 0.71 ± 0.02 (form III). K_R values for the DNAs examined were found to be reproducible and characteristic of the molecular weight and conformation. A more extensive analysis of K_R as a function of several molecular parameters will be presented elsewhere.

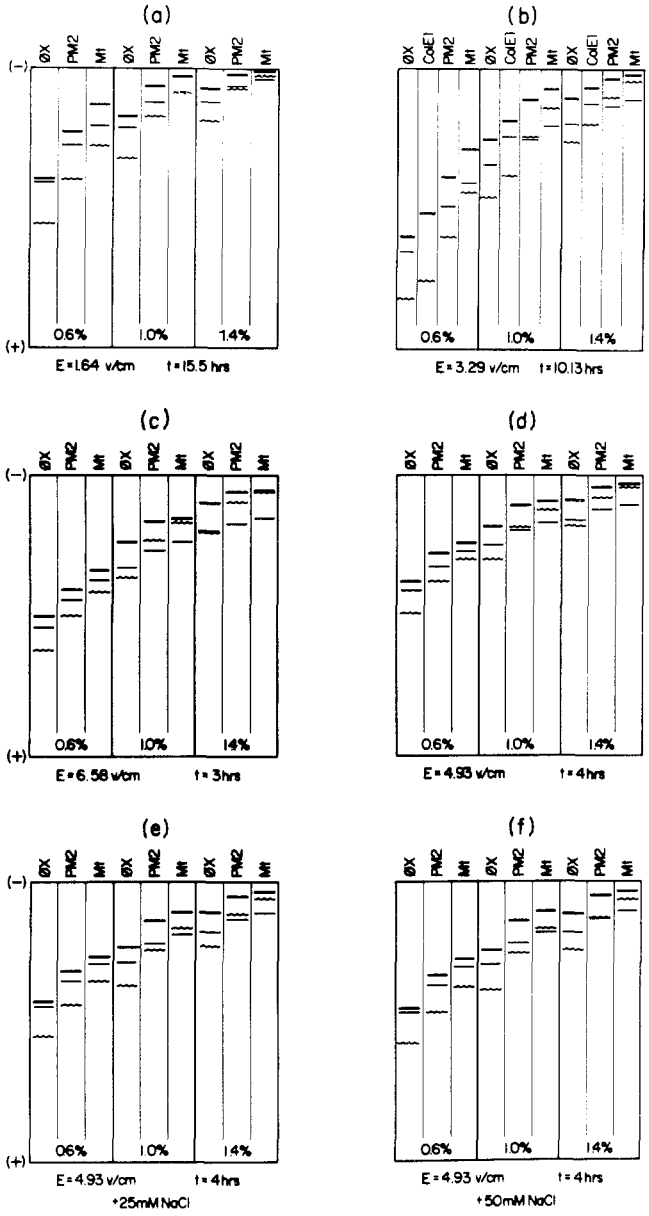

FIGURE 3: Electrophoresis of DNA conformational isomers as a function of agarose gel concentration, voltage gradient, and ionic strength. The figure shows schematic representations of gel electrophoresis experiments at three gel concentrations, four voltage gradients, and three ionic strengths ((a-d) TAE buffer; (e) TAE + 25 mM NaCl; (f) TAE + 50 mM NaCl). The identity of the DNA species is indicated at the top of the gel. Symbols: form I (〰); form II (▬); form III (—). The gel length represented is 15 cm.

Figure 2 also demonstrates that a crossover in the mobilities of forms I and III occurs at 0.92% agarose. This isomobility point defines a gel concentration above which there will be a reversal in the migration order of two species. Linear plots of log μ vs. $\%T$ were also obtained for all forms of ϕX174, ColEl, and mitochondrial DNAs between agarose gel concentrations of 0.6 and 2.0%.

(*b*) *Optimizing the Separation of Duplex DNAs*

Figure 3 is a schematic representation of the relative separation of all conformational forms of the DNAs used in this study at various electric-field strengths. Each slab gel representation demonstrates simultaneous DNA separations at three gel concentrations. Improved separation conditions for complex mixtures of DNAs of varying conformation and molecular weight may thus be evaluated by direct inspection of these diagrams. Several general principles for achieving optimal separations can be defined.

(1) DNA forms I and II of the same molecular weight can be separated under any of the conditions investigated, provided the time of electrophoresis is sufficiently long. Form I DNA is the more compact of the two and thus has the greater mobility and a lower retardation coefficient.

(2) For a given molecular weight, form III DNA has a mobility relative to forms I and III which is highly dependent on gel concentration, as illustrated by the Ferguson plot for PM2 DNA (Figure 2). The results depicted in Figure 3 show that a relative increase in the mobility of form III over forms I and II as the gel concentration is raised takes place at any field strength for the DNA species used in this study. For example, at 1.64 V/cm mtDNA forms I and III reverse in migration order when the gel concentration is raised from 0.6 to 1.4%. A reversal also takes place for ϕX174 forms I and III at 6.58 V/cm between 1.4 and 2% T (data not shown).

(3) Electrophoretic mobility (μ) is a measure of distance migrated at unit field strength and unit time. At a constant field strength, μ is independent of time for DNA in agarose gels (unpublished observations). We have calculated from the results of Figure 3 that the apparent mobilities of all DNAs increase with increasing field strength at a given gel concentration. Table I summarizes these calculations by tabulating the fractional increase in electrophoretic mobility between 1.64 and 6.58 V/cm. For ϕX174 DNA, form III shows the largest fractional increase in μ with increasing field strength, followed by form II, then form I. However, for the higher molecular weight DNAs form II shows the largest increase in μ with an increase in field, followed by form III, and then form I. The practical consequences of these results are illustrated by two examples: (a) for PM2 at 1.0% T, forms I and III reverse in migration order when the electric field is increased from 1.64 to 6.58 V/cm (Figure 3a,b,c); (b) for mtDNA at 1.4% T, an increase in the electric field from 1.64 to 6.58 V/cm dramatically increases the relative separation between forms I and III (Figure 3a,b,c); however, the separation between forms I and II becomes poorer. It seems likely that a further increase in field strength at this gel concentration may reverse the migration order of mtDNA forms I and II.

(4) The apparent electrophoretic mobility of all species examined increases when NaCl is added to the standard

TABLE I: The Effect of Field Strength on the Electrophoretic Mobility of DNA.

DNA form	Fractional increase in μ[a] 0.6% T	1.0% T	1.4% T
ϕX174			
I	1.46	1.53	1.45
II	1.67	1.93	2.00
III	1.78	2.15	2.25
PM2			
I	1.61	1.82	1.83
II	2.31	3.70	3.68
III	2.07	3.10	3.46
mtDNA			
I	1.94	2.64	3.25
II	3.32	6.80	8.13
III	2.32	3.64	5.32

[a] Calculated as the ratio of mobility at 6.58 V/cm to mobility at 1.64 V/cm. Mobilities were calculated from single time-point measurements as presented in Figure 3, and are not corrected for effects resulting from possible delays in the entry of the DNA into the gel matrix.

TABLE II: The Effect of NaCl on the Electrophoretic Mobility of DNA.

DNA form	Fractional increase in μ^a 0.6% T	1.0% T	1.4% T
ϕX174			
I	1.19	1.31	1.42
II	1.20	1.33	1.39
III	1.14	1.19	1.19
PM2			
I	1.25	1.42	1.67
II	1.22	1.30	1.28
III	1.15	1.15	1.10
mtDNA			
I	1.28	1.42	1.51
II	1.18	1.19	1.17
III	1.14	1.11	1.05

[a] Calculated as the ratio of mobility in TAE buffer + 50 mM NaCl to the mobility in TAE buffer, as described in the legend to Table I.

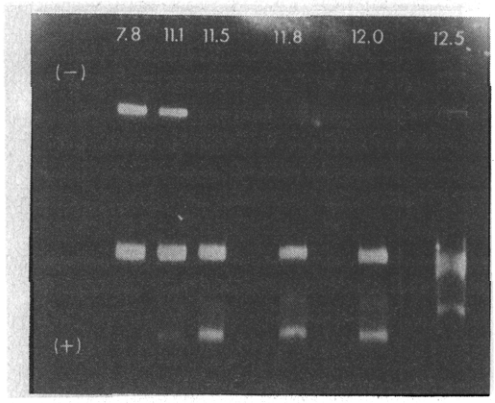

FIGURE 4: Analysis of an alkaline titration of ϕX174 RF DNA by agarose gel electrophoresis. Thirty-microliter volumes of ^{32}P-labeled samples of ϕX174 DNA were adjusted to the pH indicated by the addition of 1 N NaOH, and layered on a 2% gel. Electrophoresis for 20 h at 3.3 V/cm was in standard TAE buffer containing 10 mM NaCl to enhance the separation between single-stranded species and form I.

electrophoresis buffer (Figure 3d,e,f). Table II indicates that each conformational isomer shows a different fractional increase in mobility with added NaCl. The magnitude of this increase is greatest for form I ≥ form II > form III. This effect can be exploited to provide further control of relative separations, as is well illustrated for PM2 DNA isomers. Form I migrates more slowly than form III at 1.0% T and 4.9 V/cm (Figure 3d). Addition of 50 mM NaCl to the electrophoresis buffer causes a reversal in their mobilities such that form I now migrates more rapidly than form III (Figure 3f). An intermediate effect can be achieved by adding a lower concentration of NaCl (Figure 3e).

The increase in electrophoretic mobility of duplex DNA with added salt was unexpected. A possible explanation for this effect as the result of the elevated gel temperature observed under these conditions is currently being investigated.

(5) From the Ferguson equation, the gel concentration which gives maximal separation (T_{max}) between two species A and B occurs when $d(\mu_A - \mu_B)/dT = 0$. It was shown (Rodbard and Chrambach, 1970) that:

$$T_{max} = \frac{\log (\mu_{0_A} K_{R_A})/(\mu_{0_B} K_{R_B})}{(K_{R_A} - K_{R_B})}$$

We calculate using the data obtained from Figure 2 that T_{max} for the separation of PM2 forms I and II is 1.0%, for II and III is 0.86%, and for I and III is 1.5%. Our experience thus far supports these calculations.

(c) *Analysis of Alkaline Denatured DNA. The Separation of Complementary Strands*

Alkaline Titration of DNA. Figure 4 shows an alkaline titration of ϕX174 DNA forms analyzed by agarose gel electrophoresis at neutral pH. The sample was adjusted to the indicated pH values between 11.1 and 12.5.

Denaturation of forms II and III is essentially complete by pH 11.5, and gives rise to two new species which migrate faster than form I. The slower migrating denatured form is indistinguishable from ϕX174 DNA isolated from purified virus, while the faster form migrates identically with viral DNA which was treated briefly with pancreatic DNase I. Denatured form III DNA comigrates with nicked viral DNA, indicating that complementary strands are not being resolved under these conditions. The migration of form I DNA remains unaltered until a pH of approximately 12.5 is reached, when a species with a slightly increased mobility is generated.

These results are consistent with the known behavior of closed and nicked circular duplex DNAs during alkaline titration. Complete titration of form I DNA occurs at pH values higher than required for form II. The greater stability of closed circular DNA results from the inability of the single strands to separate (Vinograd et al., 1968). Exposure of form I DNA to hydroxyl ion concentrations sufficient to fully titrate and denature the molecule leads, on neutralization, to a denatured closed species, called denDNA I, which has a higher sedimentation coefficient in neutral sucrose gradients (Pouwels et al., 1968; Rush and Warner, 1970; Grossman et al., 1974). These previous results suggest a denatured structure which is more compact than native form I, and are consistent with a species having an increased electrophoretic mobility. The faster migration in gels of denDNA I than form I was confirmed using purified denDNA I.

Figure 4 shows two additional results. (1) Under the conditions of this experiment, form II DNA is not completely denatured. We have ruled out the possibility that the species migrating at the form II position in a denatured sample represents a small amount of closed circular DNA containing no superhelical turns, since it behaves indistinguishably from form II DNA during electrophoresis in the presence of ethidium bromide (see next section). This material may represent rapidly renaturing form II DNA. Similar results were observed with ColEl and PM2 DNAs. We note that Grossman et al. (1974) pointed out that a proportion of their PM2 form I DNA, whose amount varied among several preparations, was found as native form I after alkali treatment and reneutralization. Thus, some native DNA preparations appear to contain a proportion of molecules which resist denaturation. (2) Alkali denaturation at pH 12.5 results in the generation of single-stranded circular and linear molecules with a reduced mobility compared to species generated at a lower pH. These results also have been observed under conditions in which form I is absent, and are not explained.

Separation of Complementary Strands. The separation of the complementary strands of PM2 DNA is presented in Figure 5, top. Slots 1–4 show PM2 DNA, denatured by exposure to pH 11.8, which has been electrophoresed for 2, 4, 6, and 8 h, respectively. Denaturation of forms II and III under these conditions generates four new bands, while the mobility of form I DNA does not change. Slot 5 shows the relative distances migrated in 8 h by forms II, I, and III, in order of increasing mobility. Control experiments, shown in slots 6 and

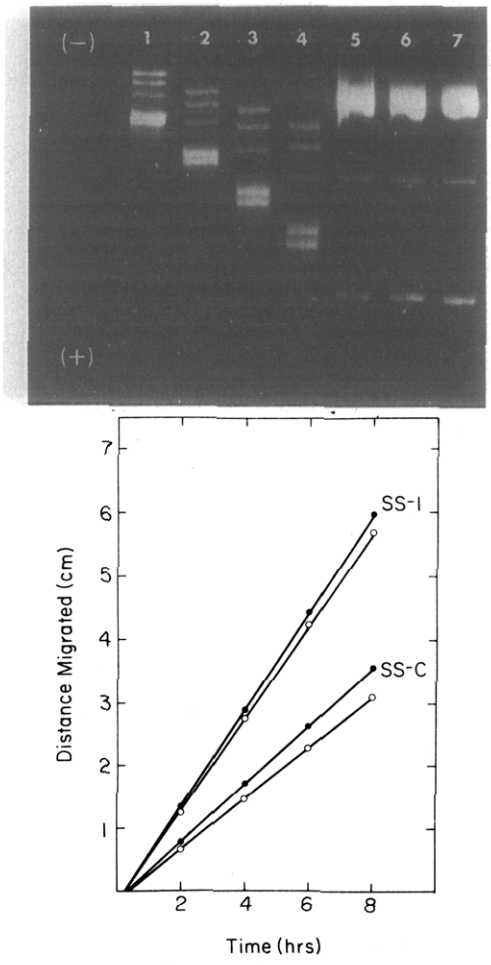

FIGURE 5: Analysis of alkaline-denatured PM2 DNA by agarose gel electrophoresis. (top) An autoradiograph of the gel. Electrophoresis in TAE buffer was through a 1.4% gel at 50 V. Slots 1–4: samples were denatured with NaOH at pH 11.8 and electrophoresed for 2, 4, 6, and 8 h. Slots 5–7: samples at neutral pH contained 0, 0.1, and 0.2 M NaCl, respectively, and were electrophoresed for 8 h. (bottom) A plot of distance migrated vs. time for the single-stranded linear (SS-1) and circular (SS-c) forms. The computed least-squares line ($r = 0.99$) has been drawn through the experimental points. The open circles represent the slower migrating species of each complementary pair.

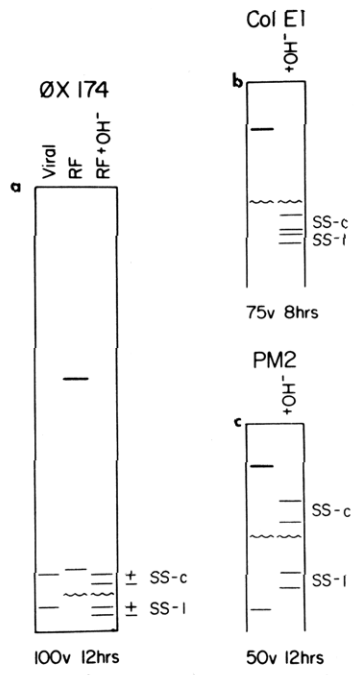

FIGURE 6: Separation of complementary strands of ϕX174 RF, ColEl, and PM2 DNAs by agarose gel electrophoresis. Gels were 1.4% in TAE buffer; times and electric-field strengths are indicated. The gel length for the ϕX174 analysis was 15.85 cm; the other gels were 15.5 cm. Samples were denatured by adding NaOH to pH 11.8 for 10 min. Symbols for duplex DNAs are given in the legend to Figure 3; single-stranded species are identified as the circular form (SS-c), linear form (SS-1), and, for ϕX174, as the viral strand (+) and the complementary strand (−).

7, indicate that there is little or no effect on the mobilities of these duplex DNAs when samples are loaded in TAE buffer containing NaCl up to 0.2 M.

The two fastest migrating species (slots 1–4) were identified as the complementary single-stranded linear forms in the following ways: (1) PM2 DNA contains a single cleavage site for the restriction endonuclease *Hpa*II; denaturation of the limit product of this enzyme gives rise to the two fastest moving bands. (2) The fastest moving bands, when isolated and reannealed, give rise to a species which comigrates with form III. We deduce that the two slowest moving bands are the complementary single-stranded circles, since they can be converted to the faster moving bands by mild endonuclease treatment (data not shown).

The separation between complementary strands increases as a function of electrophoresis time (Figure 5, top, slots 1–4; Figure 5, bottom); thus, the factor(s) responsible for the differential mobility of complementary strands is maintained during electrophoresis, and does not result from loading effects in the presence of alkali. Furthermore, an identical pattern is observed whether or not the denatured sample is neutralized before being applied to the gel.

The mobilities calculated from the data in Figure 5, bottom, are ($\times 10^5$ cm^2/V·s) 1.76 and 1.99 for the circular complementary strands and 3.15 and 3.33 for the linear complementary strands. This experiment documents two further conclusions: (1) migration is a linear function of time for single-stranded DNA and (2) the lines in Figure 5, bottom, do not extrapolate to the origin, suggesting a delay for the entry of DNA into the gel matrix. We have observed similar results for double-stranded DNAs. The significance of entry delays and their effects on calculated mobilities will be presented elsewhere.

Figure 6 shows a schematic representation of complementary strand separations for ϕX174 RF, ColEl, and PM2 DNAs under our best conditions. The required conditions are seen to depend on the molecular weight of the DNA species. Panel a shows the complementary strand separation of ϕX174 RF DNA. Digestion of the duplex circle with the restriction enzyme *Pst*I generates form III as a limit product; alkali denaturation of this linear produces the two fastest migrating species, thus identifying these as single-stranded linears. The viral (+) circular and linear strands are identified by their comigration with viral-extracted DNA. The viral (+) strand is the slower migrating species of the complementary pair for both linear and circular forms.

Panel b of Figure 6 shows the four alkaline denaturation products of ColEl DNA. This DNA is cleaved by *Eco*RI at one site, generating a linear duplex which denatures to give the two fastest migrating species. Results with PM2 DNA similar to that presented in Figure 5 are summarized in panel c.

(*d*) *Electrophoresis of DNA in the Presence of Ethidium Bromide*

The relative migration order of duplex conformational isomers of a given molecular weight DNA depends on field strength and gel concentration (Figure 3). An unambiguous method for identifying closed circular DNA from other con-

formational forms involves electrophoresis in the presence of the intercalating dye EthBr; this procedure also offers additional control over the relative separations of forms I, II, and III.

Figure 7, top, presents an autoradiogram of a series of agarose slab gels in which ϕX174 and PM2 DNA forms have been electrophoresed in the presence of increasing concentrations of EthBr. No dye was present in the running buffer; since the positively charged EthBr molecules move toward the cathode, electrophoresis was terminated before the dye boundary passed the fastest moving DNA species (De Leys and Jackson, 1976; Espejo and Lebowitz, 1976). The mobility of each DNA species shown in Figure 7, top, is plotted as a function of EthBr concentration in Figure 7, bottom. In the absence of dye, each form I DNA has the highest mobility among its isomers. With increasing dye concentration, the mobility of form I decreases to that of form II and then increases. The shape of this curve is analogous to that obtained by sedimentation velocity analysis (Gray et al., 1971).

The minima in the dye titration curves for form I DNAs (Figure 7, bottom) represent the free dye concentrations of EthBr necessary to remove all negative superhelical turns. Such critical free dye concentrations are well known to be related to superhelix density (Bauer and Vinograd, 1968). We calculate a superhelix density for PM2 DNA equal to -0.044,[2] using an association constant $K = 4.53 \times 10^5\ M^{-1}$ interpolated from the data of Hinton and Bode (1975), and their value for the maximum ratio of bound dye to nucleotide, $\nu_m = 0.18$. This value is in good agreement with that determined by Espejo and Lebowitz (1976) using agarose gel electrophoresis and, after correction for salt concentration (Wang, 1969), with the value determined by hydrodynamic methods (Gray et al., 1971; Révet et al., 1971).

Figure 7, bottom, also shows that both form II DNAs and the form III DNA of PM2 decrease in mobility with increasing dye concentration. This effect is analogous to that observed in dye titrations performed by sedimentation velocity, where bound EthBr causes a buoyant effect on the DNA–dye complex (Gray et al., 1971). However, the decrease in electrophoretic mobility of these DNAs in the presence of EthBr is likely to result from charge neutralization and from an increase in the length and stiffness of the DNA molecules.

Several additional effects of EthBr on the electrophoretic migration of DNA are demonstrated in Figure 7, bottom. (a) At high dye concentrations, forms I and II of both species are better separated than in the absence of dye. (b) The absolute mobilities of all species decrease at high dye concentration. We note particularly that the approximate magnitude of this decrease is 33% for ϕX174 form II and 39% for PM2 form II; thus, the position of form II DNA during an EthBr titration does not represent a constant internal reference point. (c) The mobility of PM2 form III decreases proportionately more than form II at increasing concentrations of EthBr, although each form binds equal amounts of dye. This may represent a proportionately greater increase in the Stokes radius of linear molecules under these conditions.

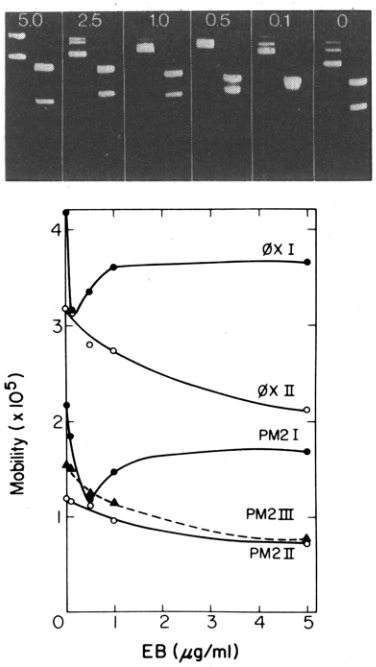

FIGURE 7: Electrophoresis of conformational isomers of ϕX174 and PM2 DNAs in the presence of EthBr. (Top) Electrophoresis through 1.4% agarose in TAE buffer +0.1 M NaCl was for 6 h at 50 V. The sections of two partitioned slab gels contained the indicated EthBr concentration in μg/mL. ϕX174 species are at the right of each panel. In the absence of dye, the species in order of decreasing mobility are ϕX174 RFI > RFII, and PM2 I > III > II. (bottom) Electrophoretic mobilities of the DNAs shown above vs. the concentration of EthBr in the gel.

Discussion

The analysis of nucleic acid structure by zone electrophoresis in agarose gels is becoming an increasingly important method in studies requiring the separation and identification of species differing in molecular weight and conformation. Although there is presently no satisfactory theory to account for the behavior of nucleic acids during gel electrophoresis, several quantitative relationships appear adequately to relate changes in electrophoretic mobility and gel concentration to various molecular parameters (Rodbard and Chrambach, 1970; Chrambach and Rodbard, 1971).

We have demonstrated in this investigation for a range of molecular weight and conformational forms of duplex DNA that the logarithm of electrophoretic mobility is a linear function of agarose gel concentration between 0.6 and 2.0%. Similar results have been obtained for single-stranded DNAs under these conditions (unpublished observations) and in the presence of methylmercury (Bailey and Davidson, 1976).

The extrapolated value of μ obtained from a Ferguson plot is the free electrophoretic mobility, which was shown to be constant for DNA in the molecular weight range 2.5×10^5 to 1.3×10^8 (Olivera et al., 1964); μ_0 values determined in agarose gels, however, are subject to correction for electroendosmotic flow of solvent (Ghosh and Moss, 1974). The determination of μ_0 would appear to be of limited value for the characterization of DNA structure.

Form III DNA has been postulated to migrate end-on during gel electrophoresis (Fisher and Dingman, 1971; Aaij and Borst, 1972; Dingman et al., 1972), an orientation which would minimize its frictional resistance to migration, and consequently yield a lower K_R. This hypothesis is supported by the example of PM2 DNA presented in Figure 2, where the K_R values for the three major conformational forms decrease in the order form II > form I > form III. This order is presumed to reflect the differences in their relative hydrodynamic dimensions during electrophoresis.

The results presented in section c under Results document the utility of agarose gel electrophoresis in analyzing single-stranded DNA. The selective denaturation of forms II and III in the presence of form I, combined with the characteristic

[2] This calculation is based on an unwinding angle of 12° for EthBr intercalation, to allow comparison with the previous literature. However, it now appears that the unwinding angle is approximately twice as large (Wang, 1974; Pulleyblank and Morgan, 1975).

effects on the mobility of form III DNA which accompany changes in gel concentration and field strength, offers a simple strategy for the identification of the major conformational isomers in a mixture of unknowns.

The separation of complementary strands of ϕX174, ColEl, and PM2 DNAs (Figure 6) was achieved most successfully at low DNA mass in the loading volume and using a low ionic strength electrophoresis buffer. Addition of NaCl to this buffer caused a dramatic increase in the mobility of all single-strand DNA species relative to their parent duplex forms, and losses in ability both to resolve complementary strands as well as circular from linear strands of either complement (data not presented).

Complementary strand separation under these conditions is presumed to result from conformational differences between the separated strands arising from differences in base composition and/or nucleotide sequence. This view is supported by the observation that separation of complementary strands increases as a function of electrophoresis time (Figure 5).

Gel electrophoresis in the presence of EthBr offers a further control over conditions which can be manipulated to enhance separations of DNA conformational isomers (see Results, section d). Equally important, it provides a simple and rapid means for determining superhelix density and for distinguishing closed circular, nicked circular and linear duplex DNAs. It is evident from Figure 7b that EthBr levels above the critical free dye concentration are most diagnostic for distinguishing PM2 forms I, II, and III. At these dye concentrations, the mobility of form I increases as positive superhelical turns are generated while the mobilities of forms II and III are differentially decreased.

This investigation further demonstrates the utility of agarose gel electrophoresis in the analysis of DNA structure. Additional studies are necessary to understand more quantitatively the effects of electric-field strength, gel concentration, and ionic conditions on DNA electrophoretic mobility and retardation coefficient, and the influence of changes in DNA size and conformation on these parameters.

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Synthesis and Isolation of DNA Complementary to Nucleotide Sequences Encoding the Variable Region of Immunoglobulin κ Chain†

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ABSTRACT: We have prepared radioactive DNAs complementary to nucleotide sequences encoding the C ($cDNA_{\kappa C}$) and V ($cDNA_{\kappa V}$) regions of the immunoglobulin κ chain produced by the mouse myeloma MOPC 41. Our procedure exploited two technical innovations. First, we used random oligodeoxynucleotides to initiate transcription of DNA from κ chain messenger RNA by RNA-directed DNA polymerase. Since initiations occurred at various sites along the messenger RNA, this procedure circumvented problems encountered when DNA synthesis was initiated on oligo(dT) bound to poly(A) at the 3′ terminus of the messenger. Second, we fractionated $cDNA_{\kappa C}$ from $cDNA_{\kappa V}$ by molecular hybridization with messenger RNA for the κ chain produced by the NP2 variant of mouse myeloma; this RNA contains a deletion affecting the entire V region and therefore hybridizes with DNA complementary to the C region, but not with DNA complementary to the V region. We characterized $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$ by molecular hybridization with RNAs from a series of mouse myelomas synthesizing κ chains with different V regions. $cDNA_{\kappa C}$ hybridized extensively with all of the RNAs tested. By contrast, the divergence of the V genes for the various κ chains was manifest in the extent and rate of hybridization with $cDNA_{\kappa V}$ and in the thermal stabilities of the hybrids. We estimate that a specific $cDNA_{\kappa V}$ can anneal appreciably with only a relatively small fraction of the DNA encoding identified V regions in mice. Consequently, the use of molecular hybridization to enumerate genes for the V region of κ chains can provide only minimum values which may be well below the total number of κ chain V genes.

The constant (C)[1] and variable (V) regions of immunoglobulin (Ig) proteins are encoded in separate genes which must be joined to produce an Ig molecule. C regions are shared by many different antibody molecules and the number of C region genes is limited accordingly. By contrast, V regions display great diversity and the mechanism which generates this diversity remains in dispute; either the entire repertoire of V genes is encoded in germ-line DNA, or a smaller number of genes undergoes somatic mutation during development of the immune system (Williamson, 1976).

Molecular hybridization can be used to study the genesis of diversity in the V region (Leder et al., 1974; Tonegawa et al., 1974; Rabbitts and Milstein, 1975; Rabbitts et al., 1975; Farace et al., 1976; Honjo et al., 1976a; Storb and Marvin, 1976; Tonegawa, 1976) and the transposition of Ig genes required to produce complete Ig proteins (Hozumi and Tonegawa, 1976). In order to facilitate these studies, we have prepared and separated radioactive DNAs complementary to at least a portion of the C region ($cDNA_{\kappa C}$) and the V region ($cDNA_{\kappa V}$) in the messenger RNA ($mRNA_{\kappa}$) for the κ light chain produced by the mouse myeloma MOPC 41 (Figure 1). Our procedure exploited two technical innovations. (i) The transcription of DNA from $mRNA_{\kappa}$ by RNA-directed DNA polymerase was initiated on random oligodeoxynucleotides (Goulian et al., 1973; Taylor et al., 1976). In this procedure, initiations occur at various positions along the mRNA; consequently, a portion of the transcripts will have initiated within the V region and will contain no DNA complementary to the C region. Previously described procedures for transcribing $mRNA_{\kappa}$ used oligo(dT) to initiate DNA synthesis at the 3′ end of the RNA template; under these circumstances, the V region is transcribed infrequently and incompletely, and the resulting transcripts from the V region are covalently linked to DNA transcribed from the C region (Stavnezer et al., 1974; Rabbitts and Milstein, 1975; Schechter, 1975; Farace et al., 1976; Honjo et al., 1976a; Smith and Huang, 1976). (ii) We separated $cDNA_{\kappa V}$ from $cDNA_{\kappa C}$ by molecular hybridization with RNA from the mouse myeloma variant NP2 (Kuehl and Scharff, 1974). The V region is deleted from all of the $mRNA_{\kappa}$ in this variant (Kuehl et al., 1975): consequently, only $cDNA_{\kappa C}$ can hybridize to the RNA, and separation of hybridized from unhybridized DNA by fractionation on hydroxylapatite (HAP) provides both $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$. We also performed a further selection by hybridization with RNA from a mouse myeloma (TEPC 15) whose κ chain is highly diverged from that of MOPC 41 (Gray et al., 1967; Barstad et al.,

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[1] Abbreviations: C and V regions, constant and variable regions of immunoglobulins, respectively; Ig, immunoglobulin; $mRNA_{\kappa}$, messenger RNA for Ig κ chain; cDNA, DNA complementary to $mRNA_{\kappa}$; $cDNA_{\kappa C}$, DNA complementary to portion of $mRNA_{\kappa}$ coding for the C region; $cDNA_{\kappa V}$, DNA complementary to portion of $mRNA_{\kappa}$ coding for the V region; C_Rt, product of concentration of RNA nucleotides in mol/L and time in s; HAP, hydroxylapatite; T_m, temperature required to denature 50% of a duplex nucleic acid; $NaDodSO_4$, sodium dodecyl sulfate; PB, buffer made of equimolar NaH_2PO_4 and Na_2HPO_4, pH 6.8; V_{κ} genes, genes for V regions of κ chains.