High-Resolution Separation and Accurate Size Determination in Pulsed-Field Gel Electrophoresis of DNA. 4. Influence of DNA Topology†

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ABSTRACT: Pulsed-field gel electrophoresis is a powerful technique for the fractionation of linear DNA molecules with sizes above 50 kilobase pairs (kb). Here it is demonstrated that this technique is also effective for separating smaller DNAs including linear, circular, and supercoiled species. The mobilities of linear DNAs larger than 8 kb can be modulated by pulse times between 0.1 and 100 s. The mobility of supercoiled DNA molecules up to 16 kb is generally unaffected by these pulse times except that 10-s pulse times cause a small but distinct increase in the mobility. The general insensitivity of small supercoiled DNAs to pulse time presumably occurs because these species reorient so rapidly that they spend most of their time undergoing conventional electrophoresis. However, the mobilities of larger supercoiled DNAs are affected by pulse times of less than 1 s, and at 0.1 s the molecules are better resolved by pulsed electrophoresis than by ordinary electrophoresis. The mobility of 3–19 kb nicked and relaxed circular DNA molecules is also affected by pulse time but in a complex way.

Pulsed-field gel electrophoresis (PFG)[1] has proven extremely powerful in the analysis of large DNA molecules from a variety of sources, including intact chromosomal DNAs from fungi (Schwartz et al., 1983; Schwartz & Cantor, 1984; Carle & Olson, 1984, 1985) and parasitic protozoa (Van der Ploeg et al., 1984; Smith et al., 1987c; Vollrath & Davis, 1987; Orbach et al., 1978) and specifically fragmented genomes of bacteria (Smith et al., 1987a) and mammals (Smith et al., 1986). In PFG, DNA molecules are subjected to electrical fields that alternate between two orientations (Schwartz & Cantor, 1984; Schwartz et al., 1983). Net translational mobility of the DNA through the gel is presumably modulated by the rate at which the molecule can reorient in response to the electric field (Schwartz & Cantor, 1984; Cantor et al., 1988; Southern et al., 1987; Deutsch, 1987; Viovy, 1987; Mathew et al., 1988a,b). Reorientation times may be expected to depend on the size and topology of the molecule in question.

Detailed analyses of the behavior of large, linear DNAs in pulsed fields have been carried out (Southern et al., 1987; Viovy, 1987; Mathew et al., 1988a,b) and reveal that reorientation times are a function of a length of the molecule and the strength of the applied electric field. Here we compare the behavior of the same DNA with four different topologies: linear, supercoiled circles, nicked circles, and relaxed circles. We concentrate on the 2–50-kb size range where we can draw correlations between the behavior of molecules in pulsed and continuous electrical fields.

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[1] Abbreviations: PFG, pulsed-field gel electrophoresis; TAE, 0.04 M Tris–acetate, 0.02 M sodium acetate, and 2 mM EDTA, pH 8.1; TBE, 0.089 M Tris–borate and 0.0025 M EDTA, pH 8.3; modified TBE, 0.1 M Tris–borate and 0.0002 M EDTA, pH 8.4; μ, mobility.

Materials and Methods

Materials. Seaplaque agarose was obtained from FMC Corp. Supercoiled samples of 11 DNA plasmids (2.1, 3.0, 4.0, 5.0, 6.0, 7.0, 8.1, 10.1, 12.1, 14.2, and 16.2 kb) and linear samples of 13 fragments (8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb) prepared by digesting bacteriophage λ DNA with different restriction enzymes were purchased from Bethesda Research Laboratories (BRL). For experiments comparing the electrophoretic behavior of linear and supercoiled DNAs, supercoiled plasmids, prepared as described below, pUC8 (2.7 kb), pLC1842 (18.9 kb), and pCS5 (21.5 kb) were added to the BRL supercoiled sample prior to electrophoresis. Linearized forms of pUC8 (2.7 kb), pBR322 (4.4 kb), and ColE1 (6.6 kb) were added to the BRL linear DNA standard. Restriction enzymes *Bam*HI and *Eco*RI were obtained from New England Biolabs and were used as recommended by the manufacturer except where otherwise indicated. A nicking–closing extract isolated from chicken erythrocytes was a gift from R. H. Morse.

Preparation of DNAs of Different Topologies. Five different plasmids, pUC8 (2.7 kb; Vieira & Messing, 1982), pBR322 (4.4 kb; Sutcliffe, 1978), ColE1 (6.6 kb; Tomizawa et al., 1977), pSC101 (9.0 kb; Meacock & Cohen, 1980), pLC1842 (18.9 kb; Sancar & Rupp, 1979), and pCS5 (21.5 kb; Smith et al., 1983), were also used to generate DNAs of different topologies. The supercoiled monomers of these plasmids were purified on 0.8% or 1.0% agarose gels in TAE buffer (0.04 M Tris–acetate, 0.02 M sodium acetate, and 2 mM EDTA, pH 8.1) (Mickel et al., 1977). The linear derivatives were prepared by digesting these plasmids with restriction enzymes that introduce unique cleavages. The nicked circular derivatives were prepared by treating with a 10-fold excess of unique-cleavage restriction enzymes in the presence of 0.4 mg/mL ethidium bromide (Brahmachari et al., 1986) and then purified as above. The relaxed circular derivatives were generated by digesting 1 μg of supercoiled DNA with 1 μL of the nicking–closing extract isolated from chicken

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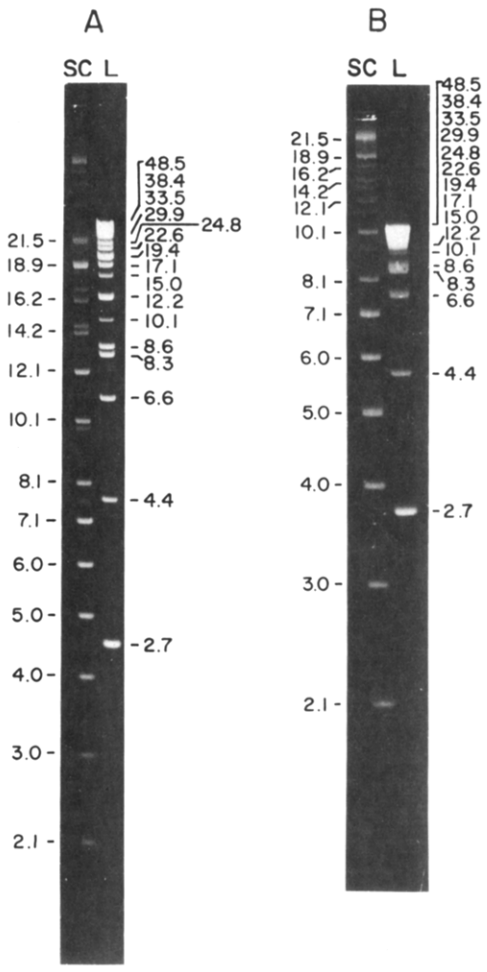

FIGURE 1: Continuous gel electrophoresis in 1% agarose at room temperature in (A) TAE buffer and (B) TBE buffer. Lanes denoted SC and L are supercoiled and linear DNAs, respectively. The faint bands in the supercoiled DNA lanes were nicked circular DNA produced during storage. Details of the species in the various samples are given under Materials and Methods.

erythrocytes (Bina-Stein et al., 1976) in the presence of 0.15 M NaCl at 37 °C for 1 h as described previously (Morse & Cantor, 1985). The reaction was stopped by adding sodium dodecyl sulfate to a final concentration of 1%. These relaxed circular derivatives were then purified on either a 0.8% or 1.0% agarose gel in TAE buffer in the presence of 0.5 μg/mL ethidium bromide.

Agarose Gel Electrophoresis. Conventional agarose gel electrophoresis was performed at room temperature, typically on horizontal slab gels 20 cm in length, either in TAE buffer at 1.3 V cm^{-1} for 21 h or in TBE buffer (0.089 M Tris–borate and 0.0025 M EDTA, pH 8.3; Maniatis et al., 1982) at 1.6 V cm^{-1} for 22 h using 1% agarose gels.

PFG electrophoresis was carried out in TBE buffer at 10 V cm^{-1} for 18 h, at 15 °C with pulse times of 0.1, 1.0, 10.0, or 100.0 s, using 1% agarose, in the double inhomogenous configuration (Schwartz & Cantor, 1983; Smith et al., 1986) in a 33-cm apparatus, the LKB Pulsaphor, and analyzed as described previously (Mathew et al., 1988a).

RESULTS

Supercoiled and linear DNAs were first analyzed by electrophoresis on conventional 1% agarose gels in TAE (Figure 1A) and TBE (Figure 1B) buffers at 1.3 and 1.6 V cm^{-1}, respectively. All the supercoiled molecules were adequately

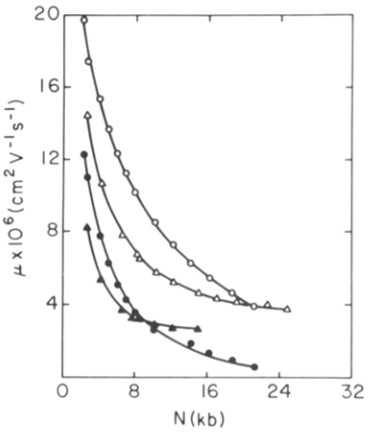

FIGURE 2: Dependence of electrophoretic mobility (μ) on DNA size from the experiments of Figure 1. Open symbols represent experiments in TAE buffer, and filled symbols are experiments in TBE buffer: (●, ○) supercoiled DNA; (▲, △) linear DNA.

resolved in both buffer systems, though performance in the former system was significantly better. This disparity was much more pronounced for linear molecules. The TAE system resolved linear molecules up to 25 kb in size, while resolution in TBE broke down above 10 kb as demonstrated in Figures 1B and 2. The crossover point, where supercoiled and linear DNAs migrate at the same rate, was 20 kb in the TAE system and 9 kb in TBE (Figure 2). This is consistent with previously published data for electrophoresis in low fields (Mickel et al., 1977). The faint bands in the supercoiled DNA lanes are due to nicked circular DNA produced during prolonged storage at 4 °C.

The inability of conventional agarose gel electrophoresis to resolve large DNA molecules is believed to be due to biased reptation. Once DNA molecules become larger than the pores in the agarose (a few hundred base pairs), they have to distort their shape to enter the gel, and then the sieving power of the matrix becomes irrelevant (De Gennes, 1971; Lerman & Frisch, 1982; Lumpkin et al., 1985). These larger molecules move through the agarose matrix by reptation. Mobility is predicted to vary with both the electric field strength and the molecular chain length (Lumpkin et al., 1985). Resolution is predicted to decrease with the square of the molecular size. At high field strengths, electrical orientation effects further reduce the size dependence of mobility. At field strengths of about 2 V cm^{-1}, resolution in 1% agarose drops off sharply for linear DNAs above 20 kb. The reduced resolving power of TBE buffer systems is probably due to the smaller pore size of gels hardened in TBE (Peats et al., 1986). Supercoiled DNAs are more compact than the corresponding linear analogues. The enhanced resolution of large supercoiled plasmids as compared to their linear analogues may be related to this compactness.

The two mixtures of DNAs of different topologies were next subjected to PFG in TBE buffer at pulse times of 0.1, 1.0, 10.0, and 100.0 s (Figure 3) and in TAE buffer at 0.1 s (data not shown). Plots of the estimated electrophoretic mobilities versus size are shown in Figure 4. Only nominal mobilities can be calculated for PFG due to the complex trajectory of the DNA molecules through the gel (Mathew et al., 1988a). Further, mobilities in both continuous field (Hervet & Bean, 1987) and pulsed-field (Mathew et al., 1988b) electrophoresis are field dependent. Hence, mobilities measured by using continuous fields of ~1.5 V cm^{-1} are not directly comparable with those measured under pulsed fields of 10 V cm^{-1}. The only com-

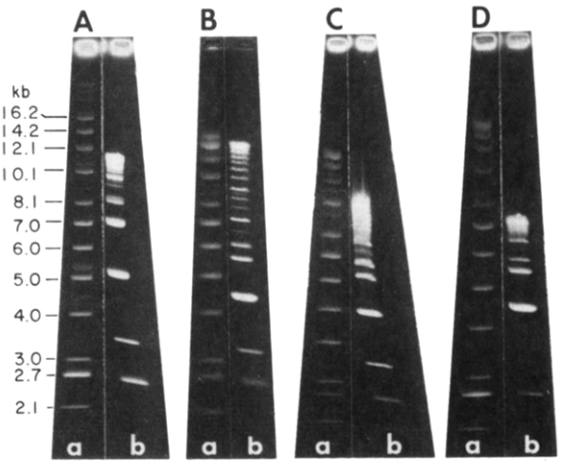

FIGURE 3: Pulsed-field gel electrophoresis of DNA in 1% agarose at 10 V cm^{-1} for 18 h in TBE buffer at 15 °C with at 0.1-, 1.0-, 10.0-, and 100.0-s pulse times. The supercoiled (a) and linear (b) samples were the same as in Figure 1 except that in these experiments supercoiled pUC8 DNA was added to all the samples as an internal standard. Sizes of the supercoiled species are shown at left.

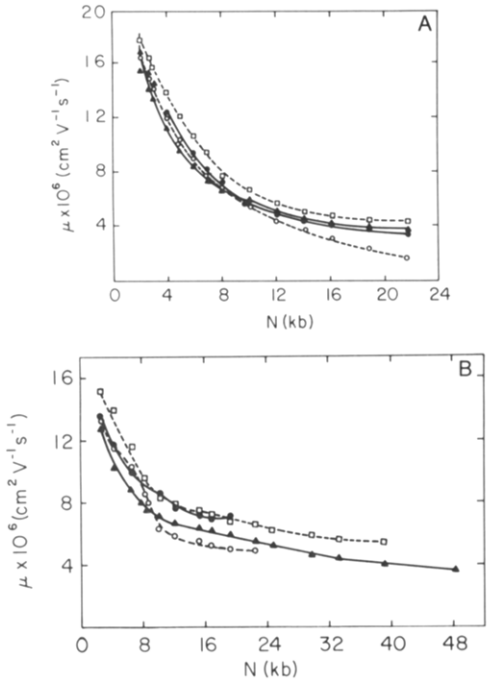

FIGURE 4: Electrophoretic mobilities of (A) supercoiled and (B) linear DNAs from Figure 3 plotted as a function of their size. The symbols represent 0.1-s (○), 1.0 s (▲), 10.0-s (□), and 100.0-s (●) pulse times.

parisons we make here are for limits of resolution between the two sets of conditions.

The resolution of most of the supercoiled species was essentially unaffected by pulse time, while that of the larger linear species is altered quite markedly. With PFG in TBE, linear DNAs in the 10–50-kb range were best resolved with pulse times ranging from 1.0 to 10.0 s. This resolution was better than that observed during continuous electrophoresis, where DNAs larger than 10 kb were not resolved. The crossover point between linear and supercoiled species at a 0.1-s pulse time was 9.0 kb, similar to that for continuous electrophoresis in TBE. The crossover points were all about 5 kb at 1.0-, 10.0-, and 100.0-s pulse times.

Resolution of the 16.2-, 18.9-, and 21.5-kb supercoiled species could only be achieved at a pulse time of 0.1 s. This is particularly apparent in the photographs of the gels (Figure

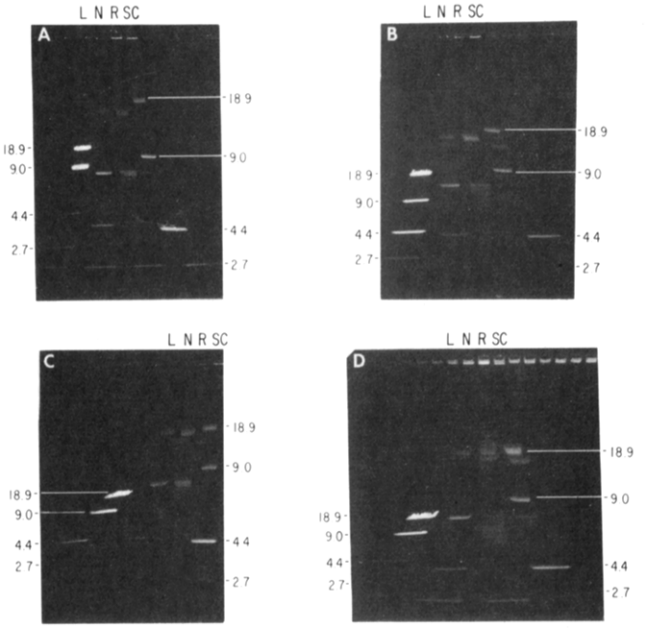

FIGURE 5: Pulsed-field gel electrophoresis of four different topological forms of plasmids pUC8 (2.7 kb), pBR322 (4.4 kb), pSC101 (9.0 kb), and pLC1842 (18.9 kb) at pulse times of (A) 0.1 s, (B) 1.0 s, (C) 10.0 s, and (D) 100.0 s. All lanes contain supercoiled pUC8 as the fastest moving band. The notations are (L) linear, (N) nicked circular, (R) relaxed circular, and (SC) supercoiled DNA. Positions of the supercoiled species are indicated on the right and the linear species on the left by the DNA sizes in kb.

3). The resolution of these large supercoiled DNAs in PFG is better than that observed with continuous electrophoresis in TBE. Linear DNAs up to 20 kb and all the supercoiled species were resolved. In fact, the resolution for both supercoiled and linear species at 0.1-s pulse times was very similar to that observed on continuous electrophoresis in TAE (see Figure 1).

One peculiar feature of PFG mobilities of supercoiled DNAs is the response of the absolute velocity to pulse time. While the resolution of molecules smaller than 16 kb is little affected, at pulse times around 10 s, all supercoiled species move somewhat faster than at either shorter or longer pulse times (Figure 4A). This effect has been seen repeatedly in two different-sized PFG apparatus being otherwise comparable electrical field shapes, strengths, gel concentration, and temperature. We do not believe it is artifactual, but we have no explanation for this apparent resonance-like behavior.

Nicked and relaxed circular molecules of pUC8, pBR322, pSC101, and pLC1842 were compared with the linear and supercoiled forms of these plasmids by PFG electrophoresis at 0.1-, 1.0-, 10.0-, and 100.0-s pulse times (Figure 5). The estimated mobilities of the nicked circular DNAs are plotted in Figure 6. The mobilities of these DNAs are affected by pulse time in a surprisingly complex way. PFG behavior of 1- and 100-s pulse times was nearly the same. However, with 0.1- and 10-s pulses, the nicked circles were significantly retarded with 0.1-s pulsing yielding the slowest mobilities of 18.9- and 9.0-kb DNA, while 10-s pulsing produced the slowest migration of smaller molecules.

Discussion

Several important, practical considerations can be drawn from the results in this paper. First, PFG electrophoresis has excellent potential for high-resolution separation of DNA molecules in the 8–50-kb size range. Thus, it should find many applications in areas like Smith–Birnstiel restriction mapping

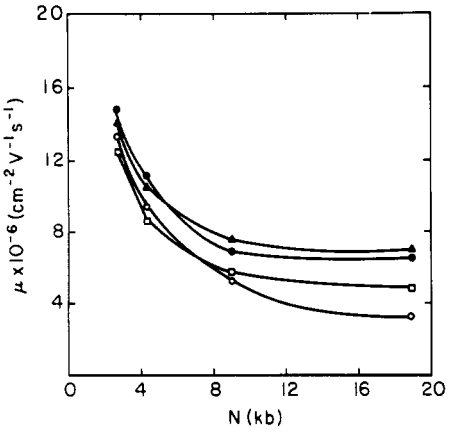

FIGURE 6: Electrophoretic mobilities of nicked circular DNAs from Figure 5 plotted as a function of their size at 0.1-s (○), 1.0-s (▲), 10.0-s (□), and 100.0-s (●) pulse times.

of cosmid clones where accurate size fractionation in this range is important (Smith & Birnstiel, 1976; Rackwitz et al., 1984).

Second, the insensitivity of small, highly supercoiled DNA to pulse time offers a simple diagnostic test for the presence of this species. It appears that this insensitivity may be due to the compactness of the supercoiled structure. Thus, the mobility of large supercoiled DNAs may still be sensitive to variation in pulse time.

Third, the relationship between PFG mobility and molecular weight is complex for DNAs in this size range. The observed mobilities presumably reflect both pulsed and continuous field electrophoresis. This complicates any attempt to interpret the data in terms of quantitative physical models. However, the plots in Figures 4 and 6 indicate that conditions can be found for the effective fractionation of almost any limited combination of DNA sizes and topologies in this size range.

In principle, the differential response to changes in pulse time by molecules of different topologies should shed some light on the mechanism of DNA reorientation in PFG. Reorientation times in the switchback theory of PFG (Southern et al., 1987) are predicted to depend exclusively on the end-to-end lengths. Other theories of PFG (Viovy, 1987; Deutsch, 1987) also predict that reorientation times are related to the length of the DNA, though it is likely that the stiffness of the molecule would also come into play. It is unlikely that even linear DNA is fully extended by fields of 10 V cm^{-1}, but equilibrium end-to-end lengths for DNA of a given molecular weight would be expected to differ for the three topologies at any electric field strength. It seems reasonable that, within the constraints of the agarose matrix and under the influence of an electrical field, nicked circular DNA should have an equilibrium end-to-end length approximately half that of the linear isomer and that highly supercoiled DNA should be much more compact. This would lead to simple predictions for the relative PFG mobility of these isomers. In practice, however, the effect of pulse time on the mobility of small supercoiled and relaxed circular DNAs is quite complex. For both classes of molecule, mobility is not a monotonic function of pulse time. To account for this behavior one must presumably take into account the stiffness of the supercoiled molecule, some aspects of its tertiary structure, and the possibility of impalement of nicked circular species on the agarose matrix (Levene & Zimm, 1987).

After this work was completed Hightower et al. (1987) reported that the mobilities of supercoiled DNAs 4–16 kb in size are essentially independent of pulse time over the range 10–120 s. The same result was seen with even larger supercoiled DNAs (Beverley, 1988). Hightower et al. also report that the mobilities of different topoisomers of small circular DNAs varied greatly. Direct comparison of results is complicated by the fact that mobilities of linear DNAs are field dependent (Mathew et al., 1988b), and the field strengths used by Hightower et al. (1987) have not been reported. Within the region of overlap, though, their results and ours are consistent.

REFERENCES

Beverley, S. M. (1988) *Nucleic Acids Res. 16*, 925–939.
Bina-Stein, M., Vogel, T., Singer, D. S., & Singer M. F. (1976) *J. Biol. Chem. 251*, 7363–7366.
Brahmachari, S. K., Shouche, Y. S., Cantor, C. R., & McClelland, M. (1986) *J. Mol. Biol. 193*, 201–218.
Carle, G. F., & Olson, M. V. (1984) *Nucleic Acids Res. 12*, 5647–5664.
Carle, G. F., & Olson, M. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3756–3760.
De Gennes, P. G. (1971) *J. Chem. Phys. 55*, 572–579.
Hightower, R. C., Metge, D. W., & Santi, D. V. (1987) *Nucleic Acids Res. 15*, 8387–8398.
Lerman, L. S., & Frish, H. L. (1982) *Biopolymers 21*, 995–997.
Levene, S. D., & Zimm, B. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4054–4057.
Lumpkin, O. J., Dejardin, P., & Zimm, B. H. (1985) *Biopolymers 24*, 1573–1593.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mathew, M. K., Smith, C. L., & Cantor, C. R. (1988a) *Biochemistry* (first of four papers in this issue).
Mathew, M. K., Smith, C. L., & Cantor, C. R. (1988b) *Biochemistry* (second of four papers in this issue).
Meacock, P. A., & Cohen, S. N. (1980) *Cell (Cambridge, Mass.) 20*, 529–542.
Mickel, S., Arena, V. Jr., & Bauer, W. (1977) *Nucleic Acids Res. 4*, 1465–1482.
Morse, R. H., & Cantor, C. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4653–4657.
Orbach, M., Vollrath, D., Davis, R. W., & Yanofsky, C. (1988) *Mol. Cell. Biol. 8*, 1469–1473.
Peats, S., Nochumson, S., & Kirkpatrick, F. H. (1986) *Biophys. J. 49*, 91a.
Rackwitz, H.-R., Zehetner, G., Frischauf, A.-M., & Lehrach, H. (1984) *Gene 30*, 195–200.
Sancar, A., & Rupp, W. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3144–3148.
Schwartz, D. C., & Cantor, C. R. (1984) *Cell (Cambridge, Mass.) 37*, 67–75.
Schwartz, D. C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M., & Cantor, C. R. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 189–195.
Smith, C. L., Siegfried, E., & Ruvolo, P. P. (1983) *DNA 2*, 291–299.
Smith, C. L., Lawrance, S. K., Gillespie, G. A., Cantor, C. R., Weissman, S. M., & Collins, F. S. (1987a) *Methods Enzymol. 151* 461–489.
Smith, C. L., Econome, J. G., Schutt, A. D., Klco, S., & Cantor, C. R. (1987b) *Science (Washington, D.C.) 236*, 1448–1453.

Smith, C. L., Tomohiro, M., Niwa, O., Klco, S., Fan, J.-B., Yanagida, M., & Cantor, C. R. (1987c) *Nucleic Acids Res. 15*, 4481–4489.
Smith, H. O., & Birnstiel, M. L. (1976) *Nucleic Acids Res. 3*, 2387–2389.
Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.
Tomizawa, J.-I., Ohmori, H., & Bird, R. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1865–1869.
Van der Ploeg, L. H. T., Schwartz, D. C., Cantor, C. R., & Borst, P. (1984) *Cell (Cambridge, Mass.) 37*, 77–84.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259–268.
Vollrath, D., & Davis, R. W. (1987) *Nucleic Acids Res. 15*, 7865–7871.

CORRECTIONS

Lymphoma–Vesicle Interactions: Vesicle Adsorption, Membrane Fragmentation, and Intermembrane Protein Transfer, by Alexandra C. Newton and Wray H. Huestis*, Volume 27, Number 13, June 28, 1988, pages 4645–4655.

Page 4646. In column 1, under Lymphoma–Vesicle Incubations, and in column 2, under Vesicle Contents, separations of protein–vesicle complexes from cell fragments were achieved by centrifugation at 200000*g* for 2 h, not 2000*g*.

Activated Conformers of *Escherichia coli* Sulfite Reductase Heme Protein Subunit, by Lawrence J. Young and Lewis M. Siegel*, Volume 27, Number 14, July 12, 1988, pages 4991–4999.

Page 4994. The assignment of EPR spectra in Figure 4 should be reversed.

Multiple Species of Myeloperoxidase Messenger RNAs Produced by Alternative Splicing and Differential Polyadenylation, by Kazuya Hashinaka, Chika Nishio, Sook-Jin Hur, Fumio Sakiyama, Susumu Tsunasawa, and Michiyuki Yamada*, Volume 27, Number 16, August 9, 1988, pages 5906–5914.

Page 5908. In Figure 2, the 5′ end of the nucleotide sequence from nucleotides 1 to 57 is missing; it should read (GACAATATCAGGTGAGCT)GTGGAGGTGGGGTCCTTGGAAGCTGGATGACAGCAGCTG 57.

Page 5913. Under Acknowledgments, Dr. Earl R. Davie should read Dr. Earl W. Davie.