1.0 g of sterilized glass microcarriers were added and the cells were allowed to attach for 4 hours; additional 1A6 cells were then added. The cells were grown to 40 to 50 percent confluency, then placed on a low serum containing medium (0.2 percent FCS) for 48 hours. The medium was harvested and the cells placed back in standard serum for 48 hours, after which there was one more cycle of harvest. The hG-CSF was then purified as described (*15*) except for the modification of the final step mentioned in the text.
22. M. W. Hunkapiller and L. E. Hood, *Science* **219**, 650 (1983).
23. E. Ohtsuka *et al.*, *J. Biol. Chem.* **260**, 2605 (1985).
24. H. Okayama and P. Berg, *Mol. Cell. Biol.* **2**, 161 (1982).
25. F. Sanger, S. Nicklen, A. R. Coulsen, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
26. B. E. Uhlin and K. Nordstrom, *Mol. Gen. Genet.* **165**, 167 (1978).
27. Bacterial cultures were grown with shaking in 2-liter flasks containing 500 ml of 2× TYE (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) at 30°C until the absorbance was 0.5 at 600 nm. The temperature was rapidly shifted to 42°C and the cultures were incubated an additional 3 to 3.5 hours. The cells were harvested by centrifugation at 5000*g* for 20 minutes in a Beckman JA10 rotor. Four grams of cells were resuspended in 25 ml of water and lysed by multiple passages through a French Press (10,000 pounds per square inch). The broken cell suspension was centrifuged at 10,000*g* for 10 minutes in a Beckman JA20 rotor and the pellet resuspended at approximately 5 to 6 mg/ml in water. The material was then solubilized in 1 percent sodium laurate, 50 m*M* tris, *p*H 8.5, and 5 percent ethanol (approximately 1 mg/ml). The solubilized pellet was centrifuged at 15,000, for 5 minutes and the supernatant loaded on a C_4 HPLC column. The C_4 column was washed in buffer A (100 m*M* ammonium acetate, *p*H 6.0 to 7.0) and a 2-propanol gradient (0 to 80 percent in buffer A) was run at a flow rate of 1 ml per minute collecting 1-ml fractions. The rhG-CSF eluted at fractions 71 to 73.
28. T. Mahmood and W. A. Robinson, *Blood* **51**, 879 (1978).
29. J. Levin and F. B. Bang, *Thromb. Diath. Haemorrh.* **19**, 186 (1968).
30. V. P. Bhavanandan and E. A. Davidson, *Biochem. Biophys. Res. Commun.* **70**, 139 (1976).
31. G. Lamblin *et al.*, *Biochem. Soc. Trans.* **12**, 599 (1984).
32. E. H. Broxmeyer, M. A. S. Moore, P. Ralph, *Exp. Hematol.* **5**, 87 (1977).
33. L. Lu *et al.*, *Blood* **61**, 250 (1983).
34. N. A. Nicola and D. Metcalf, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3765 (1984).
35. D. Metcalf, *Int. J. Cancer* **25**, 225 (1980).
36. F. Tejedor and J. P. G. Ballesta, *Anal. Biochem.* **127**, 143 (1982).
37. The WEHI-3B(D^+) cells and freshly obtained human peripheral blood myeloid leukemic cells were washed three times with phosphate-buffered saline (PBS) containing 1 percent bovine serum albumin (BSA). WEHI-3B(D^+) cells (5×10^6) or fresh leukemic cells (3×10^6) were incubated in duplicate in PBS/1 percent BSA (100 μl) in the absence or presence of various concentrations (volume: 10 μl) of unlabeled hG-CSF, rhG-CSF, or GM-CSF and in the presence of ^{125}I-hG-CSF (approximately 100,000 cpm or 1 ng) at 0°C for 90 minutes (total volume: 120 μl). Cells were then resuspended and layered over 200 μl of ice-cold FCS in a 350-μl plastic centrifuge tube and centrifuged (1000*g*; 1 minute). The pellet was collected by cutting off the end of the tube, and the pellet and supernatant were counted separately in a gamma counter (Packard).
38. E. Platzer, in preparation.
39. A. J. Hapel *et al.*, *Cell* **25**, 179 (1981).
40. J. W. Schrader and R. M. Crapper, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6892 (1983).
41. J. Gabrilove and K. Welte, in preparation.
42. J. Lotem and L. Sachs, *Int. J. Cancer* **28**, 375 (1981).
43. D. Metcalf, *J. Natl. Cancer Inst. Monogr. No. 60* (1982), p. 123.
44. We thank J. Gasson for human GM-CSF, J. Bennett, J. Fitzgerald, and J. Heuston for assistance in the preparation of this manuscript. We also thank Z. Arlin for M3 leukemic cells and helpful discussions. G. Potter provided photographic assistance. M.A.S.M. and J.G. are supported by NCI grants CA20194, CA32516, and CA00966. K.W. is supported by NCI grant CA33484.

20 December 1985; accepted 14 February 1986

Electrophoretic Separations of Large DNA Molecules by Periodic Inversion of the Electric Field

GEORGES F. CARLE, MARK FRANK, MAYNARD V. OLSON

In gel electrophoresis, nucleic acids and protein-detergent complexes larger than a threshold size all migrate at the same rate. For DNA molecules, this effect can be overcome by the simple procedure of periodically inverting the electric field. Tuning the frequency of the field inversions from 10 to 0.01 hertz, makes it possible to resolve selectively DNA's in the size range 15 to >700 kilobase pairs.

GEL ELECTROPHORESIS, IN WHICH ions are separated by size as they migrate through a gel medium in response to an applied electric field, is widely used to separate complex mixtures of macromolecules (*1*). Although it has been successfully adapted to an enormous range of biochemical applications, electrophoresis remains subject to a number of significant practical limitations. For example, in conventional electrophoretic separations of native DNA molecules on agarose gels, the largest molecules that can be separated readily are approximately 15 to 20 kb (~10^7 daltons) (*2*); larger molecules exhibit nearly size-independent mobilities, a phenomenon that is thought to be associated with an end-on, reptile-like mode of migration known as "reptation" (*3*). In 1983, Schwartz *et al.* addressed this problem by employing a complex electrode geometry that allowed alternate application of two transverse electric fields that were spatially inhomogeneous (*4*). The idea behind this method was to force large molecules to turn corners periodically in the gel matrix, thereby introducing a source of size-dependence that is absent during simple migration in one dimension. Various implementations of this technique have been used to study many previously uncharacterized DNA molecules, including the intact chromosomal DNA molecules of yeast and several protozoans (*5–8*).

In attempting to make further improvements in large-DNA electrophoresis, we have experimented with simpler electrode geometries than those employed either by Schwartz *et al.* (*4, 6*) or in our own implementation of "orthogonal-field-alternation gel electrophoresis" (OFAGE) (*5*). These experiments have led to the unexpected discovery that the electrophoresis of large DNA molecules can be made strongly size-dependent simply by periodically inverting a uniform electric field in one dimension. Typical results for molecules in the size range 2 to 400 kb are shown in Fig. 1. In these experiments, for which we used 1 percent agarose gels at a voltage gradient of 10.5 V/cm, net migration in the "forward" direction was achieved when a longer portion of each switching cycle was set for "forward" than for "reverse" migration.

In a control experiment in which only forward electrophoresis was carried out, we obtained good fractionation of molecules up to about 15 kb, slight fractionation in the 15- to 100-kb range, and size-independent mobilities for molecules >100 kb (Fig. 1A). When the switching cycle involved a forward interval of 0.5 second followed by a reverse interval of 0.25 second (Fig. 1B), we obtained a "window" of good fractionation between 15 to 30 kb, while the largest molecules still comigrated. The window of enhanced resolution can be shifted to the 50- to 125-kb region by lengthening the switching cycle (Fig. 1C). Similar results were obtained with a constant switching interval in combination with different forward and reverse voltages (*9*).

The experiment in Fig. 1C also illustrates a common feature of field-inversion gel electrophoresis (FIGE): the mobility goes through a minimum as a function of size; in this case, the minimum mobility lies in the neighborhood of 200 kb. The existence of a minimum mobility is demonstrated by the slower mobility of T4 DNA compared both to T5 DNA and to the mixture of yeast chromosomal DNA's; furthermore, in the yeast sample, the faint bands that trail behind the main front arise from the smaller yeast chromosomes, whose sizes are in the range of 260 to 290 kb (*5*).

In larger size ranges, the existence of a domain in which mobility increases with

Department of Genetics, Washington University School of Medicine, St. Louis, MO 63110.

size is even more striking. This point is illustrated in Fig. 2, in which the chromosomal DNA molecules of yeast have been separated by OFAGE in the first dimension and then by FIGE. Under the FIGE conditions used, the chromosomal molecules of intermediate size do not migrate in the FIGE dimension, while both much larger and much smaller molecules have high mobility. The molecules that have zero mobility in the FIGE dimension of Fig. 2 are approximately 600 to 700 kb (*5, 7*). It should also be noted that there is no assurance that OFAGE mobilities in this size range are strictly monotonic with size, although major deviations from this rule are unlikely (*5–7*).

The large molecule limb of Fig. 2 offers a new resource for the fractionation of extremely large DNA molecules, because molecules such as those corresponding to chromosomes IV and XII, which have been barely separated in the OFAGE dimension, are widely separated by FIGE. However, the double-valued relation between mobility and size is a drawback for many applications since molecules that differ greatly in size can unexpectedly comigrate in one-dimensional FIGE separations. This problem can be overcome with the use of switching-interval ramps (Fig. 3). In this experiment, the forward switching interval in the FIGE dimension varied linearly from 9 seconds at 0 hours to 60 seconds at 18 hours; throughout the run, the reverse interval was one-third the forward interval. The use of switching-interval ramps to carry out convenient one-dimensional FIGE separations of yeast chromosomal DNA molecules is illustrated in Fig. 4, which also displays an

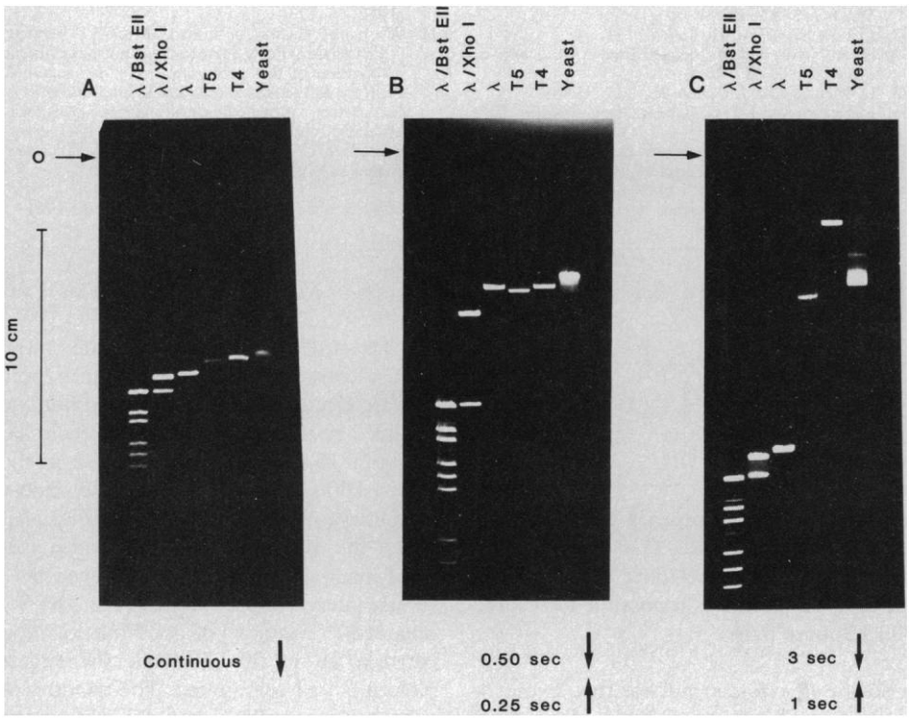


Fig. 1. Enhanced resolution of DNA molecules in the size range 15 to 200 kb by field-inversion gel electrophoresis. The DNA's used were BstE II–cleaved bacteriophage λ, ≤14.1 kb; Xho I–cleaved λ, 15.0 and 33.5 kb; λ, 48.5 kb; T5, 125 kb; T4, 170 kb; yeast chromosomal DNA's, 260 to >700 kb. (A) A control experiment in which forward electrophoresis was continuous for 4 hours, leading to little size dependence for molecules larger than 14 kb; (B) An experiment in which resolution of the two λ/Xho I fragments was enhanced by periodic inversion of the electric field; the switching regime involved repetition of a cycle comprising 0.5 second of forward migration and 0.25 second of reverse migration for 12 hours. (C) An experiment in which resolution between λ, T5, and T4 DNA's was enhanced. Electrophoresis was for 12 hours, with a switching cycle comprising 3 seconds of forward migration and 1 second of reverse migration. In all three experiments, the applied voltage was 300 V, giving rise to voltage gradients in the gel of 10.5 V/cm; with the exception of the switching regimes and total run times, other conditions were invariant both here and throughout this report. The gels contained 1% agarose, cast and run in 0.5 TBE (*5*). The gel apparatus was a conventional table-top design for running submerged agarose gels (*11*); the gel dimensions were 21.5 cm long, 20.5 cm wide, and 0.4 cm thick. The electrophoresis chamber was equipped with recirculation ports at both ends to allow recirculation of the buffer through a heat exchanger at a rate of 300 ml/min; the heat exchanger was adjusted to maintain the temperature of the running buffer and the gel at approximately 13°C. In general, the heat exchanger and switching circuitry were identical to those described for our OFAGE apparatus (*5*) except that the wiring between the relay and electrodes was changed to produce inversion of the polarity of a two-electrode system on each switching event instead of alternate selection of two different electrode pairs; (O), origin.

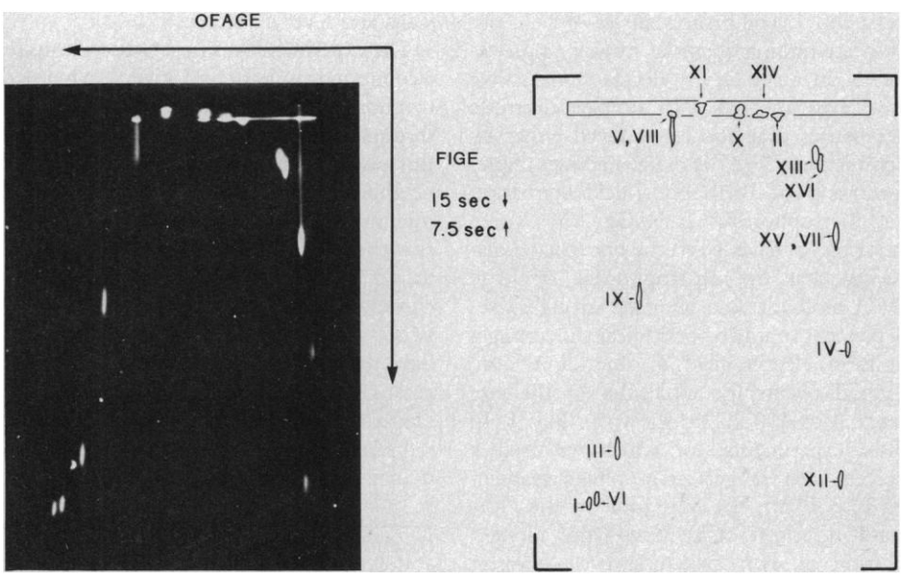


Fig. 2. A two-dimensional gel separation of the yeast chromosomal DNA molecules in which the first dimension was by orthogonal-field-alternation gel electrophoresis (OFAGE) and the second dimension was by field-inversion gel electrophoresis (FIGE). The yeast strain was AB972, for which the correlation between the OFAGE bands and the genetically defined chromosomes has been described (*7*); in the particular OFAGE implementation used for this experiment, chromosome XII, whose OFAGE behavior is erratic (*6, 7*), gives a normal band with slightly higher mobility than that for chromosome IV. In the FIGE dimension, the applied voltage was 260 V and the running time was 20 hours. Other conditions were as described for Fig. 1.

OFAGE fractionation of these molecules from the two different yeast strains employed (Fig. 4A). By varying the switching regime, it is possible either to separate the smaller molecules at high resolution (Fig. 4B) or the entire set of molecules at lower resolution (Fig. 4C).

In general, there are two reasons why switching-interval ramps often eliminate the double-valued relationship between mobility and size. First, when molecules that are much larger than those at resonance have substantially slower mobilities than molecules that are much smaller than those at resonance (for example, Fig. 1C), smaller molecules move farther than larger molecules because they spend more of the gel run moving at the faster of the two nonresonant mobilities. Second, the width of the resonances in the time domain increases with molecular size; consequently, during a gel run with a linear ramp, larger molecules spend a greater fraction of the run at resonance than do smaller molecules.

There are a number of potential applications of the FIGE technique. Our results provide an immediate means of expanding the size range over which DNA separations can be achieved on ordinary agarose gels. Furthermore, it is likely that the FIGE principle will find application in combination with various other electrophoretic methods since the phenomenon of a limiting, size-independent mobility for molecules larger than a threshold size is a universal characteristic of the electrophoresis of macromolecules. Because FIGE can be carried out in an ordinary electrophoresis chamber, it is readily compatible with nearly all the apparatus designs that are commonly used for electrophoretic separations.

The success of field inversion in imparting size dependence to the electrophoretic migration of large DNA molecules suggests that during steady-state electrophoresis the molecules have "directional" conformations. Presumably the effective radius of leading segments of the molecule is less than that of trailing segments, giving rise to an overall wedge-shaped conformation. When the field is inverted, a series of local conformational changes must propagate from one end of the molecule to the other before the wedge is inverted and the molecule acquires significant mobility in the new direction. If the switching interval is well matched to the characteristic time for wedge inversion, the molecules remain in intermediate conformations that have low mobility. The resonant switching frequency is strongly size-dependent, giving rise to the practical utility of FIGE.

The reason that large DNA molecules adopt wedge-shaped conformations during steady-state electrophoresis may be largely stochastic. The farther a segment is from the leading end of the molecule, the more likely it is to be penetrating the gel matrix along a path that is counterproductive to overall

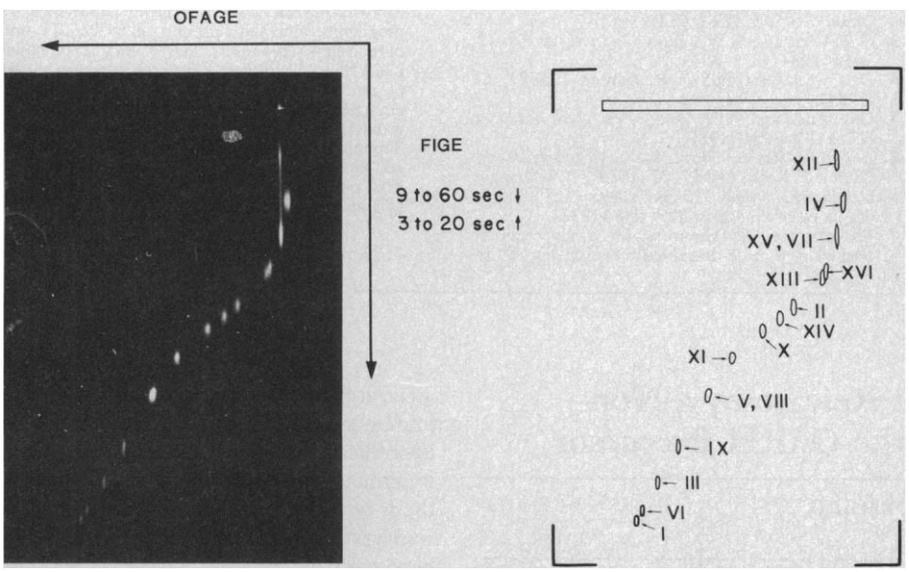
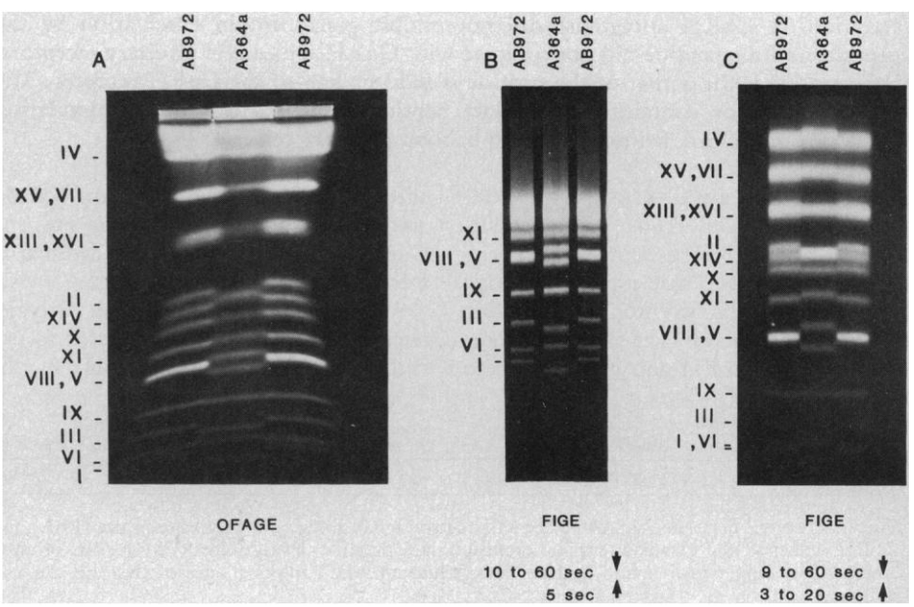


Fig. 3. A two-dimensional gel separation of the yeast chromosomal DNA molecules in which a double-valued relation between mobility and size has been avoided with the use of a switching-interval ramp. With the exception of the switching regime, the experimental conditions were the same as described for Fig. 2. To create the switching-interval ramps, an IBM PC was employed with an external interface that allowed programmed control of when line voltage was supplied to the coil of the power relay that distributes the output of the power supply to the electrodes (*12*).

Fig. 4. One-dimensional OFAGE and FIGE separations of the yeast chromosomal DNA's. (A) An OFAGE separation of DNA samples from the two yeast strains AB972 and A364a, which contain some chromosome-length polymorphisms relative to one another (*7*). (B) A FIGE separation of DNA samples from the same two yeast strains analyzed in (A); the region of the smaller yeast chromosomes (260 to 700 kb) has been expanded by using a switching-interval ramp in which the forward-migration interval varied linearly from 10 seconds at $t = 0$ hour to 60 seconds at $t = 12$ hours with a constant reverse interval of 5 seconds. (C) A FIGE separation in which the entire size range of the yeast chromosomal DNA molecules has been expanded by using a switching-interval ramp in which the forward-migration interval varied linearly from 9 seconds at $t = 0$ hour to 60 seconds at $t = 20$ hours with a constant 3:1 ratio between the forward and reverse intervals. The OFAGE separation was carried out as described (*5, 7*). For the FIGE separations, experimental conditions other than the switching regime were as described for Figs. 2 and 3. In none of the three gels was the location of chromosome XII determined.



translocation of the molecule, and the larger the radius from which such counterproductive paths can be selected. Net translocation of the molecule must require constant extrication of trailing segments from paths that are favored locally, but which are not sufficiently favorable to lead to rerouting of the entire molecule. This heuristic view is compatible with previous data indicating that DNA molecules adopt a high-mobility conformation during steady-state electrophoresis and that relaxation from this conformation is both slow and size-dependent (*10*).

With respect to the bearing of these observations on transverse-field experiments, it is notable that all published transverse-field geometries have involved variable, but generally obtuse, angles between the applied fields (*4–6*). Indeed, we were led to attempt FIGE experiments after having observed that acute angles between transverse fields are ineffective while obtuse angles give rise to strong size-dependence. It is likely that much of the fractionation observed in transverse-field alternation experiments arises at wide angles of intersection between the transverse fields and is associated with the same head-to-tail conformational change that is the basis of the FIGE technique.

REFERENCES AND NOTES

1. R. C. Allen, C. A. Saravis, H. R. Maurer, *Gel Electrophoresis and Isoelectric Focusing of Proteins: Selected Techniques* (de Gruyter, Berlin, 1984); D. Rickwood and B. D. Hames, Eds., *Gel Electrophoresis of Nucleic Acids: A Practical Approach* (IRL Press, Oxford, 1982).
2. W. L. Fangman, *Nucleic Acids Res.* **5**, 653 (1978); P. Serwer, *Biochemistry* **19**, 3001 (1980).
3. P.-G. deGennes, *J. Chem. Phys.* **55**, 572 (1971); L. S. Lerman and H. L. Frisch, *Biopolymers* **21**, 995 (1982); O. J. Lumpkin, P. Dejardin, B. H. Zimm, *ibid.* **24**, 1573 (1985).
4. D. C. Schwartz *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **47**, 189 (1983).
5. G. F. Carle and M. V. Olson, *Nucleic Acids Res.* **12**, 5647 (1984).
6. D. C. Schwartz and C. R. Cantor, *Cell* **37**, 67 (1984).
7. G. F. Carle and M. V. Olson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3756 (1985).
8. L. H. T. Van der Ploeg, D. C. Schwartz, C. R. Cantor, P. Borst, *Cell* **37**, 77 (1984); D. J. Kemp *et al.*, *Nature (London)* **315**, 347 (1985); L. H. T. Van der Ploeg *et al.*, *Science* **229**, 658 (1985).
9. For example, the 15- to 50-kb window can be opened even more dramatically than in Fig. 1B by employing applied voltages of 350 V forward/250 V reverse with a constant switching interval of 0.3 seconds for 18 hours.
10. T. Jamil and L. S. Lerman, *J. Biomolec. Struct. Dynamics* **2**, 963 (1985).
11. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), p. 153.
12. The computer control was achieved by using the parallel port on the IBM PC's Monochrome Display and Printer Adapter. The pins of the adapter's 25-pin D-shell connector provide a readily programmed source of standard transistor-transistor logic (TTL) output. Pins 2 to 9 are at +5 V relative to ground (pins 18 to 25) when bits 0 to 7, respectively, are set at I/O address hex 3BC, and they are at ground when the corresponding bits are not set. For example, the BASIC statement OUT &H3BC,&HA places pins 3 and 5 at +5 V and the remaining pins at ground. Since BASIC allows access to the PC's clock via the TIMER function (Versions 2.0 and later releases), it is straightforward to program switching-interval ramps. Each programmed pin can control a separate 110-V circuit by connecting the programmed pin and a ground pin to the TTL side of a Sigma (Braintree, MA) Series 226 model 226R1-5A1 Solid-State Relay; the line-voltage side of the relay will then be closed when the programmed pin is at +5 V and open when it is at ground.

13. Supported by a grant from the Monsanto Company and by a graduate research assistantship to G.C. from the Washington University Division of Biology and Biomedical Sciences.

21 November 1985; accepted 24 January 1986

Stimulation of Gonadotropin Release by a Non–GnRH Peptide Sequence of the GnRH Precursor

ROBERT P. MILLAR,* PATRICIA J. WORMALD, RAYMOND C. DE L. MILTON

The human gonadotropin-releasing hormone (GnRH) precursor comprises the GnRH sequence followed by an extension of 59 amino acids. Basic amino acid residues in the carboxyl terminal extension may represent sites of processing to biologically active peptides. A synthetic peptide comprising the first 13 amino acids (H · Asp-Ala-Glu-Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val · OH) of the 59–amino acid peptide was found to stimulate the release of gonadotropic hormones from human and baboon anterior pituitary cells in culture. The peptide did not affect thyrotropin or prolactin secretion. A GnRH antagonist did not inhibit gonadotropin stimulation by the peptide, and the peptide did not compete with GnRH for GnRH pituitary receptors, indicating that the action of the peptide is independent of the GnRH receptor. The GnRH precursor contains two distinct peptide sequences capable of stimulating gonadotropin release from human and baboon pituitary cells.

GONADOTROPIN-RELEASING HORmone (GnRH) is a decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly · NH_2, that stimulates release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH and its analogs also affect the central nervous system, gonads, and placenta, and GnRH-binding sites and GnRH-like peptides have been detected in these tissues (*1*).

Although in early reports an enzyme-template mechanism was suggested to underlie the biosynthesis of GnRH (*2*), the identification of higher molecular weight immunoreactive GnRH in sheep and pig hypothalamus (*3*) and human placenta (*4*) implicated conventional ribosomal biosynthesis of a prohormonal form. This conclusion was supported by cell-free translation of hypothalamic messenger RNA (mRNA) into peptides immunoprecipitable by GnRH antiserum (*5*). Our proposal that the pyroglutamic acid (pGlu) residue at the amino terminus of GnRH is derived from glutamine while the Gly · NH_2 of the carboxyl-terminus results from enzymic processing of glycine followed by a pair of basic amino acids in the GnRH precursor (*3*) was recently definitively demonstrated by the elucidation of the nucleotide sequence of a human placental GnRH mRNA (*6*). The GnRH sequence is followed by a cleavage and amidation site (Gly-Lys-Arg) and then a 53–amino acid sequence and a second potential cleavage site (Lys-Lys-Ile) (Fig. 1). In addition to cleavages at these pairs of basic amino acid residues, processing may occur at single basic amino acids (Fig. 1), as such cleavages occur in several peptide precursors (*7*).

Posttranslational processing of several peptide precursors gives rise to different peptide fragments with biological functions

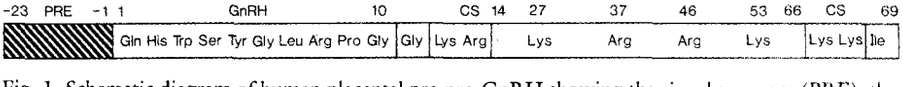


Fig. 1. Schematic diagram of human placental pre-pro-GnRH showing the signal sequence (PRE), the GnRH sequence, the carboxyl terminal extension, and putative cleavage sites (CS) at pairs of basic amino acids. Single basic amino acid residues, which may be additional sites of cleavage, are also indicated.

MRC Regulatory Peptides Research Unit, Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa.

* To whom correspondence should be addressed.