vulgaris and other legume seeds is to protect them from attack by insect seed-predators. Such a conclusion is of conspicuous significance to agriculturalists. Selection for those strains of black beans free of phytohemagglutinins (*21*) so as to reduce bean processing costs would very likely produce a crop plant on which many wild bruchids could then feed.

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References

1. I. E. Liener and M. L. Kakade, in *Toxic Constituents of Plant Foodstuffs*, I. E. Liener, Ed. (Academic Press, New York, 1969); A. C. Leopold and R. Ardrey, *Science* **176**, 512 (1972).
2. I. E. Liener, *Agr. Food Chem.* **22**, 17 (1974).
3. W. G. Jaffé, in *Toxicants Occurring Naturally in Foods* (National Academy of Sciences, Washington, D.C., 1973).
4. N. Sharon and H. Lis, *Science* **177**, 949 (1972); H. Lis and N. Sharon, *Annu. Rev. Biochem.* **42**, 541 (1973).
5. D. H. Janzen, In *Evolutionary Strategies of Parasitic Insects and Mites*, P. W. Price, Ed. (Plenum, New York, 1975), p. 154.
6. F. Zacher, *Z. Angew. Entomol.* **33**, 460 (1952).
7. Unpublished records in a general study of Costa Rican bruchids and their host plants.
8. There are other defensive compounds in *P. vulgaris* as well, but they are not the subject of this report. Heteropolysaccharides and saponins that are toxic to bruchids occur in *P. vulgaris* seeds [S. W. Applebaum, U. Tadmor, H. Podoler, *Entomol. Exp. Appl.* **13**, 61 (1970)].
9. However, wild bruchids (*Mimosestes sallaei*, *M. immunis*, and *Stator limbatus*) from Guanacaste Province, Costa Rica, have oviposited on mature and shelled black beans in the laboratory, and the larvae always died very shortly after mining into the seeds.
10. Misnamed a "weevil" long ago, this animal is in fact a member of the family Bruchidae, a family long known as "seed weevils."
11. Stock cultures are maintained in quart mason jars full of mature commercial cowpeas, *Vigna unguiculata* (L.) (a synonym of *Vigna sinensis*).
12. If seeds of black beans or other cultivars of *P. vulgaris* are mixed with the stock cowpeas, eggs are laid on them but all larvae die shortly after mining into the *P. vulgaris* beans.
13. Determined in the laboratory of I.E. Liener.
14. See (*5*) for detailed methods.
15. Prepared according to P. M. Honovar, C.-V. Shih, I. E. Liener, *J. Nutr.* **77**, 109 (1962).
16. I. E. Liener, *Annu. Rev. Plant Physiol.*, in press.
17. Hemagglutinating activity was destroyed by autoclaving a concentrated solution of the phytohemagglutinin at 15 pounds pressure (1 atm) at 130°C for 20 minutes followed by lyophilization.
18. M. M. Ventura and J. X. Filho, *An. Acad. Bras. Cienc.* **38**, 553 (1966).
19. S. W. Applebaum, *J. Insect Physiol.* **10**, 783 (1964).
20. Trypsin inhibitors (soybean and ovomucoid) were purchased from Sigma Chemical Company, St. Louis, Mo.
21. O. Brucher, M. Wecksler, A. Levy, A. Palozzo, W. G. Jaffé, *Phytochemistry* **8**, 1739 (1969); W. G. Jaffé, O. Brucher, A. Palozzo, *Z. Immununitaetsforsch. Allerg. Klin. Immunol.* **142**, 439 (1972).
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DNA Structure in Sheared and Unsheared Chromatin

Abstract. *Shearing chromatin, by either sonication or vortex homogenization, introduces significant structural artifacts. These may be detected by the anomalously large increase in the number of ethidium bromide binding sites and the large alteration of the circular dichroism spectra of chromatin. Structural alterations are also suggested by the disappearance of differential light scattering after shearing.*

The term chromatin in this report refers to the isolated chromosomes (*1*) and has only an operational definition since chromatin preparations vary from one laboratory to another. Chromatin studies would be facilitated if the material could be obtained in a homogeneous solution, and for this reason most workers have studied the functional and structural properties of chromatin after shearing it, either with a motor-driven homogenizer (*2*) or by sonication (*3*). However, it has been reported that shearing causes a dramatic change in template activity (*4*) and loss of the repeating units structure of chromatin (*5*). Unfortunately, unsheared chromatin forms a suspension and gives rise to large light-scattering artifacts. Since our previous data on chromatin changes in proliferating cells (*6*) were obtained with unsheared chromatin, we have developed and present here a method to correct for light-scattering artifacts (*7*). With this method we have compared sheared and unsheared chromatins from various cell types, using circular dichroism (CD) spectra (*6, 8*) and ethidium bromide binding (*6, 9–11*) as indications of chromatin structure.

The following cells were used: (i) WI-38 human diploid fibroblasts in confluent monolayers, either resting or stimulated to proliferate (*10*); (ii) AF-8 cells, a temperature-sensitive mutant of BHK cells (*12*), which grow at 34°C but are arrested in G_1 at 39°C; and (iii) HeLa S-3 cells maintained in suspension and synchronized by selective detachment (*13*). Chromatin was prepared as previously described (*10, 13*). Unsheared chromatin was prepared by gently resuspending the viscous chromatin pellet, with or without a few strokes of a Dounce homogenizer; sheared chromatin was prepared by resuspending the pellet and either sonicating at 50 watts for 20 seconds or more or homogenizing with a motor-driven homogenizer for 15 seconds or more. In all three cases, the solvent was 0.01*M* tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), *p*H 8.0. Circular dichroism spectra were obtained with a Jasco model J-40 recording spectropolarimeter as previously described (*10, 11, 13*). The mean ellipticity, θ, is expressed as degrees times centimeters squared per decimole of nucleotide residue, assuming a mean molecular weight of 330. The ellipticity obtained directly from the recorder chart is designated as ψ.

The CD instrument is very sensitive to scattering artifacts. Because of its particulate nature, chromatin tends to form an intensely light-scattering suspension.

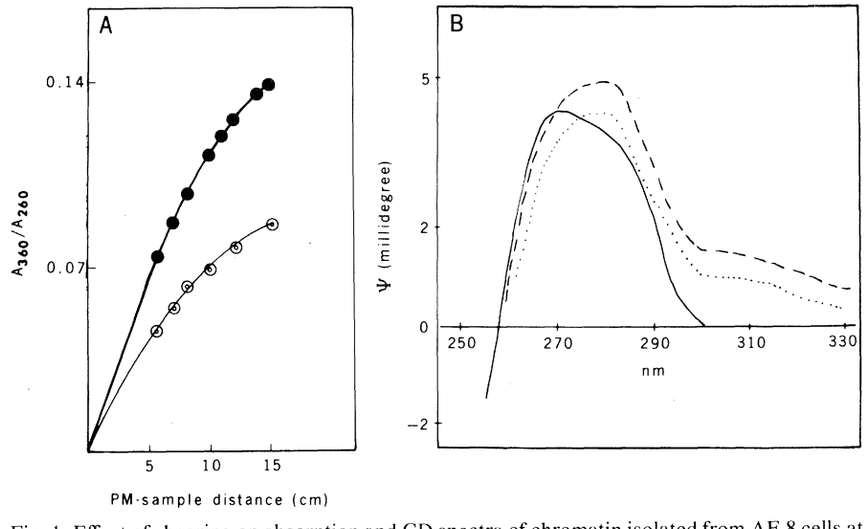

Fig. 1. Effect of shearing on absorption and CD spectra of chromatin isolated from AF-8 cells at 39°C. (A) Absorbance ratio as a function of PM-sample distance for unsheared (closed circles) and sheared (open circles) chromatins. The unsheared chromatin was subjected to a few strokes of a Dounce homogenizer and the sheared chromatin was sonicated for 20 seconds at 50 watts. (B) Observed CD spectra of unsheared chromatin at D = 14 cm (dashed line) and 7 cm (dotted line) and of sheared chromatin at D = 14 cm (solid line). Standardization and calibration are described elsewhere (*7, 17*).

Scattered light deviates from the beam axis at an angle great enough to miss the detector, which results in (i) anomalously high absorbance, and (ii) differential light scattering—that is, scattering of left and right circularly polarized light with different efficiency out of the photomultiplier (PM) window. We modified the J-40 by equipping it with a new cell holder with which the sample–PM window distance could be varied from 22 to 5.5 cm (the closest spacing physically possible with the cuvette on the monochromator side of the cuvette holder); thus it was possible to progressively increase the solid angle of detection and capture scattered photons missed in the normal J-40 configuration. Figure 1B shows the CD spectrum of unsheared chromatin from AF-8 cells at two distances from the PM. Even outside the absorptive band of chromatin a strong CD signal is present, which increases in magnitude with the distance of the sample from the PM. This can be explained in terms of differential light scattering, and corrected for as described below.

At every wavelength in the range 310 to 350 nm (Fig. 1B), where only the scattered light is presumably present, the CD data (ψ_{obs}) obtained at five distances D_i (5.5, 11.0, 14.0, 18.0, and 22.0 cm) are fitted by $\psi_{obs} = BD_i^n$, where B is a constant. The values of n that best fit the data are wavelength-dependent, and the relation between wavelength (λ) and the optimized $n(\lambda)$ is obtained by least-squares fit (*7*). Therefore, the extrapolated values of chromatin ellipticity at zero distance, $\psi_{true}(\lambda,0)$, where all scattered light is treated as transmitted, are found for every λ by least-squares fit to the observed ellipticities $\psi_{obs}(\lambda, D_i)$, using

$$\psi_{obs}(\lambda,D_i) = \psi_{true}(\lambda,0) + kD_i^{n(\lambda)} \quad (1)$$

where k is a constant for the regression. The "true" CD spectra estimated by this procedure indicate that all anomalous features, like a nonzero CD signal outside the absorption band ($\geq$ 310 nm), disappear (*7*) (see Fig. 2A).

Figure 1B shows the CD spectrum of sheared chromatin isolated from AF-8 cells at 39°C, taken at 14 cm from the PM. At this distance sheared chromatin scatters sufficient light to register $A_{360}/A_{260} = 0.08$, where A is absorbance and the subscripts indicate wavelength (Fig. 1A). However, the scattering leads to no detectable signal outside the absorption band when CD spectra are measured. For the same sample-PM distance, unsheared chromatin from the same cells scatters light at $A_{360}/A_{260} = 0.14$ (Fig. 1A) and exhibits a positive CD tail at long wavelengths, extending well into the visible spectrum (Fig. 1B). Figure 1B also shows that even at the 7-cm spacing from the PM, where the same amount of light is scattered by the unsheared chromatin as by the sheared chromatin at 14 cm, the CD spectrum of the unsheared chromatin has a nonzero signal outside the absorption band. As the solid angle is increased by positioning the sample progressively closer to the PM, the 280-nm peak and the long-wavelength tail (> 300 nm) are reduced for the CD spectra of unsheared chromatin. In contrast, the CD spectra of sheared chromatin suspensions show essentially no dependence on sample-PM distance and no tail at wavelengths > 300 nm.

Table 1 shows the mean molecular weight of a single strand of DNA, as determined by alkaline sucrose gradient (*14*), in intact cells, intact nuclei, unsheared chromatin, and sheared chromatin. It seems reasonable to conclude from these data that the length of the DNA molecule is substantially reduced by shearing, as previously reported by Noll *et al.* (*5*), while unsheared chromatin more closely approaches in vivo configuration.

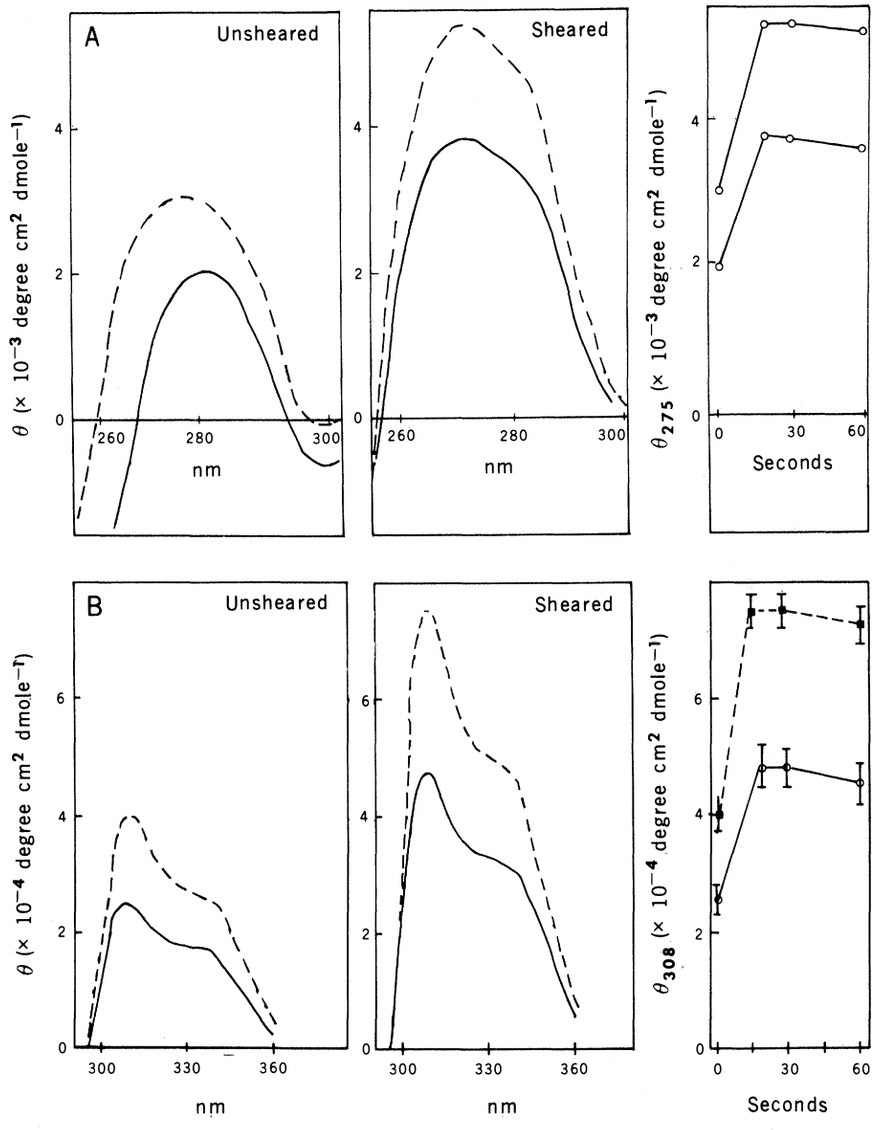

Fig. 2. (A) Molar ellipticity spectra corrected for light scattering, of unsheared and sheared chromatins from AF-8 cells at 39°C (solid line) and 34°C (dashed line). Chromatins were prepared as described in the legend of Fig. 1 and in the text. The last panel shows the dependence of the molar ellipticity at 275 nm on the duration of shearing by sonication at 50 watts. Our corrected molar ellipticity values for unsheared chromatins (*6, 10, 11, 13*) are considerably lower than those reported by most authors (*8, 20*), which closely resemble our values for sheared chromatins. (B) Molar ellipticity spectra, corrected for light scattering, of ethidium bromide–chromatin complexes (AF-8 cells) at 39°C (solid line) and 34°C (dashed line). Chromatin preparations were prepared as outlined above. The last panel shows the dependence of the ethidium bromide molar ellipticity at 308 nm on the duration of shearing by sonication at 50 watts.

To further investigate the postulated conformation change induced by shearing, we isolated chromatins from several cell lines (HeLa in different cell cycle phases, AF-8 mutants at 34° and 39°C, rat liver cells, and unstimulated and stimulated WI-38), obtained their CD spectra between 330 and 200 nm, and determined the number of primary binding sites for ethidium bromide (*10, 11, 13, 15*). Figure 2A shows the CD spectra, corrected for light scattering, of chromatins isolated from the AF-8 mutants at 34°C (permissive) and 39°C (nonpermissive). Figure 2B shows the CD spectra of complexes of the same AF-8 cell chromatins (sheared and unsheared) with ethidium bromide at a constant ethidium bromide/DNA ratio of 0.25 ± 0.01. At this constant ratio no optical activity is induced in ethidium bromide by neighboring molecules (*15*), and the optical activity of ethidium bromide bound to chromatin, measured at 308 nm, at saturation, can be used to compute the number of primary binding sites available in either DNA or chromatin (*10, 11, 13, 15*). The increase in the number of primary binding sites for ethidium bromide after chromatin shearing parallels the increase in positive ellipticity at 280 nm, the blue shift in CD spectra, and the disappearance of differential light scattering.

Figure 2, A and B, also shows the dependence of the structural alteration, determined by the molar ellipticity at 280 nm (Fig. 2A) or by ethidium bromide intercalation (Fig. 2B), on the duration of shearing by sonication. Identical results are obtained when the same chromatins are sheared in a motor-driven homogenizer for 20 seconds or more (not shown).

Finally, Table 2 summarizes the effects of shearing on 11 different chromatins isolated from various cell lines described above. A drastic alteration in chromatin conformation is indicated by the larger increases (about a factor of 2) in both primary binding sites for ethidium bromide and molar ellipticity at 275 nm (an indication of DNA conformation), properties of chromatin that have been related to the number of gene sites available for transcription (*11*). Our results agree with previous findings on the increase in chromatin template activity (*4*) by a factor of about 1.8 and on the loss of the repeating units structure (*5*) when chromatin is prepared by shearing. It must be emphasized that the chromatin disruption is independent of the methods of shearing used.

Thus, while sheared chromatin should not be used in studies of chromatin structure, it is possible to use unsheared chromatin if a correction for light scattering is applied. Artifacts in ultraviolet spectroscopy (*16*), mainly associated with large particles, have been corrected for in studies of T2 bacteriophage (*17*) and membrane structure (*18*). We emphasize from our work with unsheared and sheared chromatin, which both significantly scatter light and contain the same amount of protein (*4*), that the ratio A_{360}/A_{260} is not an indication of the presence or absence of artifacts due to light scattering in CD spectra of chromatin. Light scattering in absorbance measurements and scattering of polarized light are two different phenomena (*17*). Differential light scattering (CD signals at wavelengths outside the absorption band) and geometry-dependent CD spectra are not observed for all particulate suspensions, but might reflect the existence of ordered asymmetry of the scattering center in unsheared native chromatin, as postulated for bacteriophage (*17*) and membranes (*19*).

Table 1. Ranges of mean molecular weights of single-strand DNA in whole cells, intact nuclei, unsheared chromatin, and chromatin from the same cell line (HeLa-S3 cells in logarithmic growth phase) sonicated for 30 seconds at 50 watts. Measurements were performed in alkaline sucrose gradients as previously described (*14*). Alkaline sucrose gradients for molecular weight determination were calibrated with freshly prepared T4 and T7 bacteriophages and freshly prepared nicked circles of SV40 (*14*). Analogous results can be obtained with other cell lines (WI-38, AF-8, and rat liver). Unsheared chromatin (A) is prepared by gentle suspension without utilizing a Dounce homogenizer. Unsheared chromatin (B) is prepared as (A), but with 20 to 30 strokes of a Dounce homogenizer (see Fig. 1).

Preparation	Molecular weight ($\times 10^6$)
Whole cell	$\geq$ 3000
Intact nuclei	50–150
Unsheared chromatin (A)	20–60
Unsheared chromatin (B)	6–9
Sheared chromatin	$\leq$ 2.2

Table 2. Effect of sonication or vortex homogenization on chromatin conformation. Ratios were computed from CD spectra corrected for light scattering by (method I) extrapolation to $D = 0$, and (method II) empirical subtraction at $D = 14$ cm. The means and standard deviations are based on independent measurements of 11 chromatins from different cell populations (WI-38, HeLa, AF-8, and rat liver). In method II the CD baseline ($\psi = 0$) is empirically assumed to correspond to the observed ellipticity value at 310 nm (where no absorption or chromophore is present). Molar ellipticity is computed by using the resulting ellipticity values, divided by A_{260} obtained at the same sample-PM distance and corrected for protein. The units of θ_{275} are millidegrees per decimole of DNA as chromatin; θ_{308} is expressed in millidegrees per decimole of ethidium bromide at a constant ethidium bromide/DNA ratio of 0.25 ± 0.01.

Ratio	Method I	Method II
θ_{275}(sheared)/ θ_{275}(unsheared)	1.84 ± 0.06	1.62 ± 0.10
θ_{308}(sheared)/ θ_{308}(unsheared)	1.88 ± 0.06	1.91 ± 0.07

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References and Notes

1. J. Bonner, M. E. Dahmus, D. Fambrough, R. C. Huang, K. Marushige, D. T. H. Tuan, *Science* **159**, 47 (1968).
2. R. J. Clark and G. Felsenfeld, *Nature (London) New Biol.* **229**, 101 (1971).
3. E. A. Arnold and K. Young, *Arch. Biochem. Biophys.* **164**, 73 (1974); C. Chesterton, B. E. Courar, P. H. Butterworth, *Biochem. J.* **143**, 73 (1974).
4. D. J. dePomerai, C. J. Chesterton, P. H. W. Butterworth, *Eur. J. Biochem.* **46**, 471 (1974).
5. M. Noll, T. O. Thomas, R. D. Kornberg, *Science* **187**, 1203 (1975).
6. R. Baserga and C. Nicolini, *Biochim. Biophys. Acta*, in press.
7. C. Nicolini and F. Kendall, in preparation.
8. T. Y. Shih and G. D. Fasman, *J. Mol. Biol.* **52**, 125 (1970); R. T. Simpson and H. A. Sober, *Biochemistry* **9**, 3103 (1970); J. Bartley and R. Chalkley, *ibid.* **12**, 468 (1973); A. J. Adler *et al.*, *J. Biol. Chem.* **249**, 2911 (1974).
9. P. F. Lurquin, *Chem. Biol. Interact.* **8**, 303 (1974); J. Lapeyre and I. Bekhor, *J. Mol. Biol.* **89**, 137 (1974).
10. C. Nicolini, S. Ng, R. Baserga, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2361 (1975).
11. C. Nicolini and R. Baserga, *Chem. Biol. Interact.* **11**, 101 (1975); V. L. Selegy and P. F. Lurquin, *Nature (London) New Biol.* **243**, 20 (1973); L. Sankaran and B. M. Pogell, *ibid.* **245**, 257 (1973).
12. S. J. Burstin, H. K. Meiss, C. J. Basilico, *J. Cell. Physiol.* **84**, 397 (1974).
13. C. Nicolini, K. Ajiro, T. W. Borun, R. Baserga, *J. Biol. Chem.* **9**, 3381 (1975).
14. S. Parodi, F. S. Sarma, C. Nicolini, E. Farber, *Biochim. Biophys. Acta* **407**, 174 (1975).
15. S. Parodi, F. Kendall, C. Nicolini, *Nucleic Acids* **2**, 477 (1975); S. Aktipis, W. W. Martz, A. Kindelis, *Biochemistry* **14**, 326 (1975).
16. L. N. M. Duysen, *Biochim. Biophys. Acta* **19**, 1 (1965); D. W. Urry, *ibid.* **265**, 115 (1972); H. J. Li and D. W. Urry, *Biochem. Biophys. Res. Commun.* **34**, 404 (1969); G. Holzwarth, D. G. Gordon, J. E. McGinness, *Biochemistry* **13**, 126, (1974); D. J. Gordon, *ibid.* **11**, 413 (1972); H. Gregory and M. Raps, *Biochem. J.* **142**, 193 (1974).
17. B. P. Dorman and F. M. Maestre, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 255 (1973); B. P. Dorman, J. E. Hearst, F. M. Maestre, *Methods Enzymol.* **27D**, 30 (1973); K. D. Philipson and K. Sauer, *Biochemistry* **12**, 3454 (1974).
18. B. J. Gordon and G. Holzwarth, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2365 (1971).
19. J. M. Wigglesworth and L. Packer, *Arch. Biochem. Biophys.* **128**, 7901 (1968).
20. S. Hanlon, R. S. Johnson, B. Wolf, A. Chan, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3263 (1972); R. P. Hjelm, Jr., and R. C. C. Huang, *Biochemistry* **14**, 1682 (1975).
21. We thank T. Borun, S. Sarma, and J. McEvilla for their support. This work was supported by NIH grants CA18258 and CA12923.

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