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Slalom Chromatography: Size-Dependent Separation of DNA Molecules by a Hydrodynamic Phenomenon†

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ABSTRACT: Slalom chromatography, a size-dependent DNA fractionation method based on a new principle [Hirabayashi, J., & Kasai, K. (1989) *Anal. Biochem. 178*, 336–341], was systematically studied in detail. In this method, larger DNA fragments are eluted much later than smaller ones from columns packed with spherical microbeads. Elution of a series of DNA fragments was systematically examined by using columns packed with polymer-based packings of different diameter and different pore size for high-performance gel permeation chromatography. Packings of smaller diameter proved to be superior for resolving the smaller size range of DNA, while the reverse was the case for larger DNAs. Application of a faster flow rate led to larger retardation of every DNA fragment, while at the lowest flow rate applied (0.067 cm/min), all the fragments were eluted almost at the void volume. When the column temperature was lowered, retardation of DNA became larger. On the other hand, differences in the chemical nature and the pore size of packings, or in the hydrophobicity of the eluting solvent, had little effect on DNA retardation. Size-dependent fractionation of DNA was also achieved even on columns packed with nonporous packings having anionic groups (cation exchangers). In conclusion, these results confirmed the previous conclusion that slalom chromatography is not based on an adsorption or equilibrium phenomenon but should be attributed to a hydrodynamic phenomenon.

High-performance liquid chromatography is useful for both analysis and separation of various materials (from small organic compounds to macromolecules, e.g., proteins, nucleic acids, polysaccharides). This method became feasible only when column packings tolerant of high pressure were developed, and these are usually made of silica or synthetic organic polymers. The method is considerably superior to conventional low-pressure chromatography using open columns in speed, accuracy, reproducibility, and quantitative precision. Various modes of high-performance liquid chromatography are now used. For the separation of biological macromolecules, gel permeation chromatography is one of the most commonly used modes. In this mode, larger molecules are eluted faster than smaller molecules, because the former have difficulty in permeating into the pores of the packing materials. Though it is a powerful tool for protein research, its application to DNA has been avoided because DNA molecules have been believed to be too large for conventional gel filtration materials. Even small DNA fragments have Stokes radii considerably larger than proteins of similar molecular weight.

Recently, we found that double-stranded DNA molecules can be separated according to their size by using a system for high-performance gel permeation chromatography, though the order of elution was opposite to that expected for gel permeation chromatography (Hirabayashi & Kasai, 1989); that is, larger fragments are eluted later than smaller ones. We proposed to name this new mode of separation "slalom chromatography". In the previous paper, we provided evidence that the separation is based on a hydrodynamic phenomenon; the separation significantly depends on the flow rate and particle size of packings, but does not depend on the pore size or the chemical nature of the packings (silica or synthetic polymer). A tentative model of DNA separation in slalom chromatography is illustrated in Figure 1. The existence of open spaces between closely packed spherical particles and also

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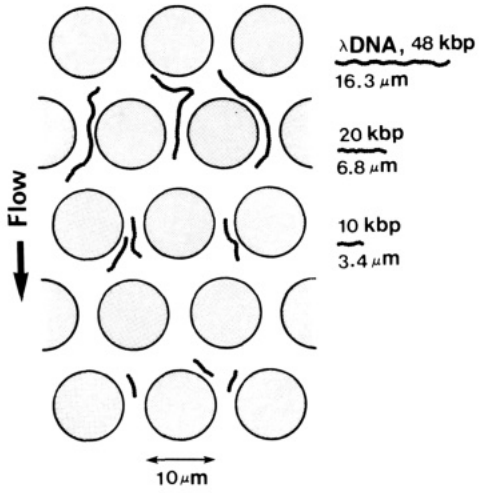

FIGURE 1: Illustration of DNA separation in slalom chromatography. DNA fragments are extended due to the occurrence of laminar flow. They turn frequently in passing through the narrow and tortuous openings between closely packed spherical particles (indicated by shaded particles). The larger the fragments, the more difficult it is for them to turn around the particles. If the DNA molecules are extended to the maximum extent, their length will be comparable to the diameter of packing particles. The distance between particles is exaggerated.

the extension (unfolding) of DNA molecules because of the high flow rate should be critical factors in this new mode of separation. When applied to the column, DNA molecules must flex quickly under a fast flow of moving phase to pass through the openings, which are very narrow and tortuous. If we use a 30-cm column packed with particles of 10-μm diameter, a DNA molecule should turn as many as 36000 times because the number of layers of particles reaches 1200 per centimeter column length. It is quite possible that the longer the DNA molecule, the more difficulty it encounters in passing through the openings, so size-dependent separation in the opposite mode to gel permeation should occur. Since this phenomenon is extremely interesting, we carried out more systematic and more detailed studies. DNA fragments of wider size range and columns of greater variety than those examined in the previous work were used. Not only columns for high-performance gel permeation chromatography but also those for cation-exchange chromatography were used. Nonporous packing was also examined. The results of all the experiments clearly indicated that the diameter of the packing material particles is the only factor that determines the order of elution. In addition, flow rate and temperature had significant effects on the elution position of DNA. This also supports the concept that the mode of separation is based on a hydrodynamic phenomenon.

Materials and Methods

Materials. Wild-type λ-phage DNA [48.50 kilobase pairs (kbp)],[1] molecular weight markers (λ/*Hin*dIII digest), and all restriction enzymes were purchased from Nippon Gene Co. Ltd. (Toyama, Japan). Intact λgt11 phage (Young & Davis, 1983) clone was isolated from a chicken liver cDNA library (Clontech) as a blue plaque on a plate containing isopropyl β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Its DNA (43.77 kbp) was purified from the lysate of infected *Escherichia coli* Y1088 as described (Maniatis et al., 1982). pUC18 (2.68 kbp), pBR322, and a replicative form of M13mp18 phage DNA (7.25 kbp) were originally purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). They were amplified in *E. coli* JM109 and purified as described (Maniatis et al., 1982).

Columns packed with porous polymer packings, Asahipak GS-220, GS-310, GS-320, GS-510, and GS-710 (Wada et al., 1985), were products of Asahi Chemical Industry Co. Ltd. (Tokyo, Japan; column size, 7.6 × 250 mm; particle diameter, 9 μm; exclusion size for pullulan determined in the gel permeation mode, 3×10^3 Da for GS-220, 4×10^4 Da for GS-310 and GS-320, 3×10^5 Da for GS-510, and 1×10^7 Da for GS-710). GS-310 columns packed with particles of different size were also prepared (average particle diameter determined by the manufacturer, 5.0, 9.0, 13.1, and 19.1 μm). Strongly acidic cation-exchange columns carrying sulfopropyl groups on either porous polymer packing, TSK SP-5PW (column size, 7.5 × 75 mm; exchanging capacity, more than 0.1 mequiv/mL gel; particle diameter, 10 μm), or nonporous polymer packing, TSK SP-NPR (column size, 4.6 × 35 mm; exchanging capacity, more than 0.15 mequiv/mL gel; particle size, 2.5 μm), were purchased from Tosoh Co. Ltd. (Tokyo, Japan).

Preparation of DNA Fragments. λ-Phage DNA was digested by restriction enzyme, *Apa*I, *Xho*I, or *Kpn*I, as described previously (Hirabayashi & Kasai, 1989). Plasmid DNAs, pUC18 and pBR322, and a replicative form of M13mp18 phage DNA were linearized by *Eco*RI digestion. After digestion, the enzymes were heat-inactivated (65 °C, 10 min) and removed by extraction with phenol–chloroform (1:1, by volume). DNA fragments were precipitated with ethanol, dried and redissolved in 10 mM sodium phosphate, pH 7, containing 1 mM EDTA (abbreviated as PE buffer) at a concentration of approximately 0.1 mg/mL, and stored at −20 °C until use. Before injection, DNA solution was warmed at 60 °C for 10 min and then placed on ice. Under these conditions, binding between DNA fragments via complementary cohesive sites (*cos* sites) was completely blocked.

Chromatography. Chromatography was carried out at room temperature (23–25 °C) unless otherwise mentioned. A conventional high-performance liquid chromatography system composed of a Tosoh computer-controlled CCPD dual pump and a Tosoh UV-8011 detector equipped with a Shimadzu C-R3A integrator was used. Solutions each containing approximately 1 μg of DNA were applied to columns equilibrated with PE buffer unless otherwise mentioned through a Rheodyne 20-μL injection valve unit, and DNA was eluted with the same buffer.

To examine the effect of temperature, a λ/*Hin*dIII digest was dissolved in 0.1 M Tris-HCl, pH 7.5, and 0.2 M NaCl and applied to an Asahipak GS-310 column packed with 9-μm packing. The column was immersed in a temperature-controlled water bath set at 15, 25, 35, or 45 °C. DNA fragments were eluted in the same buffer at a flow rate of 0.6 mL/min.

To compare the data obtained from various columns differing in column size and/or packing ratio, relative retention time ($R_{RT} = R/R_0$) was calculated for each DNA fragment as described previously (Hirabayashi & Kasai, 1989). R_0 (the retention time corresponding to the void fraction from each column) was experimentally determined by using 4.4 kbp λ/*Hin*dIII fragment, which is always eluted at the void volume.

Results

DNA Elution from a Series of Porous Polymer Columns for High-Performance Gel Permeation Chromatography. In order to study slalom chromatography in detail, DNA fragments of various sizes were prepared, and their elution from various columns for high-performance gel permeation chro-

[1] Abbreviations: kbp, kilobase pair(s); PE, 10 mM sodium phosphate, pH 7.0, containing 1 mM EDTA; R_{RT}, relative retention time.

Table I: Columns Used in Studies on Slalom Chromatography

	column packing						
	GS-220	GS-310	GS-320	GS-510	GS-710	SP-5PW	SP-NPR
expected separation mode	gel permeation	gel permeation	gel permeation	gel permeation	gel permeation	cation exchange	cation exchange
exclusion size (Da)	3×10^3	4×10^4	4×10^4	3×10^5	1×10^7		
average particle size (μm)	9.0	5.0, 9.0, 13.1, 19.1	9.0	9.0	9.0	10.0	2.5
porous or nonporous	porous	porous	porous	porous	porous	porous	nonporous
ligand, if any	none	none	none	none	none	sulfopropyl	sulfopropyl
column size (mm i.d. × mm)	7.6 × 250	7.6 × 250	7.6 × 250	7.6 × 250	7.6 × 250	7.5 × 75	4.6 × 35

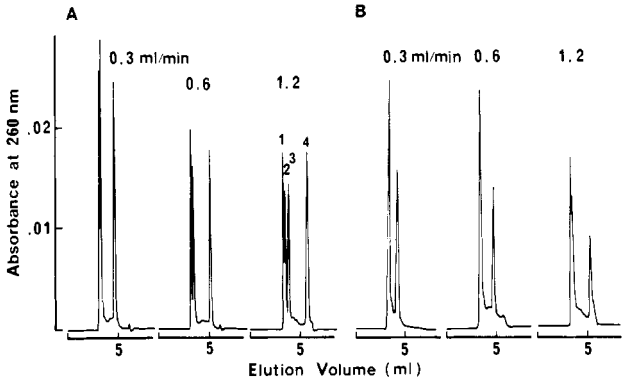

FIGURE 2: Chromatography of λ/*Hin*dIII digests on Asahipak GS-310 columns packed with 5-μm (A) and 9-μm packings (B). DNA fragments were eluted in PE buffer at a flow rate of 0.3, 0.6, or 1.2 mL/min. Fragments contained in peak 1 are 4.36, 2.32, 2.03, and 0.13 kbp. Peaks 2, 3, and 4 correspond to 6.56, 9.42, and 23.13 kbp, respectively.

matography was systematically examined. For this purpose, a series of Asahipak GS columns, which are packed with porous polymer particles developed for high-performance gel permeation chromatography (Wada et al., 1985; see Table I), were used.

First, to examine the effect of particle size, four Asahipak GS-310 columns, packed with packings of different size (average particle diameter, 5.0, 9.0, 13.1, and 19.1 μm), were used (see Table I). Chromatograms obtained for columns packed with 5-μm and 9-μm packings are shown as examples in Figure 2. It is evident that when we use the 5-μm packing and apply a flow rate of 1.2 mL/min, resolution of the fragments of less than 10 kbp is considerably improved. In most cases, retardation of DNA was normalized in terms of the relative retention time as described under Materials and Methods. Figure 3 summarizes the data. All relative retention times are plotted versus DNA length. Size-dependent separation opposite in character to that expected for gel permeation occurred in all columns examined. Figure 3 also shows that the four columns have different ranges of resolution; for example, at a flow rate of 0.6 mL/min (middle curves in Figure 3A–D), the 5-, 9-, 13-, and 19-μm columns resolved DNA fragments larger than 7, 9, 13, and 17 kbp, respectively, from the fragments that appeared at the void volume (R_{RT} = 1.0). In addition, smaller packings showed better resolution for smaller DNA fragments, while larger ones were better for larger fragments. Thus, high-resolution zones can be assigned as 9–17, 15–30, 23–40, and 35–50 kbp for the 5-, 9-, 13-, and 19-μm packings, respectively, at a flow rate of 0.6 mL/min. Each curve can apparently be divided into three zones according to the magnitude of slope, that is, poor-resolution zone, high-resolution zone, and moderate-resolution zone from left to right in Figure 3. The high-resolution zone (middle zone) shows the steepest slope. In the third or final zone, the relationship between relative retention time and DNA length seems to be almost linear. This means that DNA fragments even larger than 50 kbp can be separated in the slalom mode. However, the upper size separation limit of DNA has not been established yet because of technical difficulty. In conclusion, each column packed with a different size of packing particles has an optimum size range for DNA resolution, which corresponds to the high-resolution zone mentioned above.

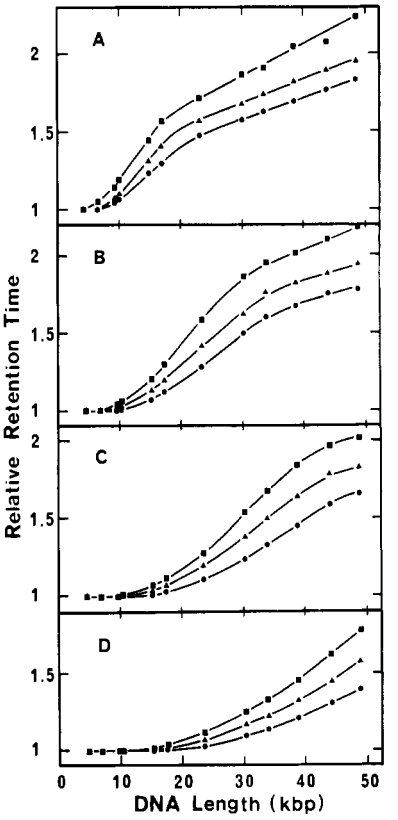

FIGURE 3: Dependence of relative retention times of DNA fragments on their lengths. Packings of different particle size were used, and different flow rates were applied. Relative retention time (R_{RT}) was plotted as a function of DNA length (kbp). Asahipak GS-310 columns were used. Particle sizes are 5 (A), 9 (B), 13 (C), and 19 μm (D). Flow rates are 0.3 (●), 0.6 (▲), and 1.2 mL/min (■).

Next, the effect of pore size and the chemical nature of packings was examined. Asahipak columns packed with packings of the same particle size (9 μm) but differing in pore size, i.e., Asahipak GS-220, -310, -320, -510, and -710 (Table I), were used. GS-310 and GS-320 are supposed to have the same pore size, but differ subtly in chemical nature (Wada et al., 1985). Relative retention times were found very similar among GS-220, -320, -310, and -510 (Table II). On the other hand, values obtained for a GS-710 column were somewhat small in terms of both absolute and relative retention times. This may be explained by partial permeation of some fragments into pores of the packing, because GS-710 has the widest

Table II: Comparison of Retention Times of Several DNA Fragments Eluted from Columns Packed with Various Packings of 9-μm Particle Size[a]

fragment (size in kbp)	flow rate (mL/min)	column packing				
		GS-220	GS-320	GS-310	GS-510	GS-710
λ/*Apa*I (10.09)	0.3	11.34 (1.00)	11.26 (1.00)	10.61 (1.01)	12.22 (1.01)	11.47 (1.00)
	0.6	5.95 (1.01)	5.87 (1.02)	5.58 (1.03)	6.33 (1.01)	6.06 (1.00)
λ/*Kpn*I (17.09)	0.3	12.58 (1.11)	12.21 (1.09)	11.75 (1.2)	13.13 (1.08)	11.94 (1.04)
	0.6	6.90 (1.17)	6.68 (1.16)	6.47 (1.19)	7.13 (1.14)	6.43 (1.06)
λ/*Hin*dIII (23.13)	0.3	14.56 (1.28)	13.78 (1.23)	13.43 (1.28)	14.91 (1.23)	13.17 (1.15)
	0.6	8.19 (1.39)	7.81 (1.36)	7.68 (1.42)	8.43 (1.34)	7.19 (1.19)
λ/*Kpn*I (29.95)	0.3	16.80 (1.48)	15.90 (1.42)	15.69 (1.49)	17.35 (1.43)	14.34 (1.25)
	0.6	9.53 (1.62)	9.13 (1.59)	9.02 (1.66)	9.28 (1.57)	8.02 (1.32)
λ/*Apa*I (38.42)	0.3	18.40 (1.62)	17.90 (1.59)	17.55 (1.67)	19.39 (1.60)	16.16 (1.41)
	0.6	10.25 (1.74)	10.08 (1.75)	9.87 (1.82)	10.75 (1.71)	9.04 (1.49)

[a] Retention times are expressed in minutes. Relative retention times calculated as described under Materials and Methods are indicated in parentheses. DNA elution was performed in 10 mM sodium phosphate, pH 7.0, and 1 mM EDTA.

Table III: Effect of Acetonitrile or Sodium Chloride on Separation[a]

fragment (size in kbp)	added reagent (concn)				
	CH_3CN (20%)	none	none[b]	NaCl[b] (0.25 M)	NaCl[b] (0.5 M)
λ/*Apa*I (10.09)	5.88 (1.00)	6.33 (1.01)	6.02 (1.00)	5.99 (1.00)	6.01 (1.00)
λ/*Kpn*I (17.05)	6.72 (1.15)	7.13 (1.14)	7.65 (1.27)	7.66 (1.28)	7.69 (1.28)
λ/*Hin*dIII (23.13)	8.04 (1.37)	8.43 (1.34)	ND[c]	ND	ND
λ/*Kpn*I (29.95)	9.64 (1.65)	9.82 (1.57)	9.25 (1.53)	9.38 (1.56)	9.46 (1.57)
λ/*Apa*I (38.42)	10.87 (1.86)	10.75 (1.71)	10.16 (1.68)	10.35 (1.73)	19.43 (1.74)

[a] Retention times are expressed in minutes. Relative retention times are indicated in parentheses. Analyses were performed on an Asahipak GS-510 column equilibrated with PE buffer containing the indicated reagent at a flow rate of 0.6 mL/min. [b] Data from Hirabayashi and Kasai (1989). [c] Not determined.

pores among the packings compared (exclusion size determined for pullulan, 1×10^7 Da). The use of packings having extremely large pores seems to be undesirable for slalom chromatography. In such a case, dual modes of separation (i.e., gel permeation mode and slalom mode) would occur at the same time, resulting in a complex separation pattern. Together with the previous results (Hirabayashi & Kasai, 1989), in which columns packed with similar size of silica or polymer packings showed almost the same separation pattern, the present results give firm support to our conclusion that in slalom chromatography neither pore size nor the chemical nature of the packing has a significant effect on the separation of DNA.

Effect of Flow Rate. As demonstrated in the previous work, slalom chromatography is unique because flow rate significantly affects the retention of DNA fragments (Figures 2 and 3). Therefore, we next studied this point in detail. Columns used were Asahipak GS-310 columns of different particle size, but that with 9-μm packing was studied in greatest detail (Figure 4B).

Application of a higher flow rate resulted in a significant increase in the relative retention time of DNA for all columns (Figure 4). This enabled us to obtain better resolution of a fragment of particular interest from the void fraction, though it is improbable that a higher flow rate increases the theoretical plate number. On the other hand, at the lowest flow rate applied (0.03 mL/min, corresponding to a linear flow rate of 0.067 cm/min), all the DNA fragments were eluted almost at the void fraction (Figure 4B). It is notable that there are two zones in the curves in Figure 4B. That is, up to a flow rate of 0.3 mL/min, relative retention time increased steeply with the increase of flow rate, while after that, the increase was gentler and almost linear. Essentially similar results were also obtained for other columns (Figure 4A,B,D). The existence of such highly flow rate dependent zones seems to correspond to a drastic conformational change of DNA molecules occurring at a certain range of flow rate. This point will be discussed later.

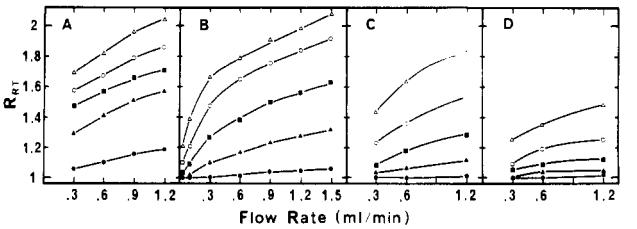

FIGURE 4: Relationship between retardation of the fragments and flow rate. Relative retention time (R_{RT}) was plotted as a function of flow rate. Asahipak GS-310 columns were used. Particle sizes are 5 (A), 9 (B), 13 (C), and 19 μm (D). The fragments used were λ/*Apa*I [10.09 kbp (●)], λ/*Kpn*I [17.05 kbp (▲)], λ/*Hin*dIII [23.13 kbp (■)], λ/*Kpn*I [29.95 kbp (○)], and λ/*Apa*I [38.42 kbp (△)].

Best separation between fragments of 10.1 and 17.5 kbp was attained by using 5-μm packing at all flow rates examined. Other columns also proved to have preferred ranges of resolution; columns packed with 9-, 13-, and 19-μm packings have optimum resolution ranges of 17.5–23.1, 23.1–30.0, and 30.0–38.4 kbp, respectively. A change in flow rate does not seem to shift the high-resolution zone characteristic of each column.

Effect of Organic Solvent. Though we have shown that the size-dependent separation of DNA is not based on electrostatic interaction between DNA and packing materials because of the absence of any effect of increasing ionic strength (Hirabayashi & Kasai, 1989; see also Table III), it remains possible that stronger retardation of larger DNA is the result of stronger hydrophobic interaction with packing materials. If such an interaction does occur, it should be abolished or greatly reduced by increasing the hydrophobicity of the eluting solvent. Therefore, we examined the effect of the addition of acetonitrile to PE buffer by using an Asahipak GS-510 column. However, the addition of up to 20% (v/v) acetonitrile had virtually no effect on the retention times of DNA fragments (Table III). Thus, in slalom chromatography, size-dependent fractionation of DNA is not attributable to hydrophobic interaction with chromatographic media.

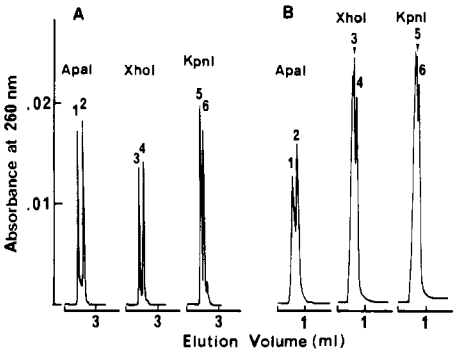

FIGURE 5: Chromatography of DNA fragments on columns for cation-exchange chromatography. The columns used were TSK-SP-5PW (7.5 × 75 mm, porous) (A) and TSK-SP-NPR (4.6 × 35 mm, nonporous) (B). Fragment size: 1, 10.09 kbp; 2, 38.42 kbp; 3. 15.00 kbp; 4, 33.50 kbp; 5, 17.05 kbp; 6, 29.95 kbp. Eluting solvent, PE buffer; flow rate, 0.29 mL/min (A) or 0.11 mL/min (B).

Ionic interaction as a cause of size-dependent separation of DNA molecules was also ruled out, because an increase in salt concentration did not affect the retention times. However, the presence of salt was advantageous from the viewpoint of recovery. Recoveries of λ/*Hin*dIII fragments fractionated by an Asahipak GS-510 column (7.6 × 500 mm) in the presence of 0.2 M NaCl (0.1 M Tris-HCl, pH 7.5, flow rate of 0.6 mL/min) were about 50% for fragments smaller than 10 kbp. These were estimated by the fluorescence intensity of bands stained with ethidium bromide after agarose gel electrophoresis of separated peaks. Since fragments contained in the peak fractions were concentrated by ethanol precipitation prior to electrophoresis, actual recoveries during slalom chromatography seemed to be more than 50%. However, the recovery of the largest fragment (23 kbp) was about 25%. In a previous report, we showed that recoveries of fragments less than 17 kbp were not significantly affected by the change in salt concentration (Hirabayashi & Kasai, 1989). On the other hand, recovery of fragments larger than 20 kbp depended significantly on salt concentration. Recovery at 0.5 M NaCl was about twice that at 0.2 M NaCl. Thus, we can expect recovery of more than 50% even for larger fragments by the addition of a high concentration of salt. The mechanism of such effect of salt has not been clarified.

Chromatography on Porous and Nonporous Cation-Exchange Columns. The above results raised the possibility that slalom chromatography could also be attained by using columns other than those for high-performance gel permeation chromatography. Since cation-exchange columns are of interest because nucleic acids are not likely to bind but rather are repelled, we used two strongly acidic cation-exchange columns bearing sulfopropyl groups, TSK-SP-5PW and TSK-SP-NPR (Table I). The latter is of special interest because it is a column packed with nonporous polymer having a particle size as small as 2.5 μm (Kato et al., 1987). Chromatography was performed at the same linear flow rate as had been applied to the Asahipak columns; i.e., a flow rate of 0.6 mL/min for Asahipak columns corresponds to flow rates of 0.58 mL/min for the SP-5PW column and 0.22 mL/min for the SP-NPR column, respectively, as regards linear flow rate.

Although the columns were very small, they were able to fractionate DNA fragments as well as the longer Asahipak columns, as shown in Figure 5. Curves of elution volume versus DNA size obtained with the SP-5PW column are essentially similar to those obtained with the Asahipak GS-310 column packed with 9-μm packing, reflecting the similar particle sizes (compare Figure 6A with Figure 3B). Both of them resolved an approximately 10 kbp DNA fragment from

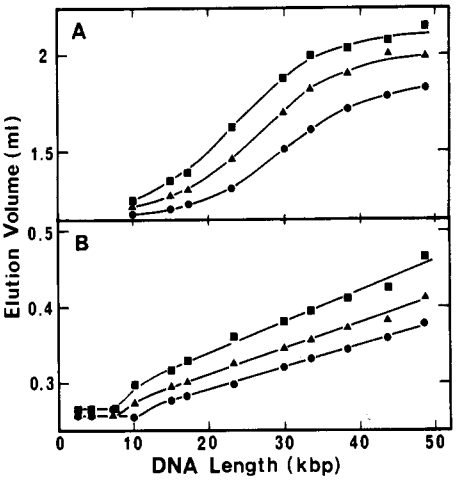

FIGURE 6: Dependence of elution volumes of DNA fragments on their lengths by using cation-exchange columns, TSK-SP-5PW (A) and TSK-SP-NPR (B). Elution volume was plotted as a function of DNA length (kbp). Flow rates for TSK-SP-5PW, 0.29 (●), 0.58 (▲), or 1.16 mL/min (■); those for TSK-SP-NPR, 0.11 (●), 0.22 (▲), or 0.44 mL/min (■).

the void fraction and showed a high-resolution zone in the size range of 15–30 kbp. On the other hand, the curves obtained for the SP-NPR column (Figure 6B) seem to resemble those for the Asahipak GS-310 column packed with 5-μm packing (Figure 3A) to some degree. Thus, we were able to demonstrate that slalom chromatography is achievable even with cation exchanger and nonporous packings. This result also confirmed there is no interaction between DNA molecules and the chromatographic media.

Though detailed experiments have not been done, DNA recovery increased about twice as much when compared with that obtained with other gel permeation columns when low ionic strength PE buffer was used. This is probably due to either the smaller size of the column or the presence of negative charges on packing materials which would prevent undesirable adsorption of DNA fragments. However, in the case of the TSK-SP-NPR column, increased retardation of DNA was observed in the presence of a high concentration (0.5 M) of NaCl (data not shown), though such a phenomenon was not observed in the case of other columns (Table III). This may be due to increased hydrophobic interaction between DNA and isopropyl groups of the cation exchanger at high ionic strength. Thus, when a cation exchanger is used, dual modes of separation (i.e., hydrophobic and slalom modes) would occur.

Effect of Temperature. We also found that temperature had a significant effect on separation. An Asahipak GS-310 column with 9-μm packing was immersed in a water bath, and elution of a λ/*Hin*dIII digest with 0.1 M Tris-HCl, pH 7.5, and 0.2 M NaCl at the flow rate of 0.6 mL/min was analyzed. Fragments smaller than 10 kbp (peak 1 in Figure 7) were all eluted at the void fraction (approximately at 6 min) at every temperature examined. Peak 4 (small compounds such as salt) was also less temperature-sensitive. On the other hand, the elution of a 23.13 kbp fragment (peak 2 in Figure 7) was strongly temperature-dependent. Its relative retention time significantly increased when the temperature was lowered; that is, relative retention times of the 23.13 kbp fragment at 15, 25, 35, and 45 °C were 1.50, 1.38, 1.34, and 1.29, respectively. Under the conditions employed, some of the 23.13 and 4.36 kbp fragments formed a complex via *cos* sites, and elution of this complex (27.49 kbp, peak 3 in Figure 7) was also found to be temperature-dependent; relative retention times at 15,

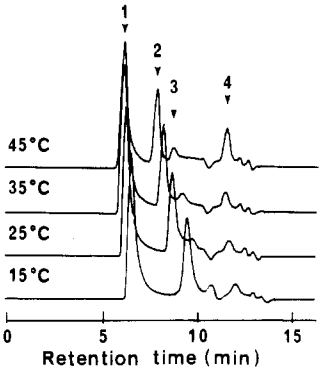

FIGURE 7: Chromatography of λ/*Hin*dIII digest at various temperatures. λ/*Hin*dIII fragments were applied to a column of Asahipak GS-310 packed with 9-μm packings. Elution was performed in 0.1 M Tris-HCl, pH 7.5, and 0.2 M NaCl at a flow rate of 0.6 mL/min. Peak 1, λ/*Hin*dIII fragments smaller than 10 kbp; peak 2, λ/*Hin*dIII fragment (23.13 kbp); peak 3, λ/*Hin*dIII complex fragment (23.13 kbp + 4.36 kbp); peak 4, small compounds such as salt.

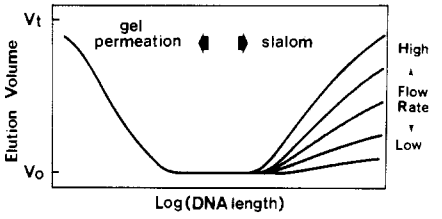

FIGURE 8: Schematic presentation of DNA separation attained by a column for high-performance gel chromatography. A possible relationship between DNA length and elution volume is shown. In slalom chromatography, DNA fragments are separated size-dependently in the opposite order to that which would be expected for the gel permeation mode, and different separation curves are obtained at different flow rates. Note that at a very low flow rate, DNA retardation becomes almost negligible.

25, 35, and 45 °C were 1.69, 1.54, 1.49, and 1.44, respectively. Thus, at lower temperature, retardation of a DNA fragment became more significant. Temperature should affect significantly various physicochemical factors such as solvent viscosity, size of packing (packing ratio), etc., and result in altered retardation of DNA molecules.

Discussion

All the experimental evidence presented in this work supports the previously proposed mechanism of DNA separation, that is, the slalom mode. Critical factors in slalom chromatography were confirmed to be DNA length, particle size, flow rate, and temperature. On the other hand, the pore size and chemical nature of the packing and the composition of the moving phase are not important. Size-dependent separation of DNA molecules is not based on either adsorption or partition but is principally based on a hydrodynamic phenomenon. DNA molecules extended in the flowing medium should turn frequently to traverse the tortuous pathway between closely packed spherical particles, and this will be more difficult for longer molecules, resulting in greater retardation (Figure 1).

The separation behavior of DNA molecules can be summarized as shown in Figure 8. If we use a column for high-performance gel permeation chromatography, very small DNA molecules will be separated in the gel permeation mode. Although retardation of DNA molecules is not significant at a very low flow rate, once the flow rate is increased, fractionation of large DNA molecules based on the slalom mode occurs. Slalom mode fractionation was also demonstrated even on columns of cation exchanger or nonporous packing. Nonporous packing of small particle size (2.5 μm) was expected to be effective for the separation of DNA fragments shorter than 10 kbp. Thus, slalom mode separation of DNA proved to be general, and prospects for its practical application seem to be very promising. This new DNA fractionation procedure may have a number of advantages over gel electrophoresis, the most popular means for DNA separation. It usually takes less than 30 min, and reproducible, accurate, and quantitative data can be obtained easily by using a conventional high-performance liquid chromatography system.

Several size-fractionation methods for macromolecules are known. Of particular interest is one called hydrodynamic chromatography, which has been developed as a size determination method of submicrometer to micrometer order colloid particles, such as lattices (Small, 1974). It is in general performed by using a column packed with nonfunctionalized, nonporous particles of around 15–20 μm in diameter. Its basis has been explained to be as follows (Small & Langhorst, 1982): large colloid particles are excluded from the interface, where the fluid velocity is lowest. The larger the particle, the higher its mean velocity. Consequently, larger particles are eluted faster than smaller ones, as in gel permeation chromatography and opposite to slalom chromatography. Both procedures are a branch of liquid chromatography. However, the role of the packing seems to be very different from that in other chromatographic procedures, because no direct interaction with the packing material is considered to be necessary for separation; all that seems to be required is that the packing should produce microscopic heterogeneity in the flow rate. The following question then arises: Is the presence of a stationary phase essential? In fact, a procedure named capillary hydrodynamic chromatography, which does not require a stationary phase, has been reported as a variation (Noel et al., 1978; Tijssen et al., 1983). Thus, we examined whether size-dependent DNA fractionation is possible or not by using a capillary. However, using a Teflon tube capillary (i.d., 0.25 mm) of 30-m length and flow rates of 0.1–0.3 mL/min, we failed to detect any sign of resolution of λ/*Hin*dIII fragments (data not shown). Though a much longer tube might have been able to attain very slight resolution, it would not be of practical use. In slalom chromatography, the frequent turns of extended DNA molecules passing through the tortuous passageways between closely packed spherical particles (ca. 100 times per second, and ca. 1000 times per 1 cm) seem to greatly facilitate the separation.

Another size fractionation method of DNA, so-called pulsed-field gel electrophoresis, has proved to be applicable to very large DNA molecules such as chromosomal ones (Schwarz & Cantor, 1984; Carle & Olson, 1984). The basis of this procedure is considered to be that, when the direction of an electric field is repeatedly changed, larger DNA molecules require a longer time to adapt to the newly formed electric field and to change their direction of movement (reorientation time). Separation depends on various physicochemical factors, i.e., agarose concentration, temperature (Mathew et al., 1988a), pulse time, electric field strength (Mathew et al., 1988b), electric field shape (Cantor et al., 1988), and DNA topology (Mathew et al., 1988c). We can point out some common features between pulsed-field gel electrophoresis and slalom chromatography; e.g., both methods are based on the fact that the ability of DNA molecules to adapt to a frequently changing environment (direction of electric field or flow) depends on molecular weight, though the frequency is very much higher in slalom chromatography. In other words, both are based on a relaxation phenomenon. It is notable that in pulsed-field gel electrophoresis three different zones of molecular weight dependency similar to those

observed in slalom chromatography (Figure 3) have been reported, though no explanation was proposed (Mathew et al., 1988a).

DNA molecules are known to show dynamic behavior in solution. This was first visualized by Yanagida et al., who developed a method for observing individual DNA molecules under a fluorescence microscope by using a DNA binding fluorescent probe, 4',6-diamino-2-phenylindole (Yanagida et al., 1982). They observed that a DNA molecule in the absence of external force takes a folded structure that fluctuates rapidly due to thermal movement. However, in the presence of laminar flow, it is extended to form a thick or thin filament. Theoretical studies on DNA movement during gel electrophoresis have also postulated DNA movement characterized by cycles of elongation and contraction (Deutsch & Madden, 1989). This was complemented by experimental observations both in a steady state and in a pulsed electric field (Schwarz & Koval, 1989; Smith et al., 1989). Thus, DNA conformation in solution is very flexible and is not fixed.

On the basis of the experimental results and above discussion, we believe that the separation mechanism of slalom chromatography proposed in the previous report is appropriate as a first approximation. Apparently, DNA molecules do not permeate into pores (Table II) or interact with the packing materials (Table III). They simply pass through narrow and tortuous openings which are formed between the closely packed spherical packing particles (Figure 1). As discussed above, when a relatively fast flow rate is applied, DNA molecules should be extended due to the occurrence of laminar flow. For example, the end-to-end distance of a 23 kbp λ/*Hin*dIII fragment is as long as 7.9 μm at maximum, comparable to the diameter of the used packing particle. Such long DNA strands must turn rapidly around packing particles. Simple calculation shows that DNA fragments turn 33 000 times if applied to a column of 250-mm length packed with 9-μm packing. Simultaneously, the 23 kbp fragment turns as frequently as 70 times per second, when a flow rate of 0.6 mL/min is applied. On each turn, DNA strands should be exposed to a significant frictional force against the solvent, and this force would increase with the increase in DNA length so that larger DNA molecules are retarded (Figure 3). Application of a faster flow rate increases the average end-to-end distance of DNA, and, therefore, DNA retardation becomes greater (Figure 4). When smaller packing materials are used, DNA strands should turn more frequently in the openings, which would be narrower and more tortuous. Thus, a stronger frictional force should be generated between DNA strands and solvent. This may explain why smaller DNA molecules are better resolved by smaller packings. Possible occurrence of a higher flow rate gradient in the laminar flow may also contribute by extending the DNA molecules more efficiently. Although the explanation presented here seems to be too much simplified and straightforward, it should be a useful model to solve a precise mechanism of slalom chromatography.

One of the alternative models is as follows. Larger DNA molecules require a longer reorientation time as in the case of gel electrophoresis. In a closely packed column, there exists macroscopic heterogeneity in the flow rate of solvent. According to the Bernoulli principle, macroscopic velocity of the solvent will be lower in the wider openings than in the narrower openings. DNA molecules may have to change their shape depending on such perturbation of flow rate. Larger DNA molecules may have more difficulty in responding to such a situation, resulting in retardation.

Both theoretical and experimental approaches will be necessary to elucidate in detail the separation mechanism of slalom chromatography. We are also trying to construct a mathematical model. From a practical point of view, optimization of DNA recovery and extension of the range of application are of particular importance. Separation of DNA molecules by their conformations, e.g., supercoiled and relaxed, should be challenged. Development of chromatographic media being able to give variety of flow properties is also awaiting.

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