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(54) Titre : FY7 POLYMERASE  
(54) Title: FY7 POLYMERASE

(57) **Abrégé/Abstract:**

A purified recombinant thermostable DNA polymerase which exhibits at least about 80 % activity at salt concentrations of 50 mM and greater, at least about 70 % activity at salt concentrations of 25 mM and greater, and having a processivity of about 30 nucleotides per binding event. An isolated nucleic acid that encodes the thermostable DNA polymerase, as well as a recombinant DNA vector comprising the nucleic acid and a recombinant host cell transformed with the vector, are also disclosed. A method of sequencing DNA using the DNA polymerase as well as a kit for sequencing DNA is also disclosed.



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(21) International Application Number:</b> PCT/US99/13741<br><b>(22) International Filing Date:</b> 17 June 1999 (17.06.99)<br><b>(30) Priority Data:</b><br>60/089,556      17 June 1998 (17.06.98)      US<br><b>(71) Applicant:</b> AMERSHAM PHARMACIA BIOTECH INC.<br>[US/US]; 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US).<br><b>(72) Inventors:</b> DAVIS, Maria; Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US). FULLER, Carl, W.; Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US). MAMONE, Joseph, A.; Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US). HUANG, Lin; Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US).<br><b>(74) Agent:</b> RONNING, Royal, N., Jr.; Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US). |           | <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>Without international search report and to be republished upon receipt of that report.</i> |
| <b>(54) Title:</b> FY7 POLYMERASE<br><br><b>(57) Abstract</b><br><br>A purified recombinant thermostable DNA polymerase which exhibits at least about 80 % activity at salt concentrations of 50 mM and greater, at least about 70 % activity at salt concentrations of 25 mM and greater, and having a processivity of about 30 nucleotides per binding event. An isolated nucleic acid that encodes the thermostable DNA polymerase, as well as a recombinant DNA vector comprising the nucleic acid and a recombinant host cell transformed with the vector, are also disclosed. A method of sequencing DNA using the DNA polymerase as well as a kit for sequencing DNA is also disclosed.                                                                                                                                                                                                                                                                                                                                          |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |

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## FY7 POLYMERASE

## BACKGROUND OF THE INVENTION

Field of the Invention

The instant disclosure pertains to thermostable DNA polymerases which exhibit improved robustness and efficiency.

Background

DNA polymerases are enzymes which are useful in many recombinant DNA techniques such as nucleic acid amplification by the polymerase chain reaction ("PCR"), self-sustained sequence replication ("3SR"), and high temperature DNA sequencing.

Thermostable polymerases are particularly useful. Because heat does not destroy the polymerase activity, there is no need to add additional polymerase after every denaturation step.

However, many thermostable polymerases have been found to display a 5' to 3' exonuclease or structure-dependent single-stranded endonuclease ("SDSSE") activity which may limit the amount of product produced or contribute to the plateau phenomenon in the normally exponential accumulation of product. Such 5' to 3' nuclease activity may contribute to an impaired ability to efficiently generate long PCR products greater than or equal to 10kb, particularly for G+C rich targets. In DNA sequencing applications and cycle sequencing applications, the presence of 5' to 3' nuclease activity may contribute to a reduction in desired band intensities and/or generation of spurious or background bands.

Additionally, many of the enzymes presently available are sensitive to high salt environments, a condition commonly

Presently available enzymes have so-so processing ability (are more distributive - fall off more often - explain in more detail)

dITP added to address compression problems - usually kills activity of enzyme

Thus, a need continues to exist for an improved DNA polymerase having increased tolerance to high salt conditions, efficient utilization of dITP, high productivity, and improved performance on GC-rich templates.

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## BRIEF SUMMARY OF THE INVENTION

The instant disclosure teaches a purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 1, as well as a purified recombinant thermostable DNA polymerase which exhibits at least about 80% activity at salt concentrations of 50 mM and greater. The instant disclosure further teaches a purified recombinant thermostable DNA polymerase which exhibits at least about 70% activity at salt concentrations of 25 mM and greater, and a purified recombinant thermostable DNA polymerase having a processivity of about 30 nucleotides per binding event.

The instant disclosure also teaches an isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence set forth in Figure 1, as well as a recombinant DNA vector that comprises the nucleic acid, and a recombinant host cell transformed with the vector.

The instant disclosure also teaches a method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase in the presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments. The instant disclosure also teaches a kit for sequencing DNA comprising the DNA polymerase.

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## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIGURE 1 depicts the amino acid sequence (and DNA sequence encoding therefor) for the FY7 polymerase.

FIGURES 2A-2B depict the DNA sequence of M13mp18 DNA sequenced using the FY7 polymerase formulated in Mn conditions, as shown by a print out from an ABI model 377 automated fluorescent DNA sequencing apparatus.

FIGURES 3A and 3B depict the DNA sequence of M13mp18 DNA sequenced using the FY7 polymerase formulated in Mg conditions, as shown by a print out from an ABI model 377 automated fluorescent DNA sequencing apparatus.

FIGURE 4 depicts the percent of maximum polymerase activity for Thermo Sequenase™ enzyme DNA polymerase versus FY7 DNA polymerase under varying KCl concentrations.

FIGURE 5 depicts the effect of high salt concentrations on DNA sequencing ability in radioactively labeled DNA sequencing reactions using Thermo Sequenase™ enzyme DNA polymerase versus FY7 DNA polymerase.

FIGURES 6A-6B, 7A-7B, 8A-8B, 9A-9B and 10A-10B depict the effect of increasing salt concentration on the performance of Thermo Sequenase. At concentrations as low as 25mM data quality is affected with the read length being decreased from at least 600 bases to about 450 bases. At 50mM salt the read length is further decreased to about 350 bases, 75mM to about 250 bases and at 100mM the read length is negligible.

FIGURES 11A-11B, 12A-12B, 13A-13B, 14A-14B and 15A-15B depict the effect of increasing salt concentration on the performance of FY7 DNA polymerase.

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There is no detrimental effect on performance to at least 75mM KCl and only a slight decrease in data quality at 100mM KCl.

FIGURE 16 depicts the processivity measured for Thermo Sequenase DNA polymerase, AmpliTaq FS DNA polymerase, compared with the processivity measured for FY7 DNA polymerase.

FIGURE 17 depicts the improved read length obtained when using FY7 polymerase versus Thermo Sequenase DNA polymerase in radioactively labeled sequencing reactions incorporating the dGTP (Guanosine triphosphate) analog dITP (Inosine triphosphate) at 72°C.

FIGURES 18A-18B, 19A-19B, 20A-20B, 21A-21B and 22A-22B show the effect of increasing extension step time on the read length and data quality produced by Thermo Sequenase DNA polymerase in fluorescently labeled terminator DNA sequencing reactions.

FIGURES 23A-23B, 24A-24B, 25A-25B, 26A-26B and 27A-27B show the effect of increasing extension step time on the read length and data quality produced by FY7 DNA polymerase in fluorescently labeled terminator DNA sequencing reactions.

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## DETAILED DESCRIPTION OF THE INVENTION

A series of polymerase mutants were constructed with the aim of obtaining an improved polymerase for DNA sequencing, by reducing the exonuclease activity found in full length *Thermus thermophilus* and *Thermus aquaticus* DNA polymerase I enzymes. Six conserved motifs (Gutman and Minton (1993) Nucleic Acids Research 21, 4406 - 4407) can be identified in the amino-terminal domain of pol I type polymerases, in which the 5' to 3' exonuclease activity has been shown to reside. Further, six carboxylate residues in these conserved regions have been shown in a crystal structure to be located at the active site of the exonuclease domain of *Thermus aquaticus* DNA pol I (Kim et al., (1995) Nature 376, 612-616). Point mutations were made by site-directed mutagenesis to carboxylates and other residues in three of six conserved motifs in Tth and Taq polymerases as follows:

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Taq D18A, Taq T140V, Taq D142N/D144N. All of these have the mutation F667Y outside of the exonuclease domain.

Tth D18A, Tth T141V, Tth D143N/D145N. All of these have the mutation F669Y outside of the exonuclease domain.

All polymerases were evaluated for exonuclease activity, processivity, strand displacement, salt tolerance, thermostability, and sequencing quality. One FY7 polymerase, Tth D18A, F669Y, is described in further detail below.

## EXAMPLES

### Methods

#### In vitro mutagenesis

PCR was employed to introduce an aspartic acid to alanine amino acid change at codon 18 (D18A) of cloned full length F669Y Tth (plasmid pMR10). Mutagenic Primer 1 (CTGTTCTGAACCCAAAGGCCGTGTCCTCCTGGTGGCCGGCCACCAC) spans nucleotides 19-60 of pMR10 including codon 18 and a *Bst*BI restriction site. Oligonucleotide Primer 2 (GAGGCTGCCGAATTCCAGCCTCTC) spans an *Eco*RI site of pMR10. pMR10 was used as template DNA. The PCR product was digested with *Bst*BI and *Eco*RI and ligated to two fragments of pMR10: a 5000 bp *Kpn*I/ *Bst*BI and a 2057 bp *Eco*RI / *Kpn*I, creating plasmid pMR12. Cells of *E. coli* strain DH1 $\lambda^+$  were used for primary transformation, and strain M5248 ( $\lambda$  cI857) was used for protein expression, although any comparable pair of *E. coli* strains carrying the cI $^+$  and cI857 alleles could be utilized. Alternatively, any rec $^+$  cI $^+$  strain could be induced by chemical agents such as nalidixic acid to produce the polymerase.

#### Purification of Polymerase

M5248 containing plasmid pMR12 was grown in one liter of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), preferably 2X LB medium, containing 100 mg/ml ampicillin at 30°C. When the OD<sub>600</sub> reached 1.0, the culture was induced at 42°C for 1.5 hours. The cultures were then cooled to <20°C and the cells harvested by centrifugation in a Sorvall RC-3B centrifuge at 5000 rpm at 4°C for 15 to 30 minutes. Harvested cells were stored at -80°C.

The cell pellet was resuspended in 25 ml pre-warmed lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl<sub>2</sub>, 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM EDTA, 0.1%, preferably 0.2% Tween<sup>\*</sup>20, 0.1%, preferably 0.2% NP40). Preferably, the lysis buffer contains 300 mM NaCl. Resuspended cells were incubated at 75 - 85°C for 10-20 minutes, sonicated for 1 minute, and

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cleared by centrifugation. The cleared lysate was passed through a 300 ml column of diethylaminoethyl cellulose (Whatman<sup>\*</sup> DE 52) equilibrated in buffer A (50mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% Tween 20, 0.1% NP40) containing 100mM, preferably 300 mM NaCl. Fractions were assayed for polymerase activity, and those demonstrating peak polymerase activity were pooled, diluted to 50 mM NaCl with Buffer A, and loaded onto a heparin sepharose column (20 ml) equilibrated with 50 mM NaCl in buffer A. The polymerase was eluted from the column with a linear salt gradient from 50 mM to 700mM NaCl in buffer A. Fractions were assayed for polymerase activity, and those demonstrating peak activity were pooled and dialyzed against final buffer (20mM Tris-HCl pH8.5, 50 % (v/v) glycerol, 0.1mM EDTA, 0.5% Tween 20, 0.5% NP40, 1mM DTT, 100mM KCl). The purified protein is designated FY7. The amino acid sequence (and DNA sequence encoding therefor) are presented in Figure 1.

#### Bacterial Strains

*E. coli* strains: DH1 $\lambda^+$  [gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44,  $\lambda^+$ ]; M5248 [ $\lambda$  (bio275, cI857, cIII+, N+,  $\lambda$  (H1))].

#### PCR

Plasmid DNA from *E. coli* DH1 $\lambda^+$  (pMR10) was prepared by SDS alkaline lysis method (Sambrook et al., Molecular Cloning 2<sup>nd</sup> Ed. Cold Spring Harbor Press, 1989). Reaction conditions were as follows: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 1uM each primer, 2.5U Taq polymerase, per 100  $\mu$ l reaction. Cycling conditions were 94°C 2 minutes, then 35 cycles of 94°C 30s, 55°C 30s, 72°C 3 minutes, followed by 72°C for 7 minutes.

#### Example 1 Formulation of the enzyme in Mn conditions

In the following "pre-mix" protocol, all the reagents are contained in two solutions; reagent mix A and reagent mix B.

#### Reagent Mix A

The following reagents were combined to make 10 ml of reagent mix A:

2.5 ml 1 M HEPPS N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid), pH 8.0

500  $\mu$ l 1 M tartaric acid, pH 8.0

50,000 units FY7 DNA polymerase

1 unit *Thermoplasma acidophilum* inorganic pyrophosphatase

100  $\mu$ l 100 mM dATP

100  $\mu$ l 100 mM dTTP

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100  $\mu$ l 100 mM dCTP

500  $\mu$ l 100 mM dITP

9.375  $\mu$ l 100  $\mu$ M C-7-propargylamino-4-rhodamine-6-G-ddATP

90  $\mu$ l 100  $\mu$ M C-5-propargylamino-4-rhodamine-X-ddCTP

6.75  $\mu$ l 100  $\mu$ M C-7-propargylamino-4-rhodamine-110-ddGTP

165  $\mu$ l 100  $\mu$ M C-5-propargylamino-4-tetramethylrhodamine-ddUTP

10  $\mu$ l 50 mM EDTA

1 ml glycerol

The volume was made up to 10,000  $\mu$ l with deionized H<sub>2</sub>O.

#### Reagent Mix B

The following reagents were combined to make 10 ml of reagent mix B:

10  $\mu$ l 1M MES 2-(N-morpholino)ethanesulfonic acid, pH 6.0

200  $\mu$ l 1M MgCl<sub>2</sub>

75  $\mu$ l 1M MnSO<sub>4</sub>

The volume was made up to 10,000  $\mu$ l with deionized H<sub>2</sub>O.

#### Example 2: Use of the formulation from Example 1

Two (2)  $\mu$ l reagent mix A, 2  $\mu$ l reagent mix B, 200 ng M13mp18 DNA, 5 pmole of primer (M13 - 40 Forward 5'-GTTTCCAGTCACGACGTTGTA), and deionized water to a total volume of 20  $\mu$ l were mixed together and subjected to 25 cycles of (95 °C 30 seconds, 60 °C 1 minute) in a thermal cycler. After cycling, 4  $\mu$ l of a solution which contained 1.5 M sodium acetate, 250 mM EDTA was added. The solution was mixed and 4 volumes (100  $\mu$ l) of ethanol added. The DNA was precipitated by incubation on ice for 15-20 minutes followed by centrifugation. The supernatant was removed and the pellet was washed with 70% ethanol, dried and resuspended in 4  $\mu$ l of formamide containing loading dye. The resuspended DNA was then run on an automated fluorescent DNA sequencing apparatus (ABI model 377 instrument). The print out from the machine of the DNA sequence is shown as Figure 2.

#### Example 3 Formulation of the enzyme in Mg conditions

In the following "pre-mix" protocol, all the reagents are contained in one solution.

#### Sequencing premix

The following reagents were combined to make 800  $\mu$ l of Sequencing premix

200  $\mu$ l of 500 mM Tris-HCl pH 9.5, 20 mM MgCl<sub>2</sub>

100  $\mu$ l 40 units/ $\mu$ l FY7 DNA polymerase, 0.0008 units/ $\mu$ l *Thermoplasma acidophilum*  
inorganic pyrophosphatase

100  $\mu$ l 10 mM dITP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP

100  $\mu$ l 0.125  $\mu$ M C-7-propargylamino-4-rhodamine-6-G-ddATP

100  $\mu$ l 1.2  $\mu$ M C-5-propargylamino-4-rhodamine-X-ddCTP

100  $\mu$ l 0.09  $\mu$ M C-7-propargylamino-4-rhodamine-110-ddGTP

100  $\mu$ l 2.2  $\mu$ M C-5-propargylamino-4-tetramethylrhodamine-ddUTP

#### Example 4 Use of the formulation from example 3

Four (4)  $\mu$ l of sequencing premix, 200 ng M13mp18 DNA, 5 pmole of primer (M13 - 40 Forward 5'- GTTTTCCCAGTCACGACGTTGTA), and deionized water to a total volume of 20  $\mu$ l were mixed together and subjected to 25 cycles of (95 °C 30 seconds, 60 °C 2 minutes) in a thermal cycler. After cycling, 7  $\mu$ l of 7.5 M ammonium acetate was added. The solution was mixed and 4 volumes (100  $\mu$ l) of ethanol added. The DNA was precipitated by incubation on ice for 15-20 minutes followed by centrifugation. The supernatant was removed and the pellet was washed with 70% ethanol, dried and resuspended in 4  $\mu$ l of formamide containing loading dye. The resuspended DNA was then run on an automated fluorescent DNA sequencing apparatus (ABI model 377 instrument). The print out from the machine of the DNA sequence is shown as Figure 3.

#### Example 5 Polymerase Activity versus Salt Concentration (KCl) for Thermo Sequenase<sup>TM</sup> enzyme and FY7 enzyme.

The percent of maximum polymerase activity was measured for Thermo Sequenase<sup>TM</sup> enzyme DNA polymerase and FY7 DNA polymerase under varying KCl concentrations. The results are depicted in Figure 4. The data indicate that FY7 has a much higher salt optimum as well as broader range of tolerance for salt in the reaction mixture than Thermo Sequenase<sup>TM</sup>. The salt concentration which gives 50% activity is five-fold higher for FY7 than for Thermo Sequenase.

The effect of high salt concentrations on DNA sequencing ability in radioactively labeled DNA sequencing reactions was also examined. The results are presented in Figure 5. At KCl concentrations of 50mM or higher Thermo Sequenase<sup>TM</sup> polymerase performance degrades to levels at which usable data cannot be extracted. FY7 DNA polymerase, however, is able to give quite good sequencing data at concentrations of KCl of 100mM.

#### Example 6 Fluorescent Sequencing Salt Tolerance

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These experiments examined the effect of the above-demonstrated polymerase activity in high salt concentrations on DNA sequencing ability in fluorescently labeled terminator DNA sequencing reactions. The results are presented in Figures 6-15.

Figures 6-10 show the effect of increasing salt concentration on the performance of Thermo Sequenase. At concentrations as low as 25mM data quality is affected with the read length being decreased from at least 600 bases to about 450 bases. At 50mM salt the read length is further decreased to about 350 bases, 75mM to about 250 bases and at 100mM the read length is negligible.

Figures 11-15 show the effect of increasing salt concentration on the performance of FY7 DNA polymerase. There is no detrimental effect on performance to at least 75mM KCl and only a slight decrease in data quality at 100mM KCl.

As it is recognized that some types of DNA preparations may be contaminated with salt (which is detrimental to DNA sequencing data quality), the use of FY7 DNA polymerase allows for a more robust sequencing reaction over a broader range of template conditions.

#### Example 7 Polymerase Processivity

The processivity (number of nucleotides incorporated per DNA polymerase binding event) has been measured, for different DNA sequencing polymerases. The results are presented in Figure 16.

Thermo Sequenase DNA polymerase has a processivity of only ~4 nucleotides per binding event. AmpliTaq<sup>\*</sup> FS DNA polymerase has a processivity of ~15 nucleotides per binding event. FY7 DNA polymerase has a processivity more than seven-fold greater than Thermo Sequenase DNA polymerase and ~two-fold greater than AmpliTaq FS DNA polymerase at ~30 nucleotides per binding event.

#### Example 8 Polymerase Extension with dITP at 72 °C

The series examined improved read length obtained when using FY7 polymerase versus Thermo Sequenase DNA polymerase in radioactively labeled sequencing reactions incorporating the dGTP (Guanosine triphosphate) analog dITP (Inosine triphosphate) at 72 °C. The results are presented in Figure 17. FY7 is able to incorporate >50-100 more nucleotides under standard <sup>33</sup>P[α-dATP] sequencing conditions than Thermo Sequenase.

#### Example 9 Effect of Extension Step Time on Length of Read

These series of experiments examined the effect of increasing extension step time of the read length and data quality of Thermo Sequenase and FY7 DNA polymerases in fluorescently labeled terminator DNA sequencing reactions. The results are presented in Figures 18-27.

Figures 18-22 show the effect of increasing extension step time on the read length and data quality produced by Thermo Sequenase DNA polymerase. This data shows that a minimum of a two

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minutes extension step is required by Thermo Sequenase in order to achieve a quality read of at least 600 bases. Signal strength generally increases to a maximum at a four minute extension (the time specified in the commercial product utilizing this enzyme and method).

Figures 23-27 show the effect of increasing extension step time on the read length and data quality produced by FY7 DNA polymerase. This data shows that a minimum of a 30 second extension step is required by FY7 in order to achieve a quality read of at least 600 bases. Signal strengths plateau at about one minute extension time. The FY7 DNA polymerase can produce data of equivalent quality to Thermo Sequenase in one-quarter to one-half the time of extension reaction.

Although the above examples describe various embodiments of the invention in detail, many variations will be apparent to those of ordinary skill in the art. Accordingly, the above examples are intended for illustration purposes and should not be used in any way to restrict the scope of the appended claims.

## SEQUENCE LISTING

<110> AMERSHAM PHARMACIA BIOTECH INC.

<120> FY7 Polymerase

<130> 30134-1

<140> 2,330,735

<141> 1999-06-17

<150> 60/089,556

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<150> 09/334,818

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cgccgggagg ccaagacggg gaactacggc gtcctctacg gcatgtccgc ccataggctc 2040
tcccaggagc tagccatccc ctacgaagaa gcggtggcct ttatagagcg ctacttccaa 2100
agcttccccca aggtgcgggc ctggatagaa aagaccctgg aggaggggag gaagcggggc 2160
tacgtggaaa ccctcttcgg aagaaggcgc tacgtgcccg acctcaacgc ccgggtgaag 2220
agcgtcaggg aggccgcgga gcgcattggc ttcaacatgc ccgtccaggg caccgcccgc 2280
gacctcatga agctcgccat ggtgaagctc tccccccgcc tccgggagat gggggcccgc 2340
atgctcctcc aggtccacga cgagctctc ctggaggccc cccaagcgcg ggccgaggag 2400
gtggcggtt tggccaagga ggccatggag aaggcctatc ccctcgccgt gcccctggag 2460
gtggaggtgg ggatggggga ggactggctt tccgccaagg gttag 2505

```

&lt;210&gt; 2

&lt;211&gt; 834

&lt;212&gt; PRT

<213> *Thermus thermophilus*

&lt;400&gt; 2

```

Met Glu Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
  1              5              10              15

```

```

Val Ala Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
      20              25              30

```

```

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
      35              40              45

```

```

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
      50              55              60

```

```

Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
      65              70              75              80

```

```

Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
      85              90              95

```

```

Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
      100             105             110

```

```

Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
      115             120             125

```

```

Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
      130             135             140

```

```

Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu
      145             150             155             160

```

```

Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Arg
      165             170             175

```

```

Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
      180             185             190

```

```

Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
      195             200             205

```

Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg  
 210 215 220  
 Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp  
 225 230 235 240  
 Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu  
 245 250 255  
 Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg  
 260 265 270  
 Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly  
 275 280 285  
 Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro  
 290 295 300  
 Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp  
 305 310 315 320  
 Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg  
 325 330 335  
 Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly  
 340 345 350  
 Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp  
 355 360 365  
 Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro  
 370 375 380  
 Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp  
 385 390 395 400  
 Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg  
 405 410 415  
 Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr  
 420 425 430  
 His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala  
 435 440 445  
 Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu  
 450 455 460  
 Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala  
 465 470 475 480  
 Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu  
 485 490 495  
 Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly  
 500 505 510  
 Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His  
 515 520 525

Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys  
 530 535 540  
 Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly  
 545 550 555 560  
 Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu  
 565 570 575  
 Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu  
 580 585 590  
 Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu  
 595 600 605  
 Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu  
 610 615 620  
 Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile  
 625 630 635 640  
 His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val  
 645 650 655  
 Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Tyr Gly Val Leu  
 660 665 670  
 Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr  
 675 680 685  
 Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys  
 690 695 700  
 Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly  
 705 710 715 720  
 Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn  
 725 730 735  
 Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn  
 740 745 750  
 Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val  
 755 760 765  
 Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln  
 770 775 780  
 Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu  
 785 790 795 800  
 Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala  
 805 810 815  
 Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala  
 820 825 830  
 Lys Gly

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CLAIMS:

1. A purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in SEQ ID NO:2.
2. An isolated nucleic acid that encodes a  
5 thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence set forth in SEQ ID NO:1.
3. A recombinant DNA vector that comprises the nucleic acid of claim 2.
- 10 4. The recombinant DNA vector of claim 3 comprising the plasmid pMR10.
5. A recombinant host cell transformed with the vector of claim 3 or 4.
6. The recombinant host cell of claim 5 that is  
15 *E. coli*.
7. The recombinant host cell of claim 6 which is *E. coli* carrying the *cI*<sup>+</sup> and *cI857* alleles.
8. The recombinant host cell of claim 6 selected from the group consisting of DH1λ<sup>+</sup> [*gyrA96*, *recA1*, *relA1*, *endA1*,  
20 *thi-1*, *hsdR17*, *supE44*, λ<sup>+</sup>] and M5248 [λ (*bio275*, *cI857*, *cIII+*, *N+*, λ (*H1*))].
9. Method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase of claim 1 in the  
25 presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments.

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10. A kit for sequencing DNA comprising the DNA polymerase of claim 1 and at least one chain terminating agent.

FETHERSTONHAUGH & CO.  
OTTAWA, CANADA

PATENT AGENTS

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/1
ATG GAA GCG ATG CTG CCG CTG TTC GAA CCC AAA GGC CGT GTC CTC CTG GTG GCC GGC CAC
M E A M L P L F E P K G R V L L V A G H
61/21
CAC CTG GCC TAC CGC ACC TTC TTC GCC CTG AAG GGC CTC ACC ACG AGC CGG GGC GAA CCG
H L A Y R T F F A L K G L T T S R G E P
121/41
GTG CAG GCG GTC TAC GGC TTC GCC AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC
V Q A V Y G F A K S L L K A L K E D G Y
181/61
AAG GCC GTC TTC GTG GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC CAC GAG GCC TAC GAG
K A V F V V F D A K A P S F R H E A Y E
241/81
GCC TAC AAG GCG GGG AGG GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG CTC GCC CTC ATC
A Y K A G R A P T P E D F P R Q L A L I
301/101
AAG GAG CTG GTG GAC CTC CTG GGG TTT ACC CGC CTC GAG GTC CCC GGC TAC GAG GCG GAC
K E L V D L L G F T R L E V P G Y E A D
361/121
GAC GTT CTC GCC ACC CTG GCC AAG AAG GCG GAA AAG GAG GGG TAC GAG GTG CGC ATC CTC
D V L A T L A K K A E K E G Y E V R I L
421/141
ACC GCC GAC CGC GAC CTC TAC CAA CTC GTC TCC GAC CGC GTC GCC GTC CTC CAC CCC GAG
T A D R D L Y Q L V S D R V A V L H P E
481/161
GGC CAC CTC ATC ACC CCG GAG TGG CTT TGG GAG AAG TAC GGC CTC AGG CCG GAG CAG TGG
G H L I T P E W L W E K Y G L R P E Q W
541/181
GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC AAC CTC CCC GGG GTC AAG GGC ATC
V D F R A L V G D P S D N L P G V K G I
601/201
GGG GAG AAG ACC GCC CTC AAG CTC CTC AAG GAG TGG GGA AGC CTG GAA AAC CTC CTC AAG
G E K T A L K L L K E W G S L E N L L K
661/221
AAC CTG GAC CGG GTA AAG CCA GAA AAC GTC CGG GAG AAG ATC AAG GCC CAC CTG GAA GAC
N L D R V K P E N V R E K I K A H L E D
721/241
CTC AGG CTC TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC CTC CCC CTG GAG GTG GAC CTC
L R L S L E L S R V R T D L P L E V D L
781/261
GCC CAG GGG CGG GAG CCC GAC CGG GAG GGG CTT AGG GCC TTC CTG GAG AGG CTG GAA TTC
A Q G R E P D R E G L R A F L E R L E F
841/281
GGC AGC CTC CTC CAC GAG TTC GGC CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC
G S L L H E F G L L E A P A P L E E A P
901/301
TGG CCC CCG CCG GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC CCC GAG CCC ATG TGG
W P P P E G A F V G F V L S R P E P M W
961/321
GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG GTG CAC CGG GCA GCA GAC CCC
A E L K A L A A C R D G R V H R A A D P
1021/341
TTG GCG GGG CTA AAG GAC CTC AAG GAG GTC CGG GGC CTC CTC GCC AAG GAC CTC GCC GTC
L A G L K D L K E V R G L L A K D L A V
1081/361
TTG GCC TCG AGG GAG GGG CTA GAC CTC GTG CCC GGG GAC GAC CCC ATG CTC CTC GCC TAC
L A S R E G L D L V P G D D P M L L A Y
1141/381
CTC CTG GAC CCC TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG GGG GAG TGG
L L D P S N T T P E G V A R R Y G G E W
1201/401
1231/411

```

Figure 1

ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC TCG GAG AGG CTC CAT CGG AAC CTC CTT AAG  
 T E D A A H R A L L S E R L H R N L L K  
 1261/421 1291/431  
 CGC CTC GAG GGG GAG GAG AAG CTC CTT TGG CTC TAC CAC GAG GTG GAA AAG CCC CTC TCC  
 R L E G E E K L L W L Y H E V E K P L S  
 1321/441 1351/451  
 CGG GTC CTG GCC CAC ATG GAG GCC ACC GGG GTA CGG CTG GAC GTG GCC TAC CTT CAG GCC  
 R V L A H M E A T G V R L D V A Y L Q A  
 1381/461 1411/471  
 CTT TCC CTG GAG CTT GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTG TTC CGC TTG GCG  
 L S L E L A E E I R R L E E E V F R L A  
 1441/481 1471/491  
 GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTG CTC TTT GAC GAG CTT  
 G H P F N L N S R D Q L E R V L F D E L  
 1501/501 1531/511  
 AGG CTT CCC GCC TTG GGG AAG ACG CAA AAG ACA GGC AAG CGC TCC ACC AGC GCC GCG GTG  
 R L P A L G K T Q K T G K R S T S A A V  
 1561/521 1591/531  
 CTG GAG GCC CTA CGG GAG GCC CAC CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC  
 L E A L R E A H P I V E K I L Q H R E L  
 1621/541 1651/551  
 ACC AAG CTC AAG AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC CAC CCG AGG ACG GGC  
 T K L K N T Y V D P L P S L V H P R T G  
 1681/561 1711/571  
 CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGG AGG CTT AGT AGC TCC GAC  
 R L H T R F N Q T A T A T G R L S S S D  
 1741/581 1771/591  
 CCC AAC CTG CAG AAC ATC CCC GTC CGC ACC CCC TTG GGC CAG AGG ATC CGC CGG GCC TTC  
 P N L Q N I P V R T P L G Q R I R R A F  
 1801/601 1831/611  
 GTG GCC GAG GCG GGT TGG GCG TTG GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGC GTC  
 V A E A G W A L V A L D Y S Q I E L R V  
 1861/621 1891/631  
 CTC GCC CAC CTC TCC GGG GAC GAA AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC  
 L A H L S G D E N L I R V F Q E G K D I  
 1921/641 1951/651  
 CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC CCC CCG GAG GCC GTG GAC CCC CTG ATG  
 H T Q T A S W M F G V P P E A V D P L M  
 1981/661 2011/671  
 CGC CGG GCG GCC AAG ACG GTG AAC TAC GGC GTC CTC TAC GGC ATG TCC GCC CAT AGG CTC  
 R R A A K T V N Y G V L Y G M S A H R L  
 2041/681 2071/691  
 TCC CAG GAG CTA GCC ATC CCC TAC GAA GAA GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA  
 S Q E L A I P Y E E A V A F I E R Y F Q  
 2101/701 2131/711  
 AGC TTC CCC AAG GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC  
 S F P K V R A W I E K T L E E G R K R G  
 2161/721 2191/731  
 TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC AAC GCC CGG GTG AAG  
 Y V E T L F G R R R Y V P D L N A R V K  
 2221/741 2251/751  
 AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC ATG CCC GTC CAG GGC ACC GCC GCC  
 S V R E A A E R M A F N M P V Q G T A A  
 2281/761 2311/771  
 GAC CTC ATG AAG CTC GCC ATG GTG AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC  
 D L M K L A M V K L F P R L R E M G A R  
 2341/781 2371/791  
 ATG CTC CTC CAG GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG GCC GAG GAG  
 M L L Q V H D E L L L E A P Q A R A E E  
 2401/801 2431/811  
 GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC GTG CCC CTG GAG  
 V A A L A K E A M E K A Y P L A V P L E

Figure 1 (continued)

|          |     |     |     |     |     |     |     |     |     |     |          |     |     |     |  |  |  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----------|-----|-----|-----|--|--|--|
| 2461/821 |     |     |     |     |     |     |     |     |     |     | 2491/831 |     |     |     |  |  |  |
| GTG      | GAG | GTG | GGG | ATG | GGG | GAG | GAC | TGG | CTT | TCC | GCC      | AAG | GGT | TAG |  |  |  |
| V        | E   | V   | G   | M   | G   | E   | D   | W   | L   | S   | A        | K   | G   | *   |  |  |  |

Figure 1 (continued)

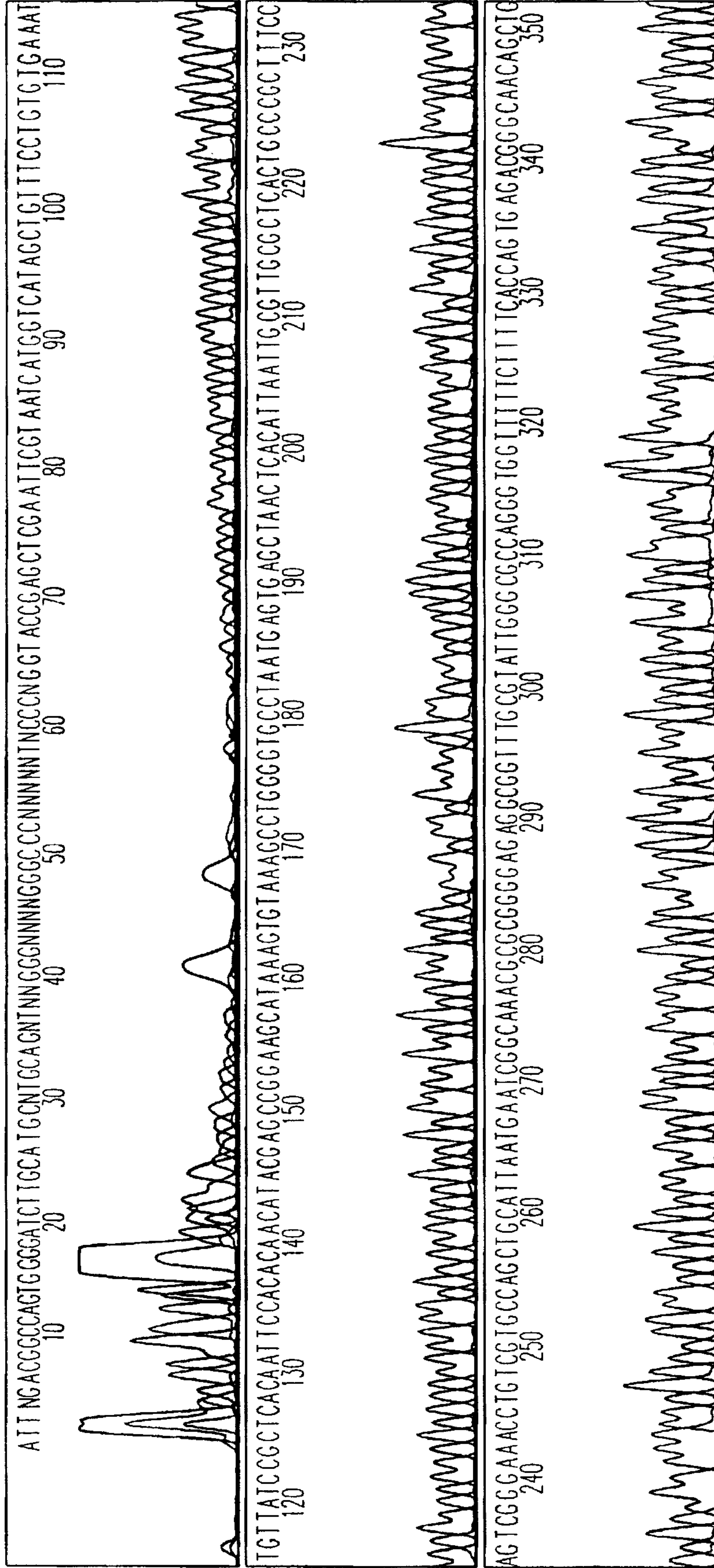


FIG.2A

FIG. 2B

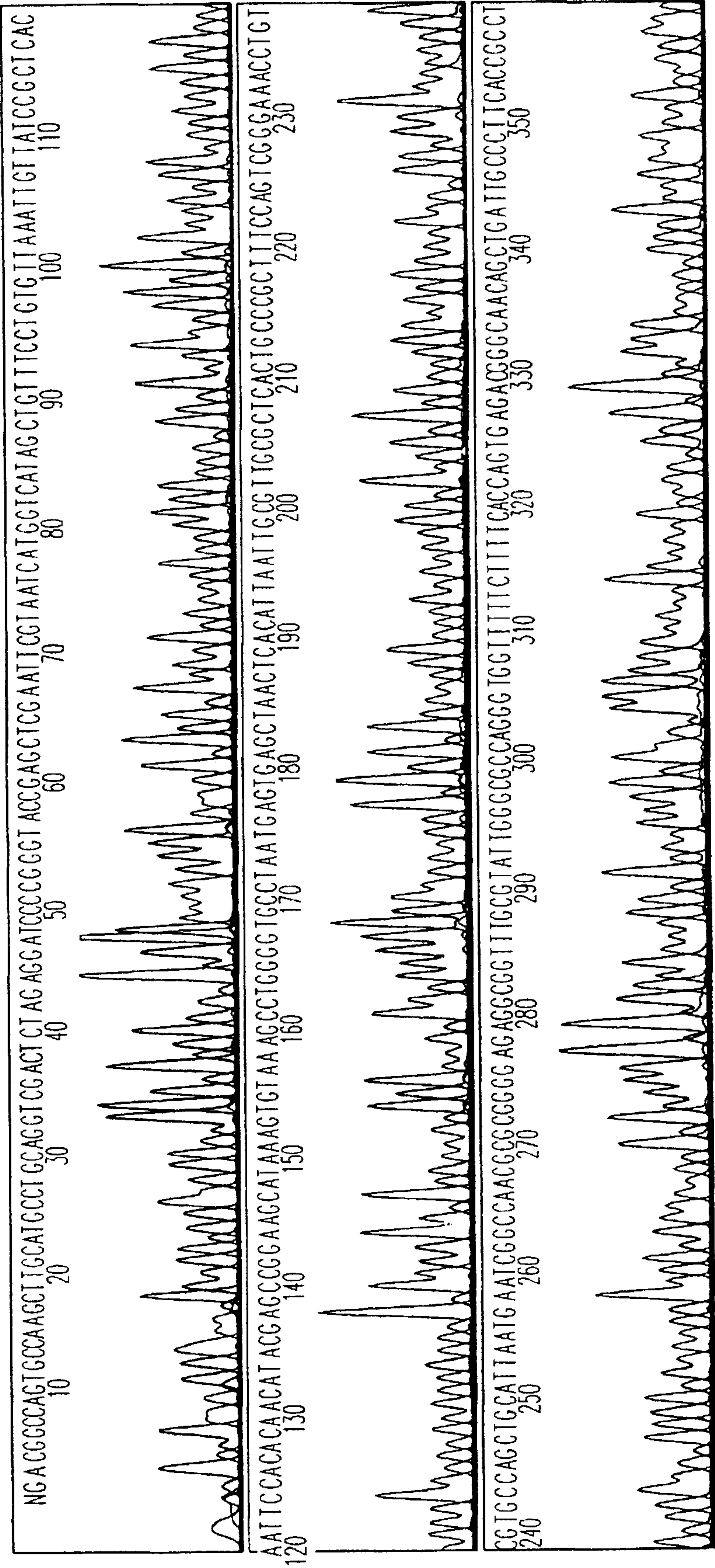


FIG.3A

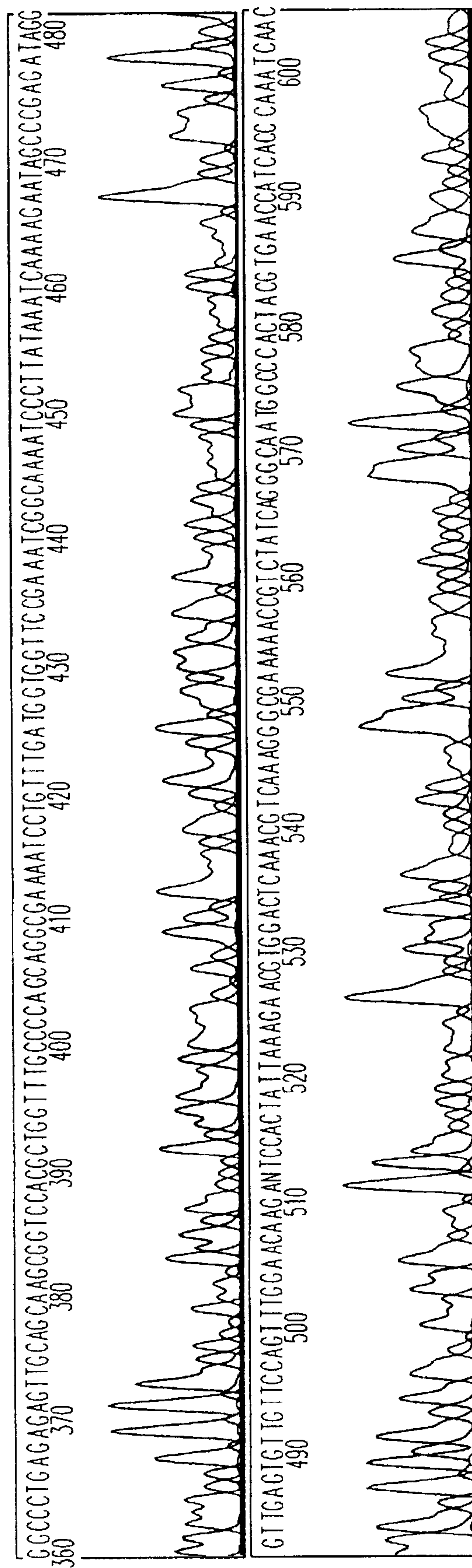


FIG. 3B

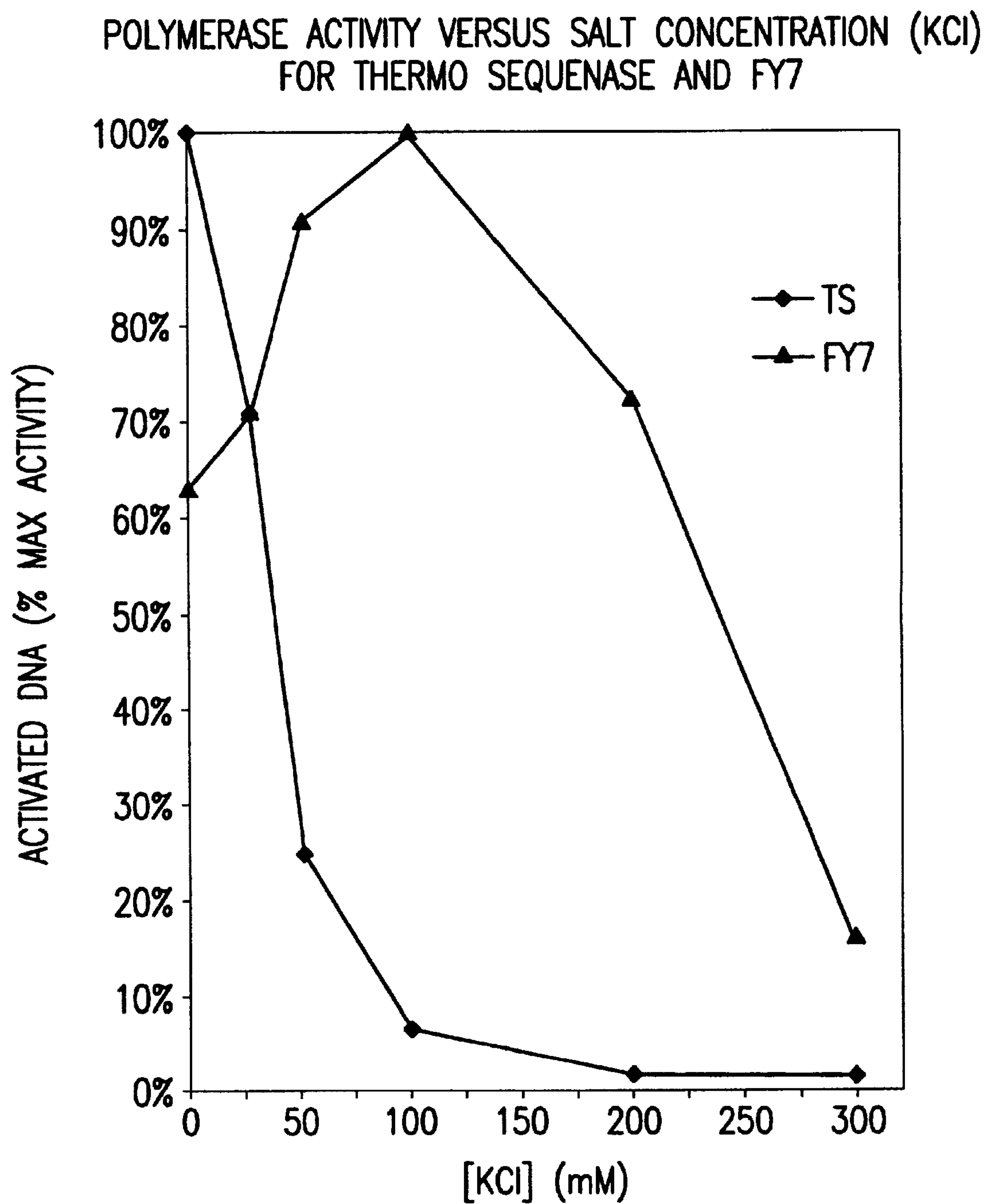


FIG.4

## SALT TOLERANCE (KCl) OF FY7 vs. THERMO SEQUENASE

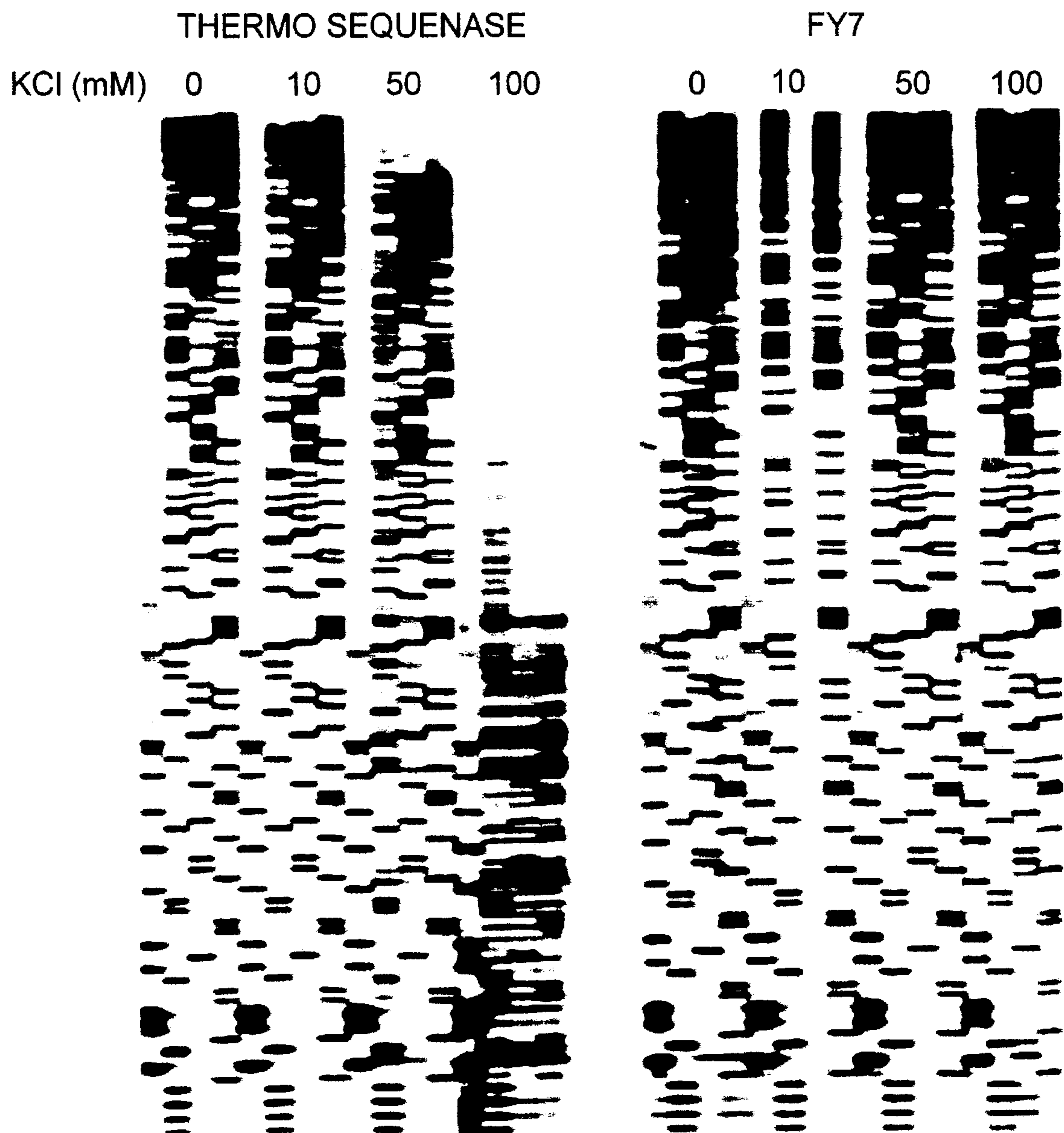


FIG.5

**FIG. 6A**

**FIG. 6B**

**FIG. 7A**

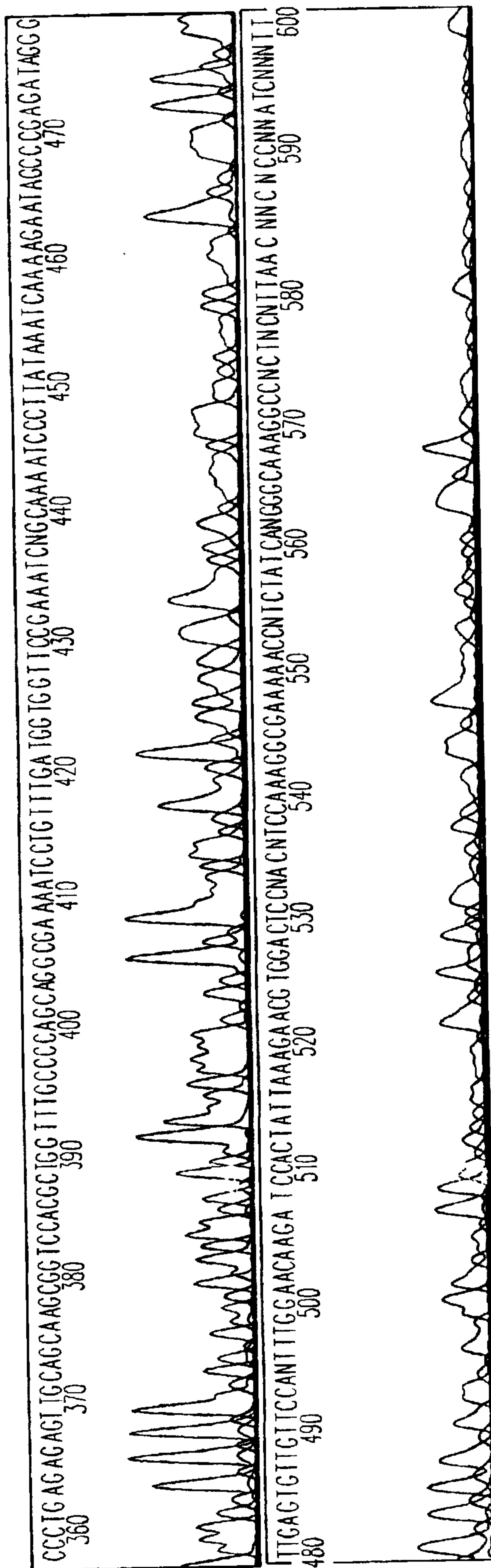


FIG. 7B

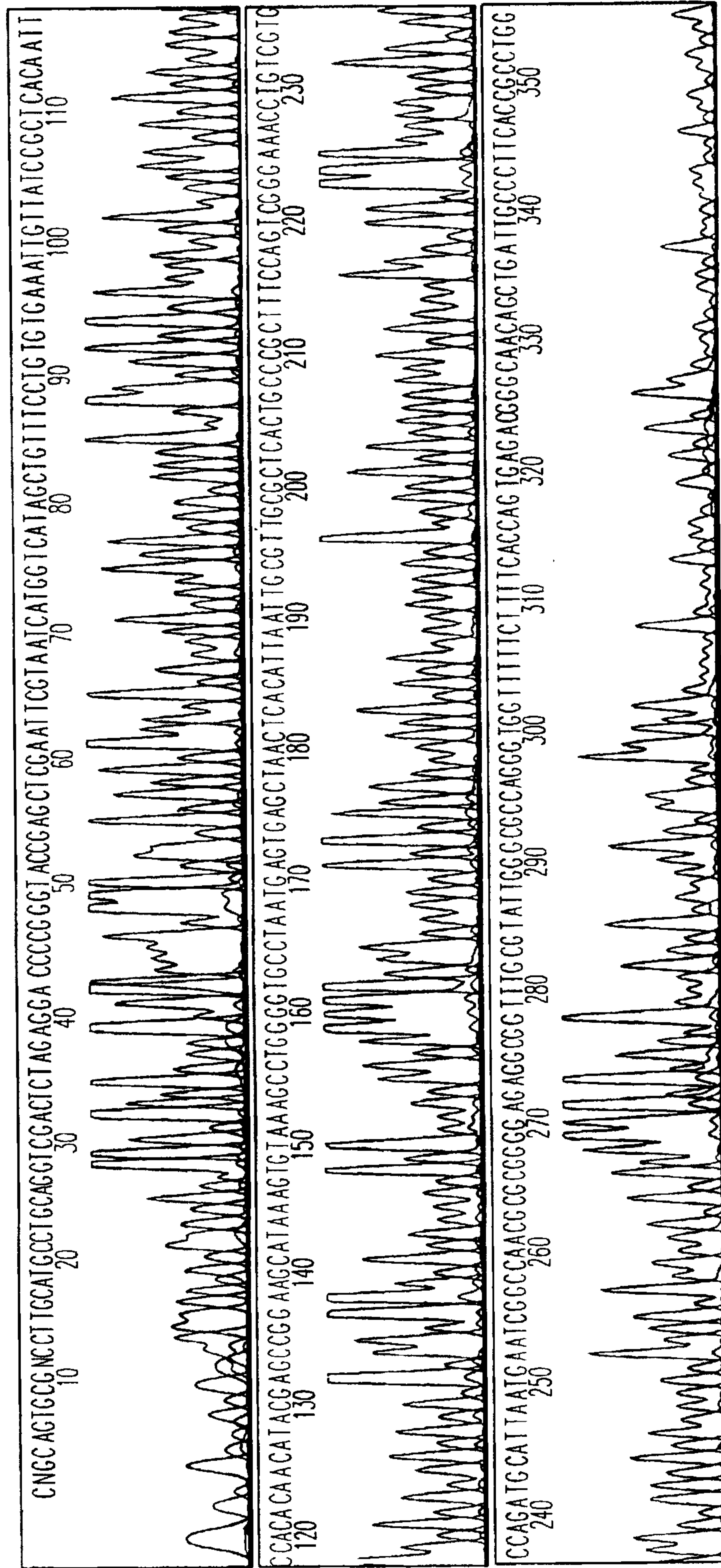


FIG.8A

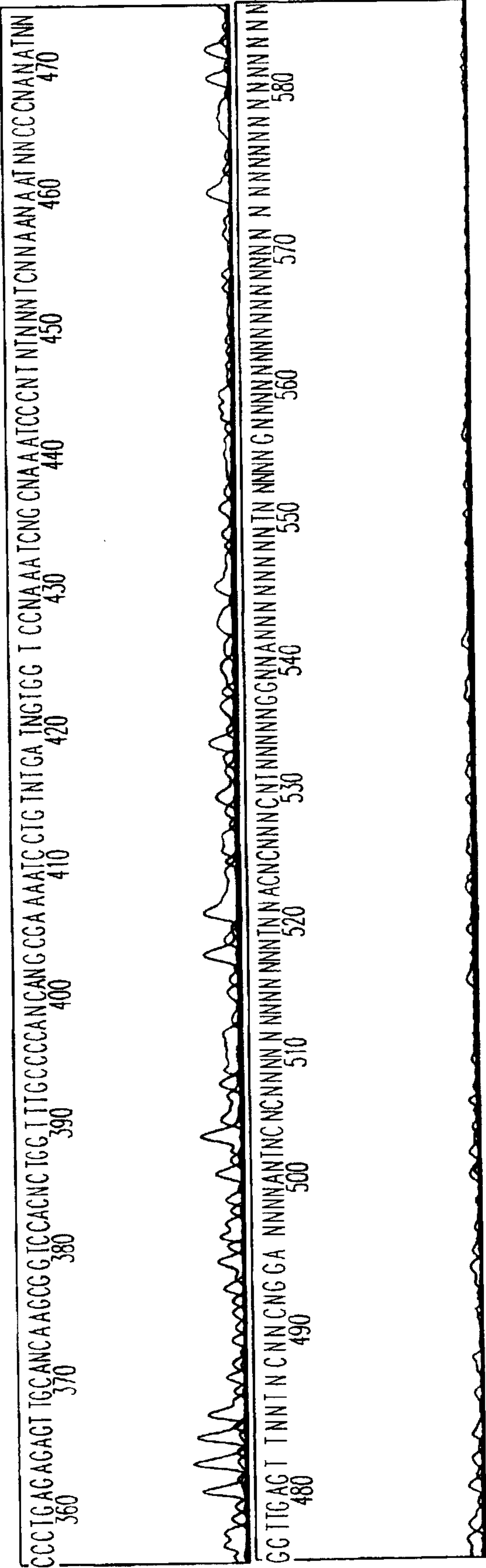


FIG.8B

**FIG. 9A**

**FIG. 9B**

ATNGACGGCAGTGCCAAGCTTGCATGGCTGCAGGTCCGACTCTAGAGGATCCCGGGGTACCGAGCICGAAATCGTAATCATGGTCAATAGCIGTTCCTGCTGAAATGCTGAAATCGCTCACAAT  
 10 20 30 40 50 60 70 80 90 100 110 120  
 TCCACACACA  
 130  
 TCCACACACA  
 140  
 TCCACACACA  
 150  
 TCCACACACA  
 160  
 TCCACACACA  
 170  
 TCCACACACA  
 180  
 TCCACACACA  
 190  
 TCCACACACA  
 200  
 TCCACACACA  
 210  
 TCCACACACA  
 220  
 TCCACACACA  
 230  
 TCCACACACA  
 240  
 TCCACACACA  
 250  
 TCCACACACA  
 260  
 TCCACACACA  
 270  
 TCCACACACA  
 280  
 TCCACACACA  
 290  
 TCCACACACA  
 300  
 TCCACACACA  
 310  
 TCCACACACA  
 320  
 TCCACACACA  
 330  
 TCCACACACA  
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 TCCACACACA  
 350  
 TCCACACACA  
 360  
 TCCACACACA  
 370  
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 390  
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 400  
 TCCACACACA  
 410  
 TCCACACACA  
 420  
 TCCACACACA  
 430  
 TCCACACACA  
 440  
 TCCACACACA  
 450  
 TCCACACACA  
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 TCCACACACA  
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 750  
 TCCACACACA  
 760  
 TCCACACACA  
 770  
 TCCACACACA  
 780  
 TCCACACACA  
 790  
 TCCACACACA  
 800  
 TCCACACACA  
 810  
 TCCACACACA  
 820  
 TCCACACACA  
 830  
 TCCACACACA  
 840  
 TCCACACACA  
 850  
 TCCACACACA  
 860  
 TCCACACACA  
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 TCCACACACA  
 880  
 TCCACACACA  
 890  
 TCCACACACA  
 900  
 TCCACACACA  
 910  
 TCCACACACA  
 920  
 TCCACACACA  
 930  
 TCCACACACA  
 940  
 TCCACACACA  
 950  
 TCCACACACA  
 960  
 TCCACACACA  
 970  
 TCCACACACA  
 980  
 TCCACACACA  
 990  
 TCCACACACA  
 1000  
 TCCACACACA

**FIG. 10B**

FIG. 11A

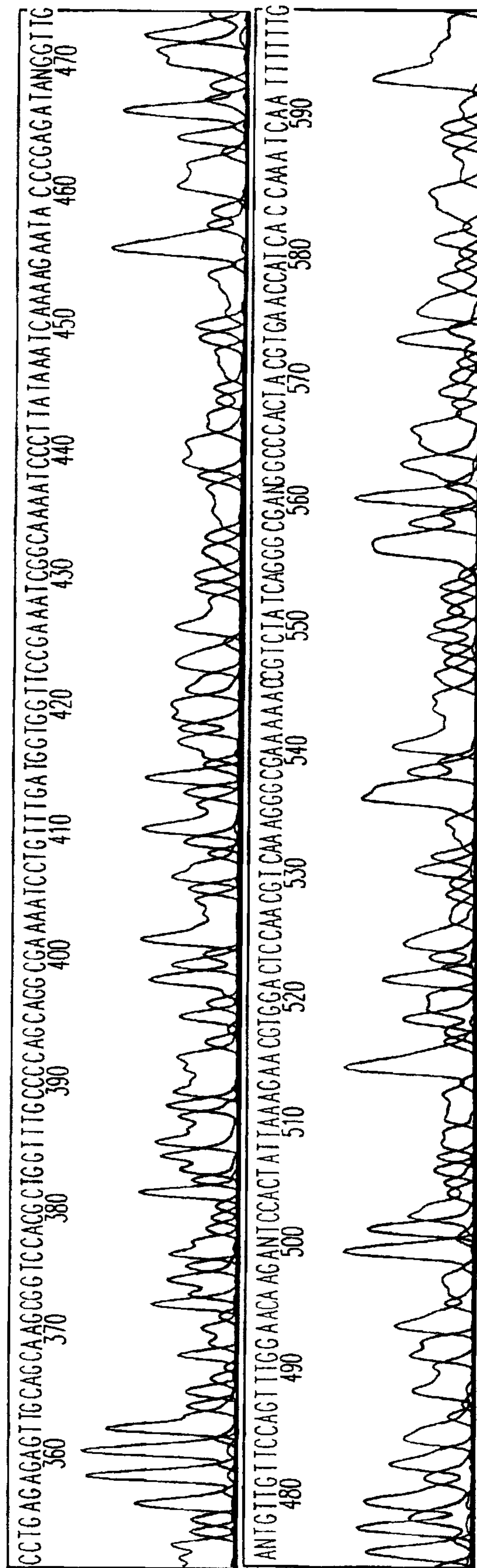


FIG.11B

FIG. 12A

**FIG. 12B**

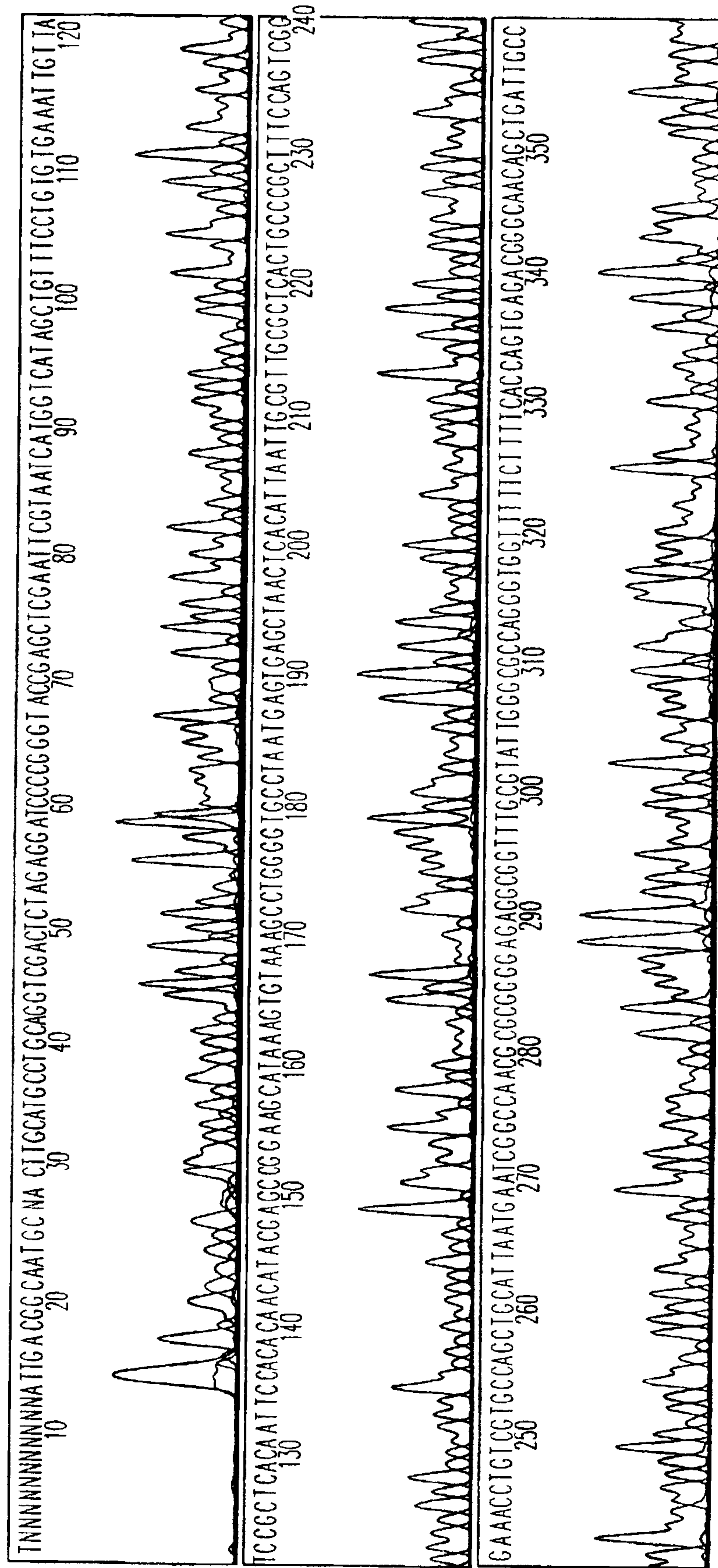


FIG.13A

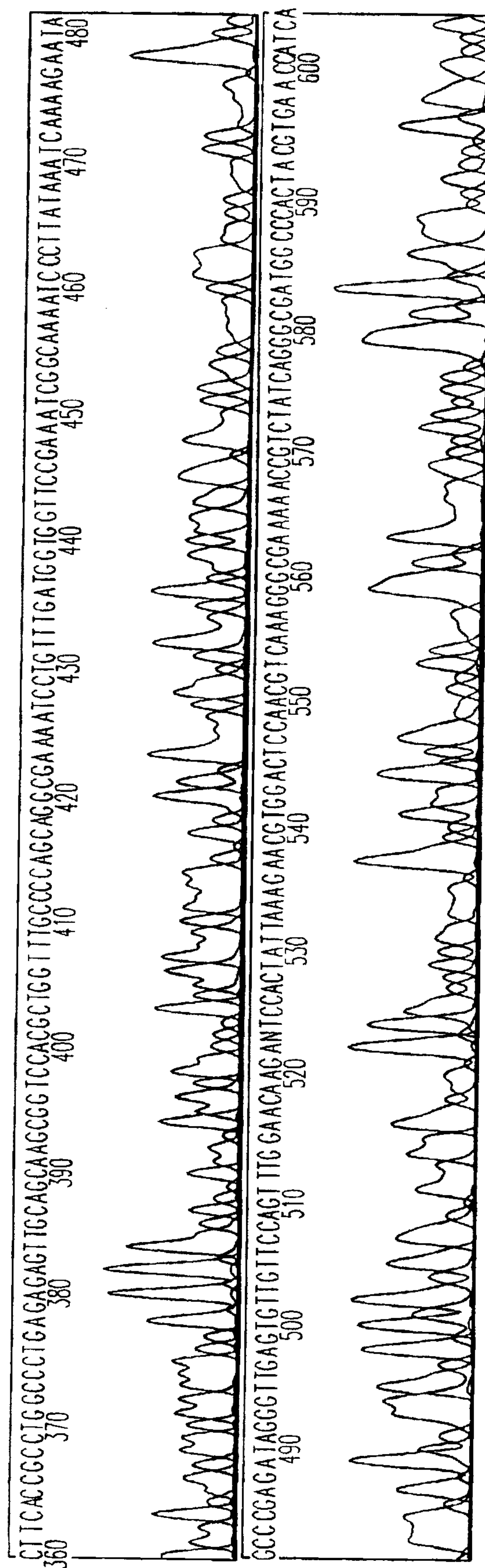


FIG. 13B

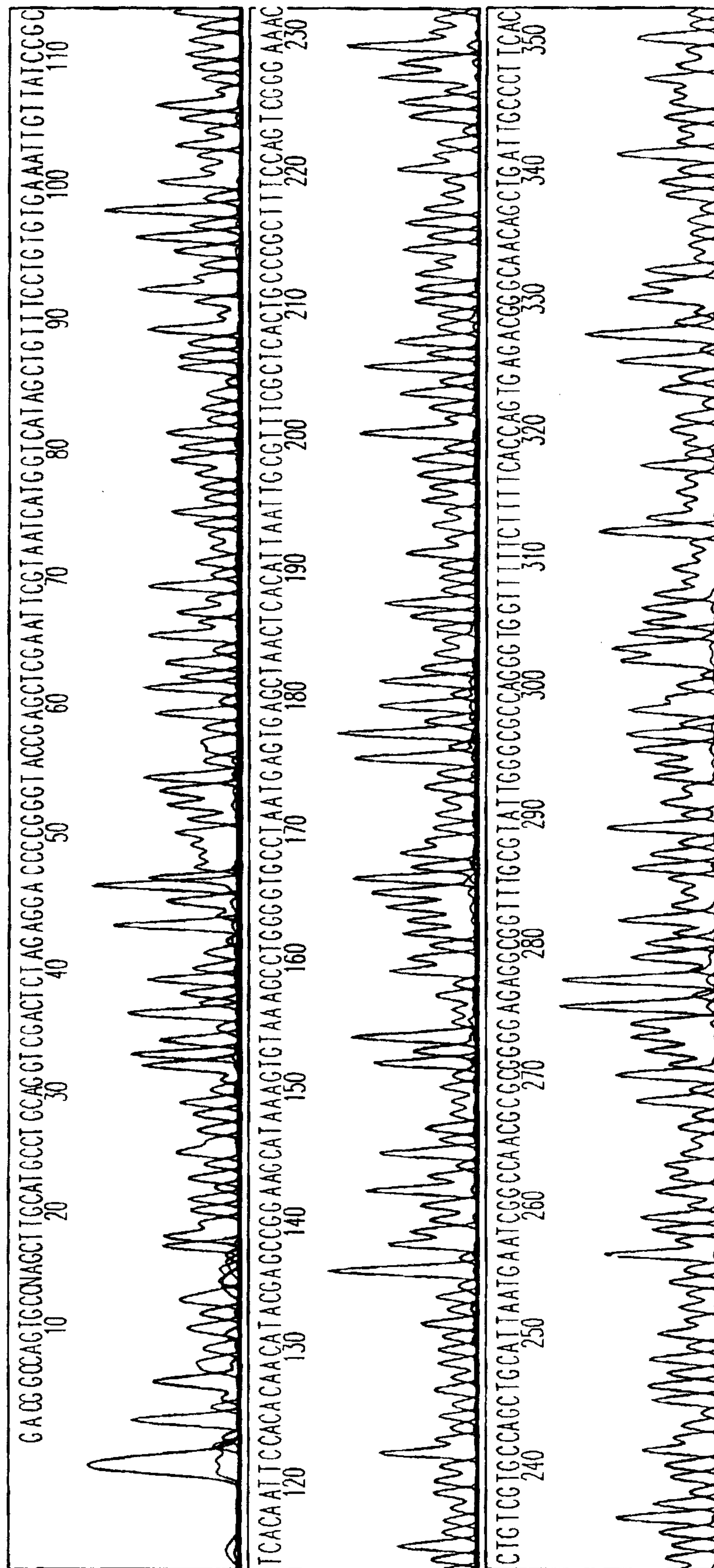


FIG.14A

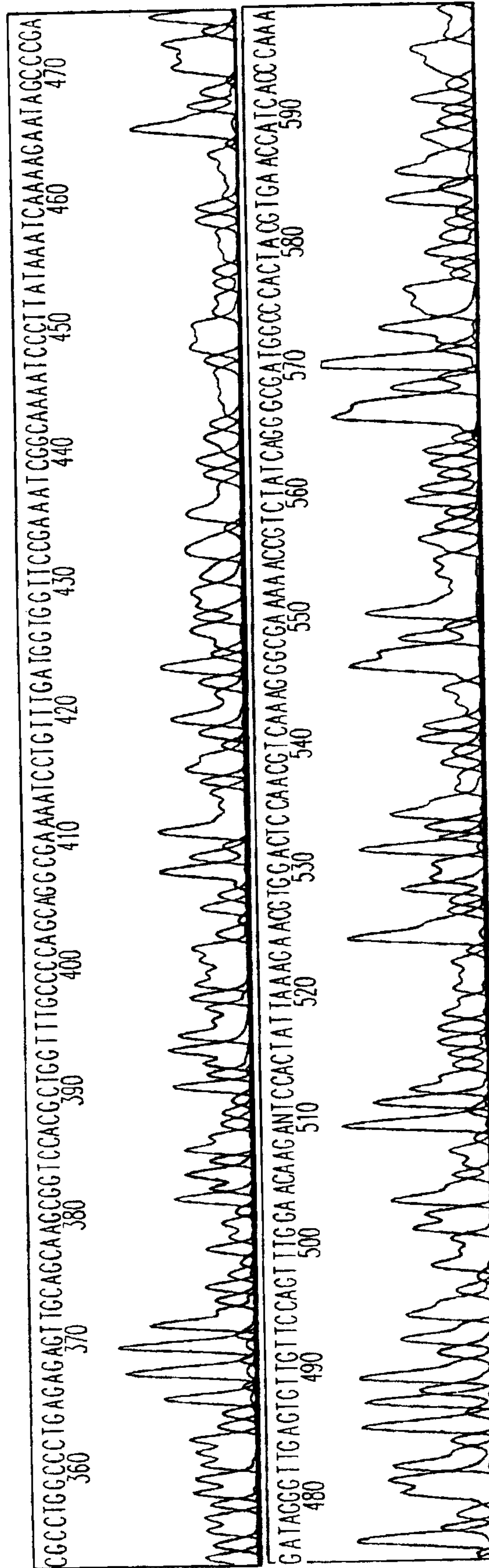


FIG.14B

**FIG. 15A**

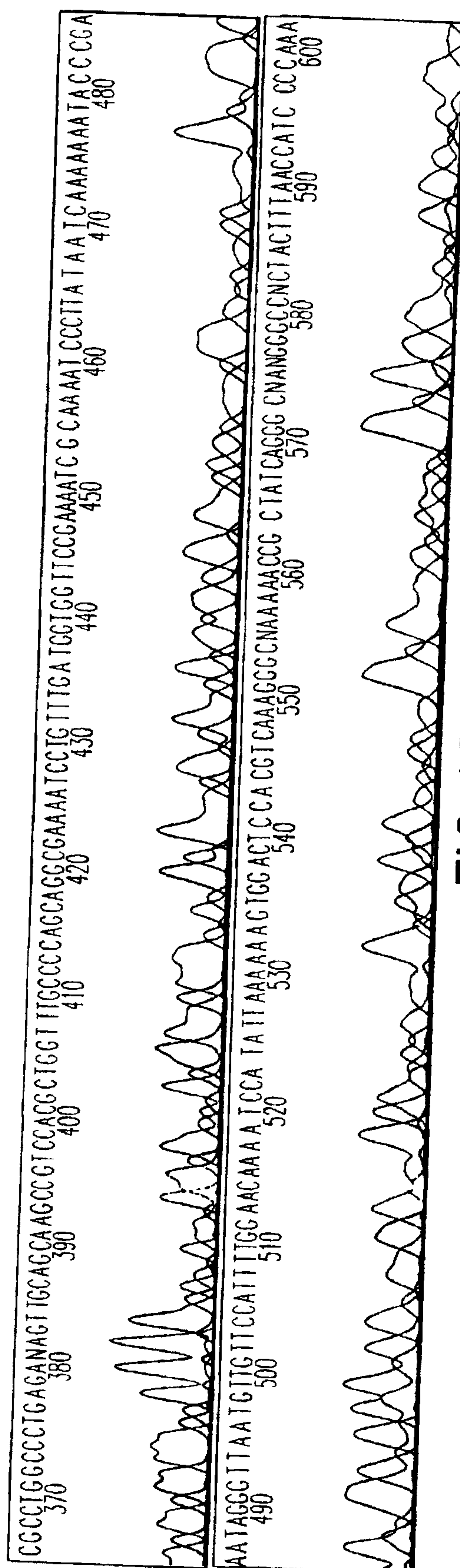


FIG.15B

| POLYMERASE       | PROCESSIVITY<br>NUMBER OF NUCLEOTIDES |
|------------------|---------------------------------------|
| THERMO SEQUENASE | 4                                     |
| AMPLITAQ FS      | 15                                    |
| FY7              | 30                                    |

FIG.16

# Polymerase Extension with dITP at 72°C



FIG.17

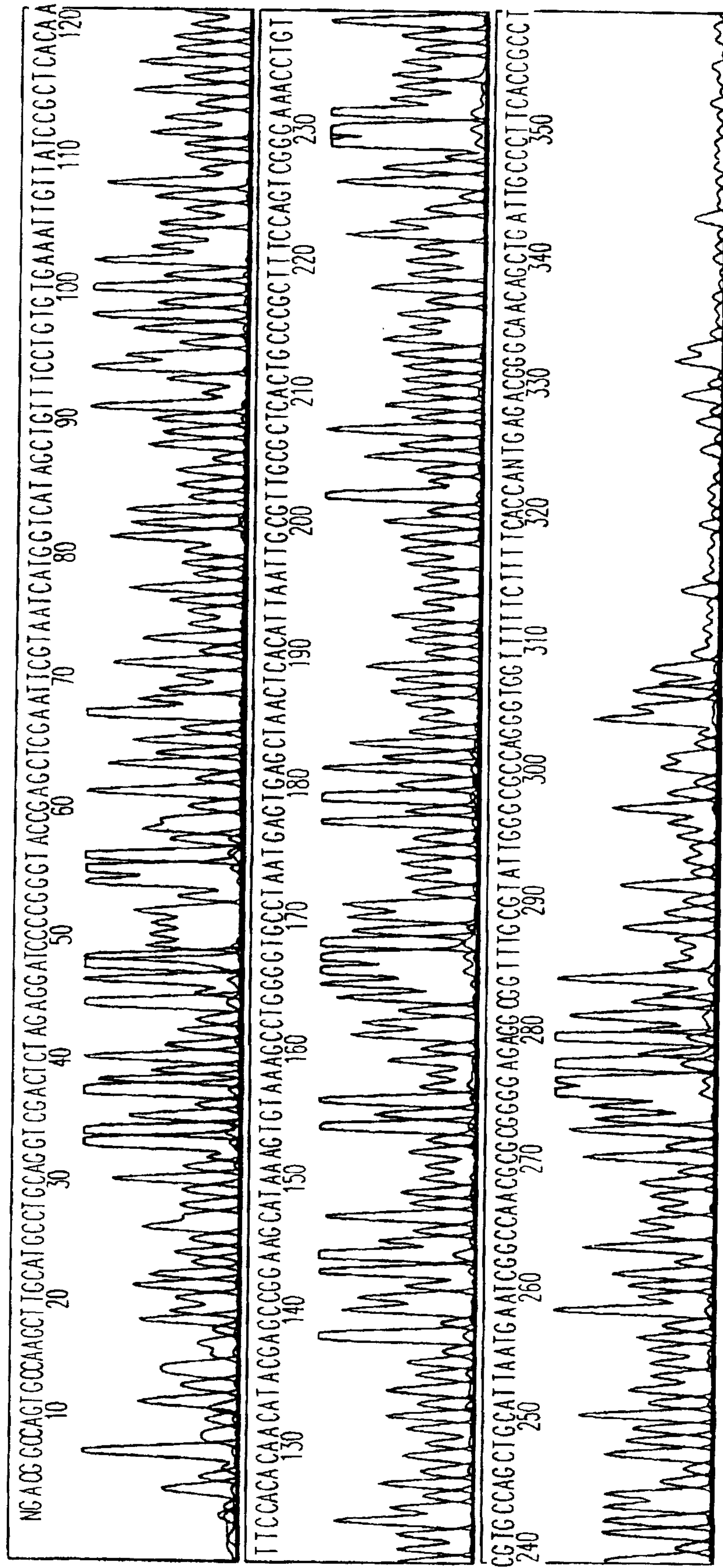


FIG.18A

**FIG. 18B**

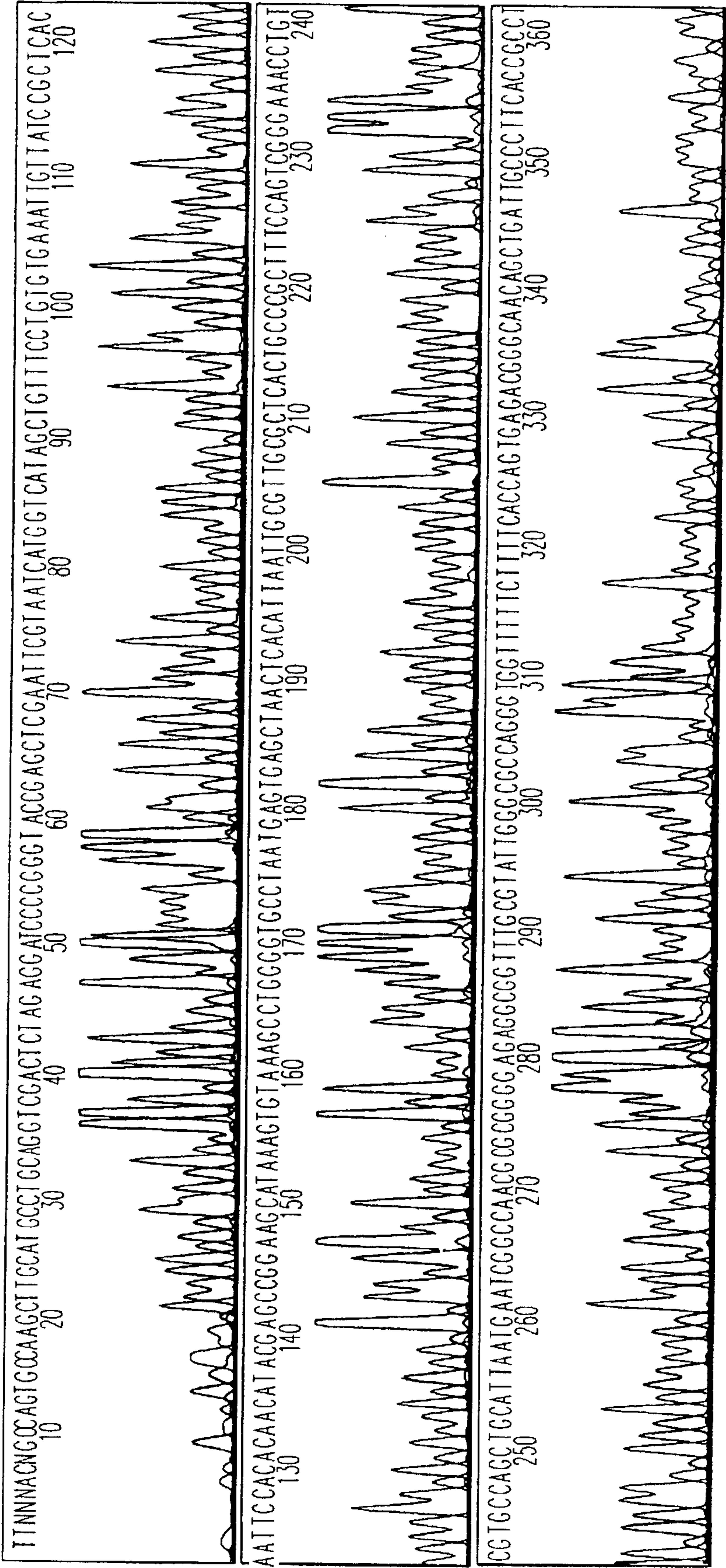


FIG.19A

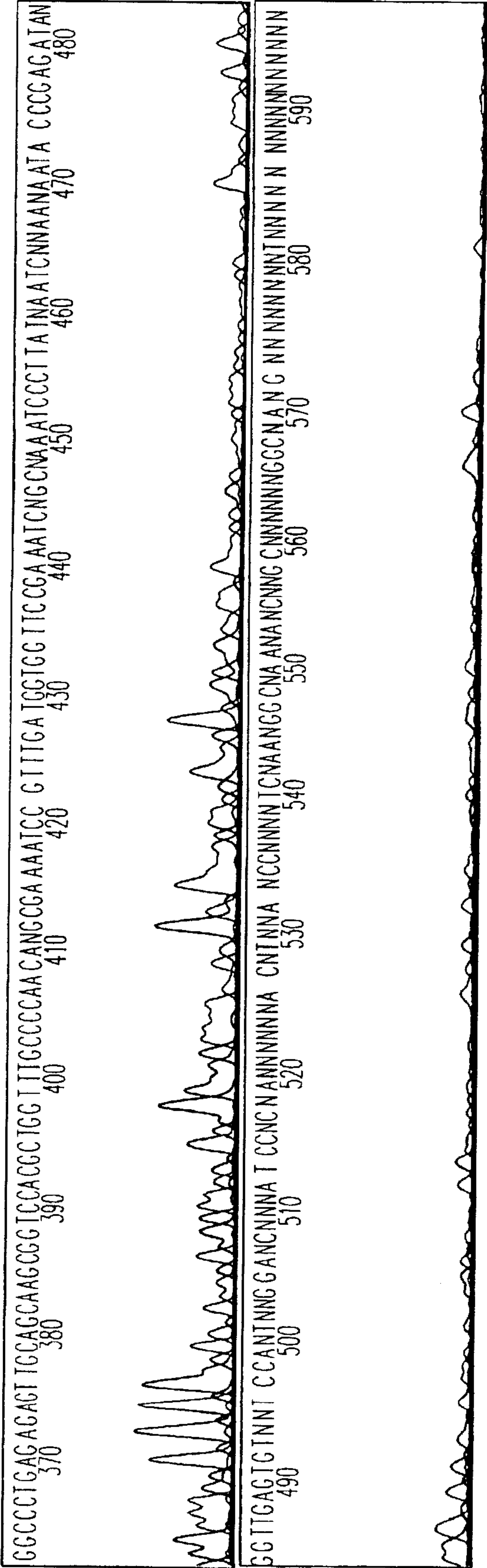


FIG.19B

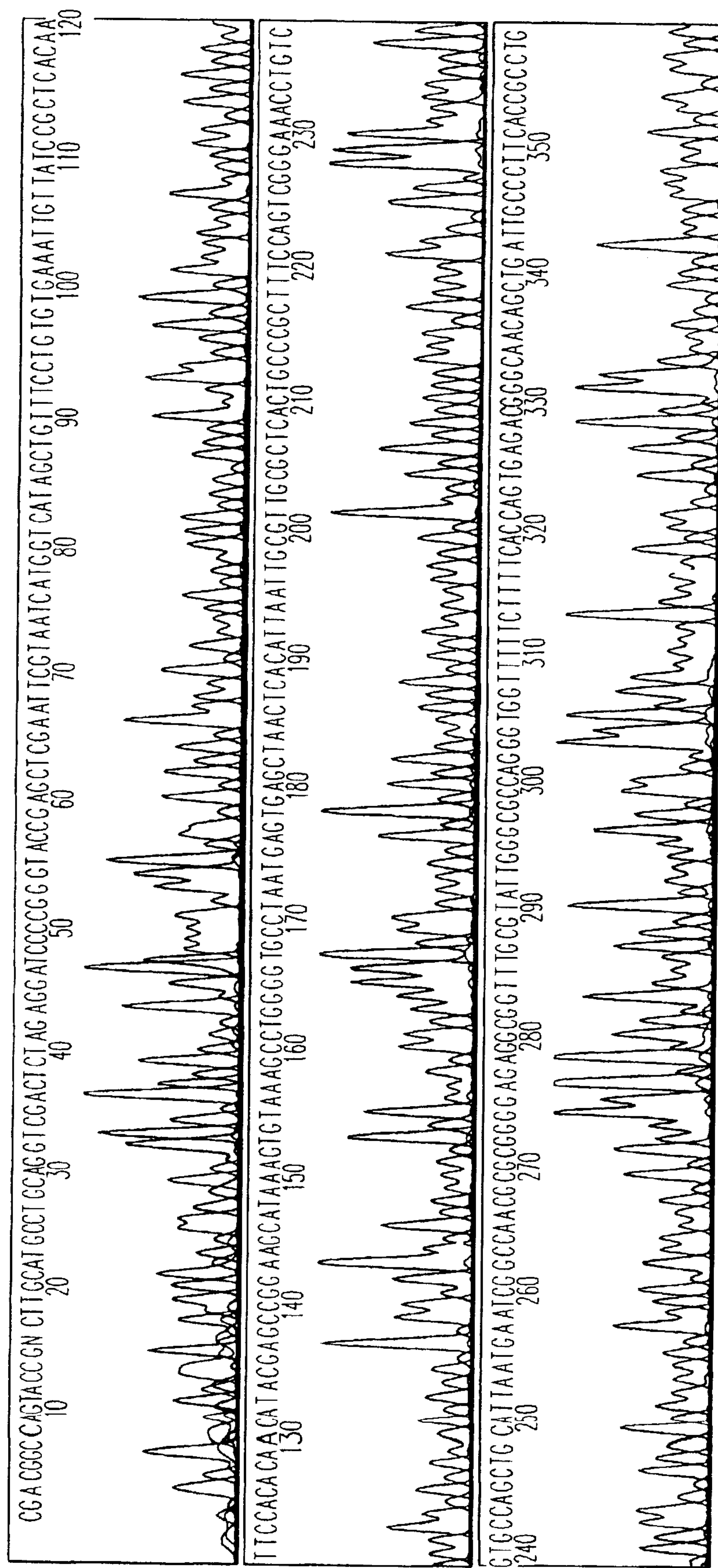


FIG. 20A

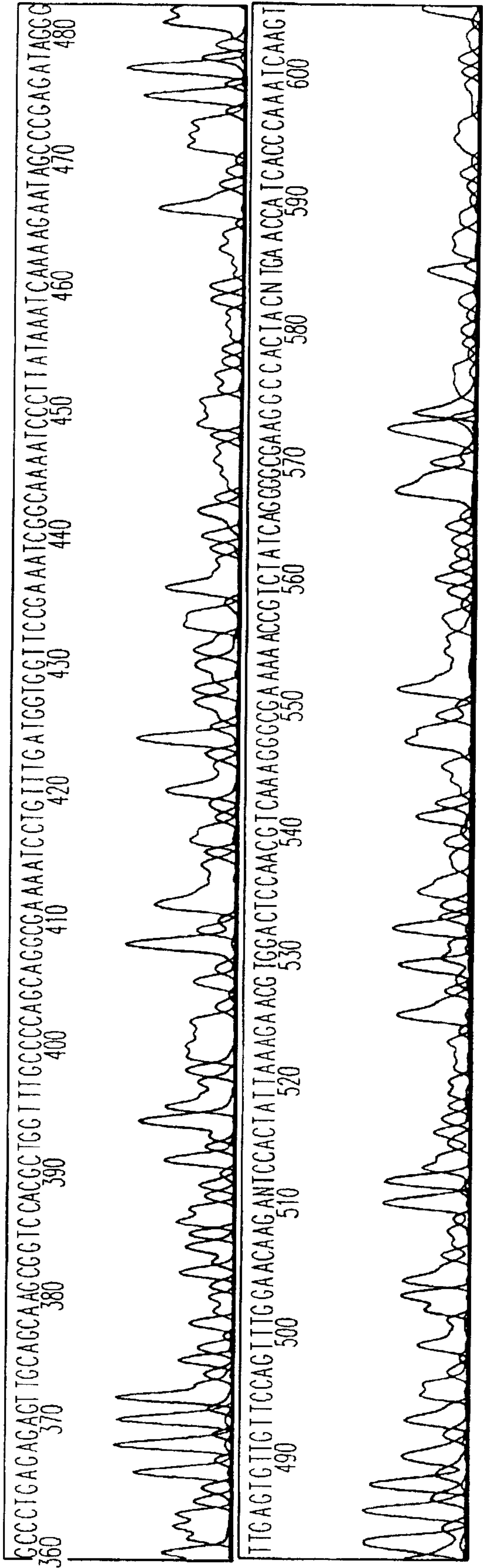


FIG.20B

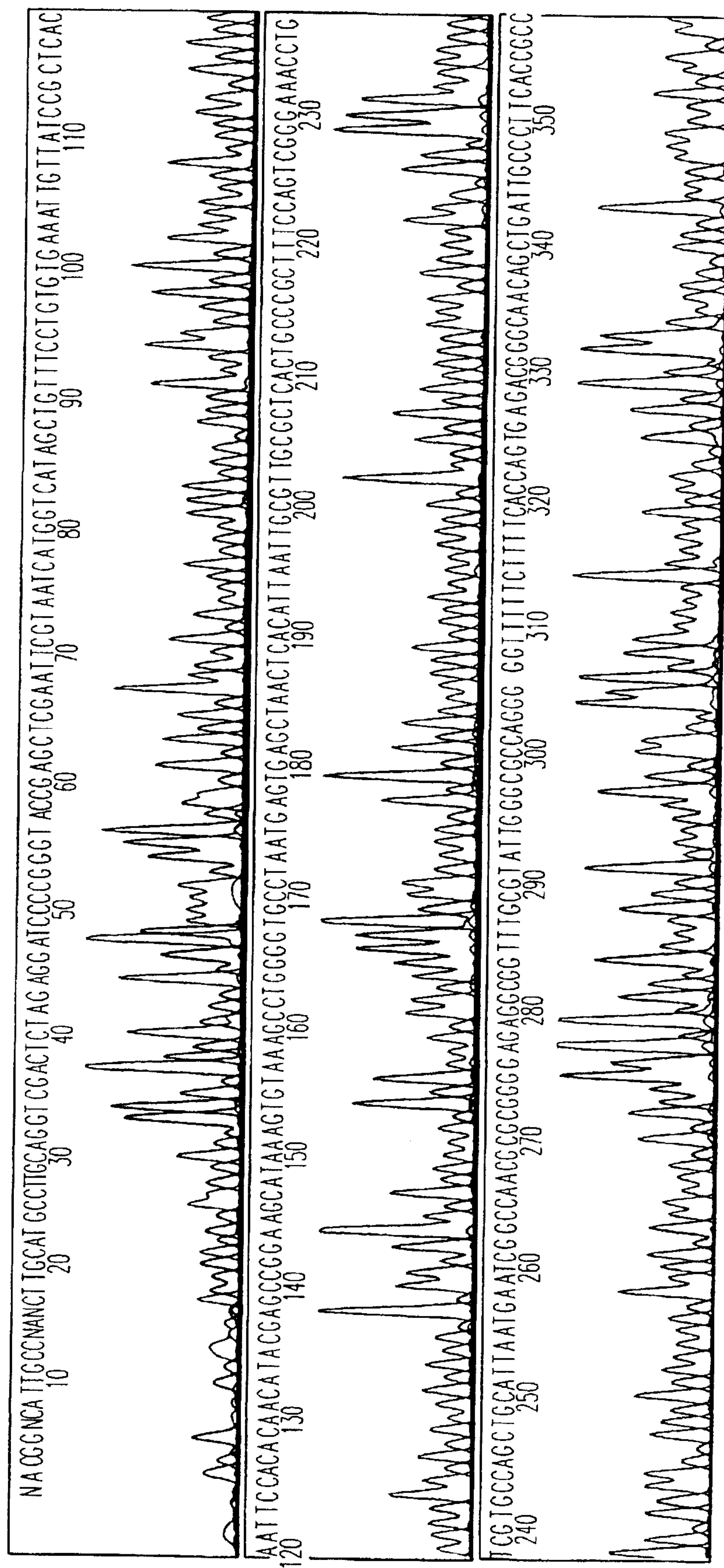


FIG.21A

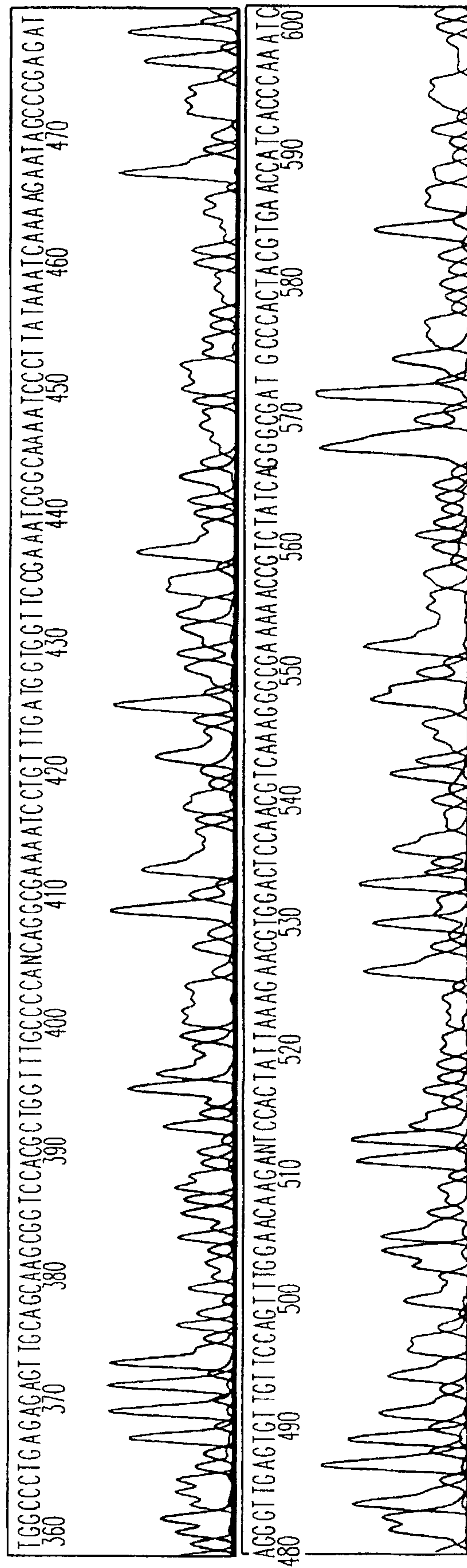


FIG. 21B

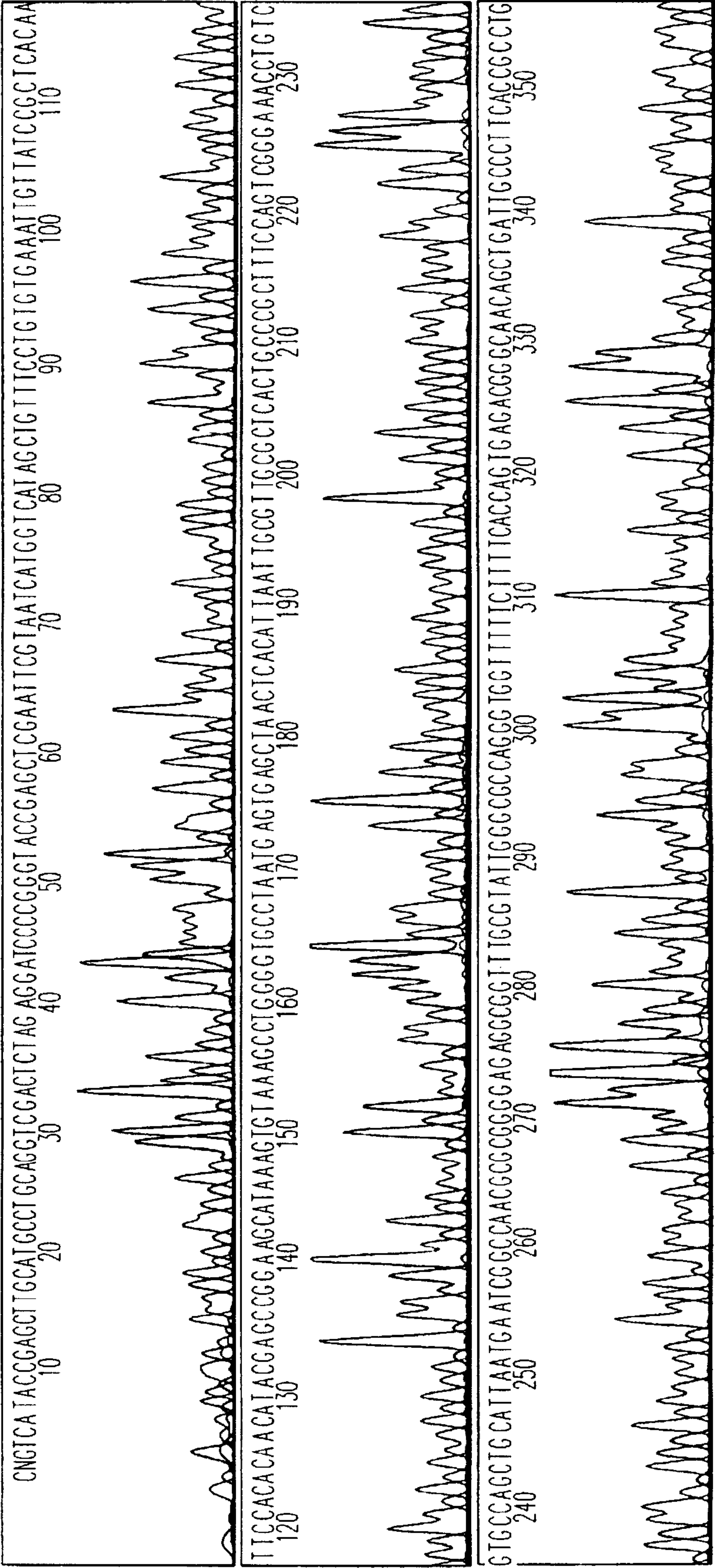


FIG.22A

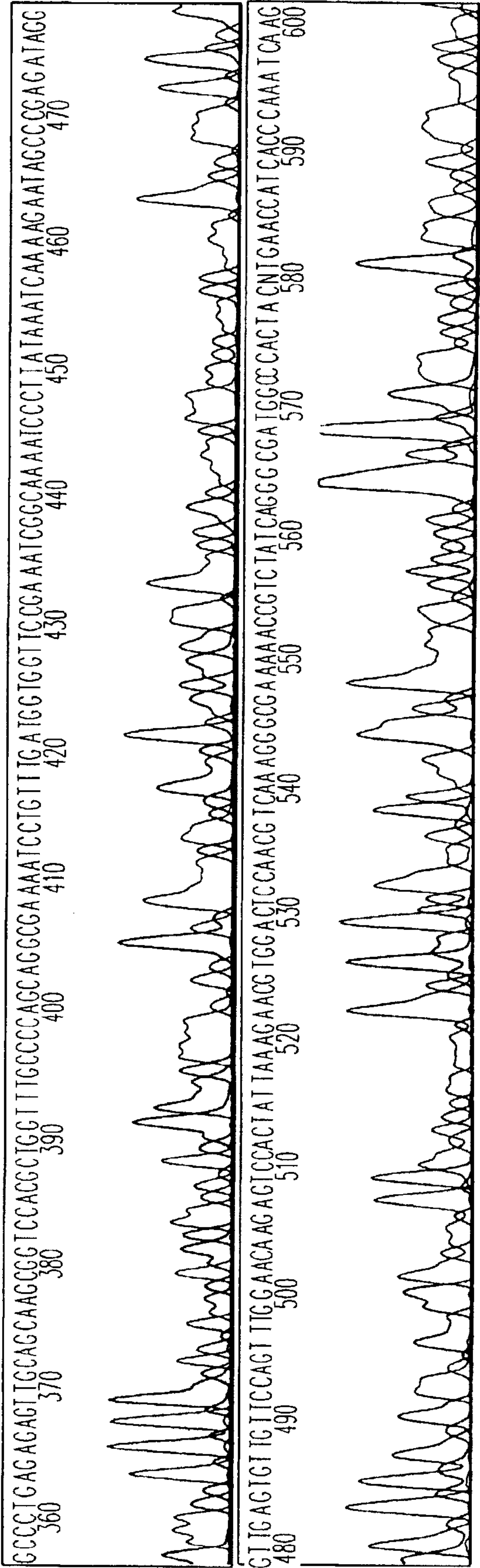


FIG.22B

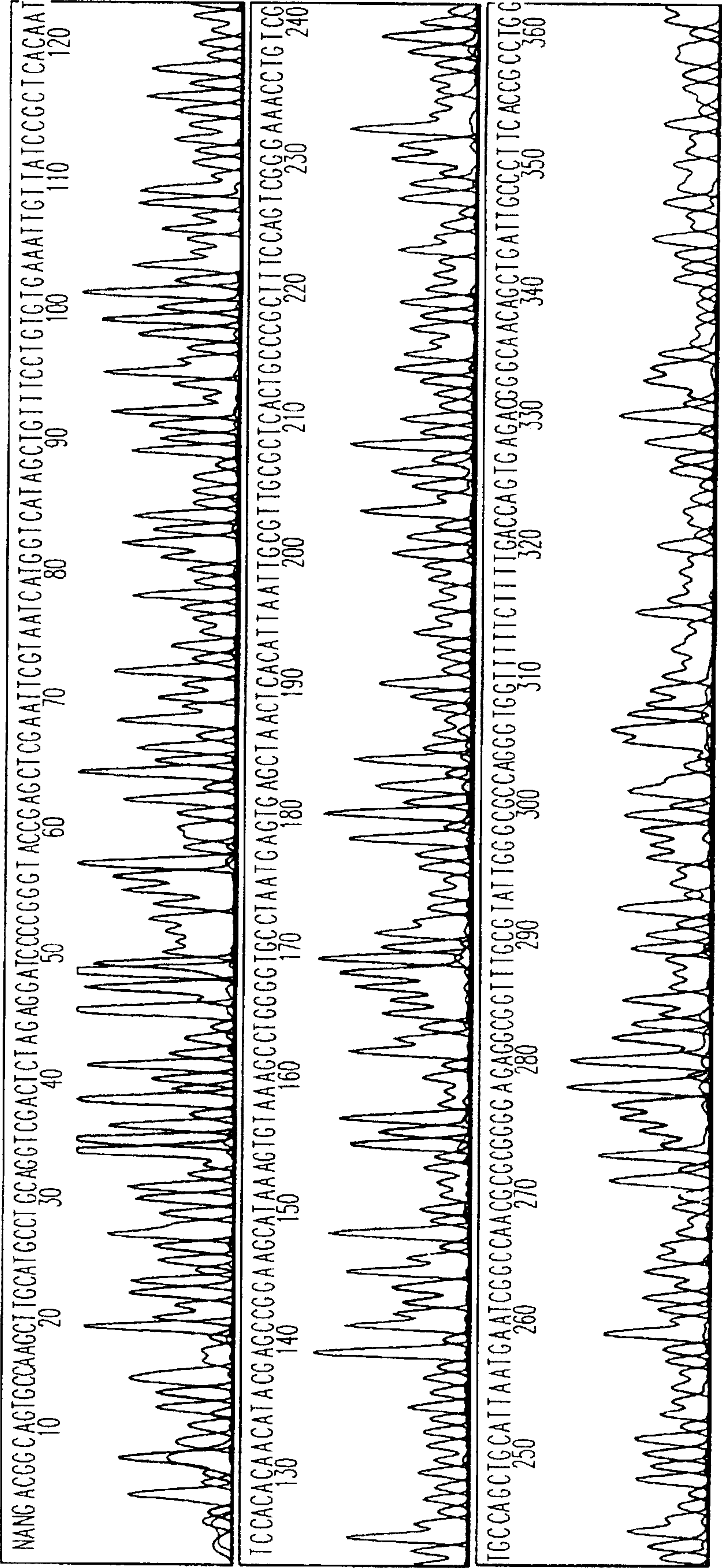


FIG.23A

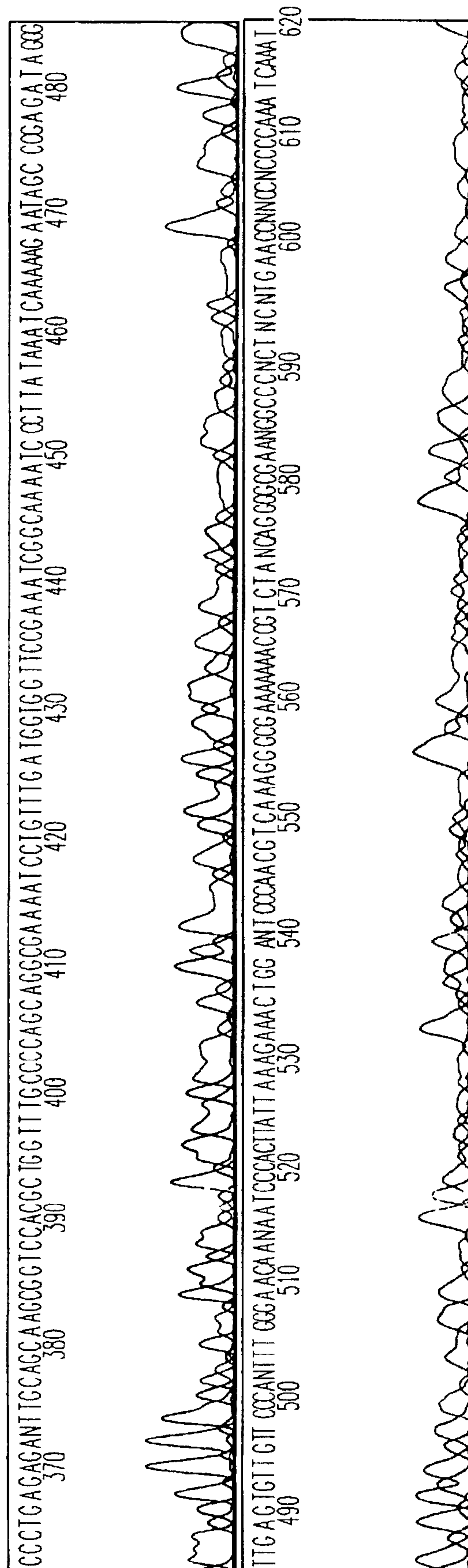


FIG.23B

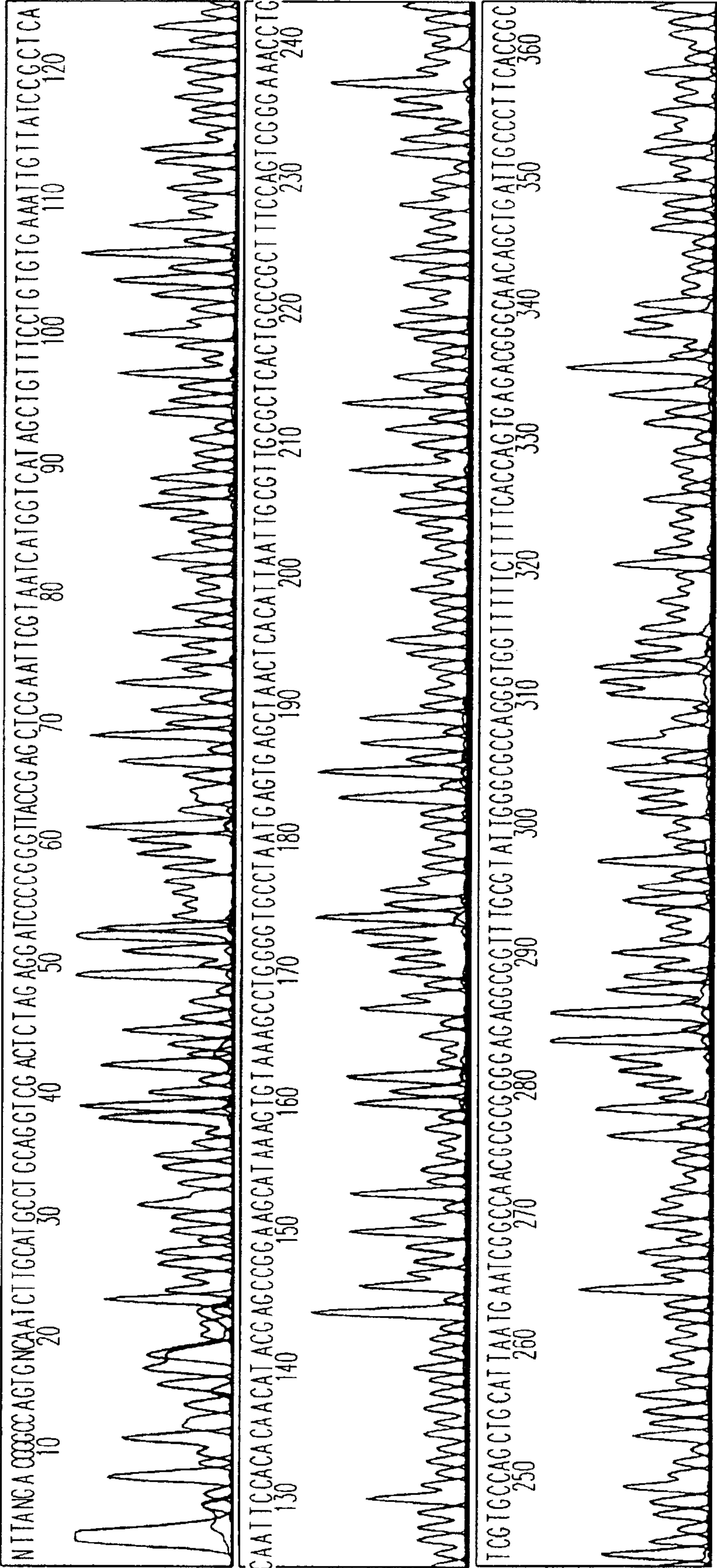


FIG.24A

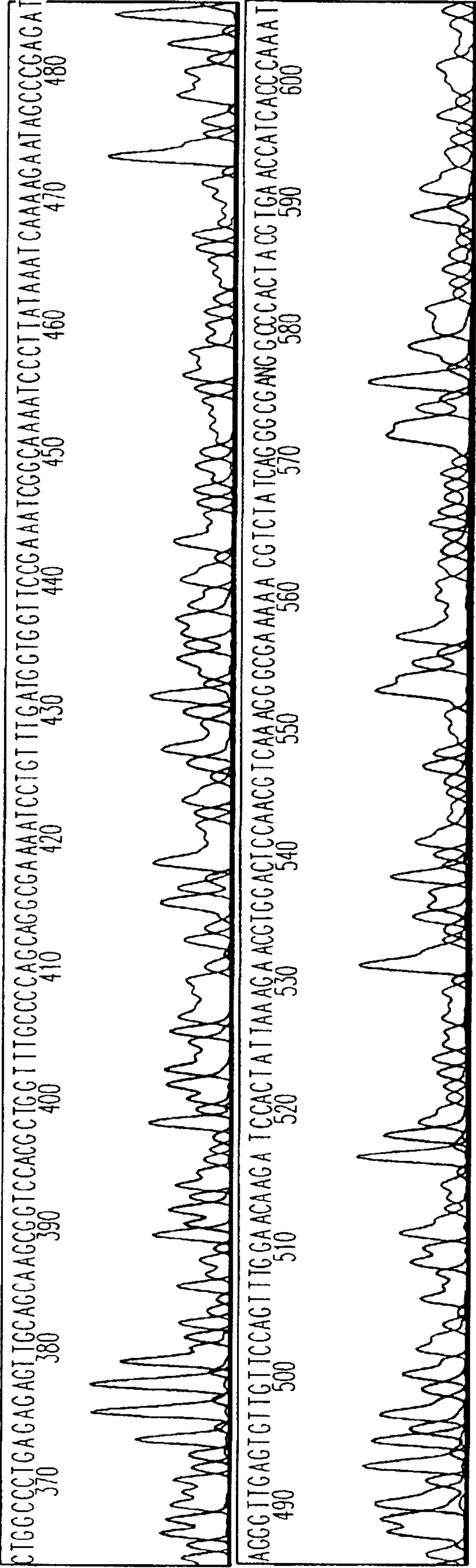


FIG.24B

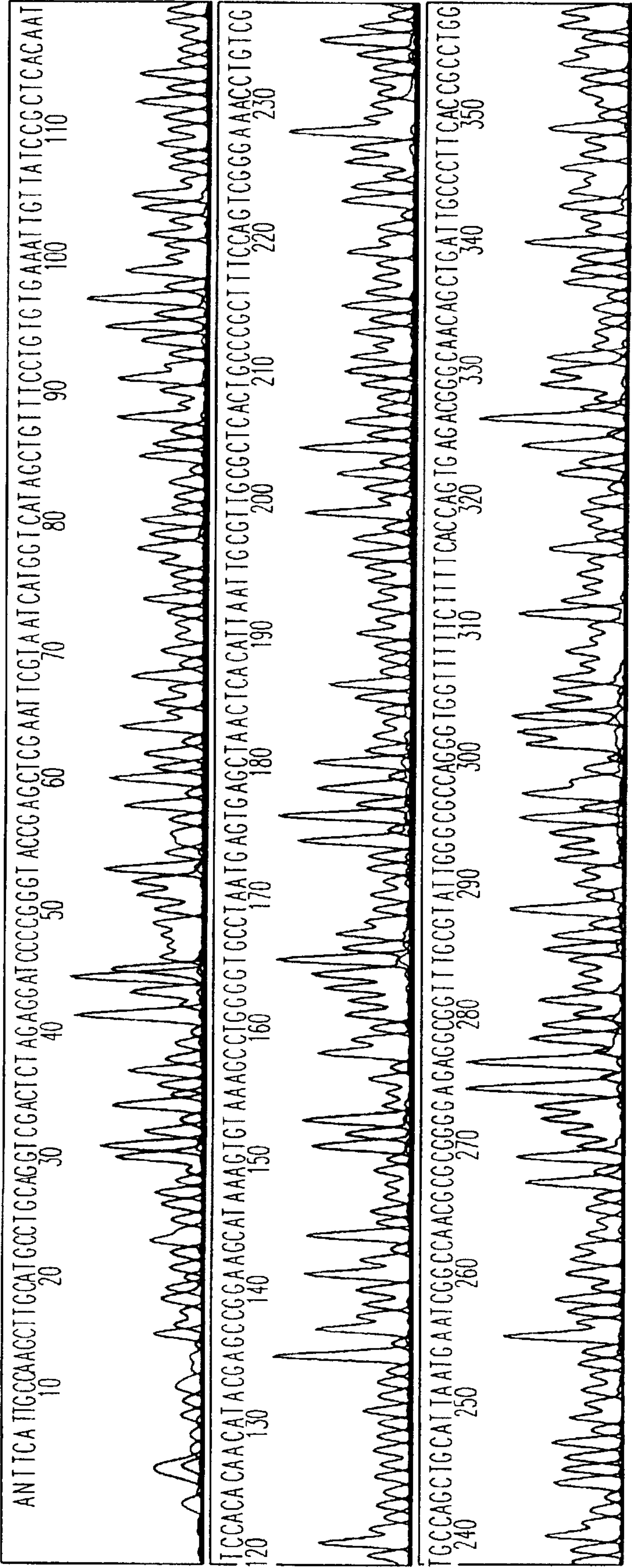


FIG.25A

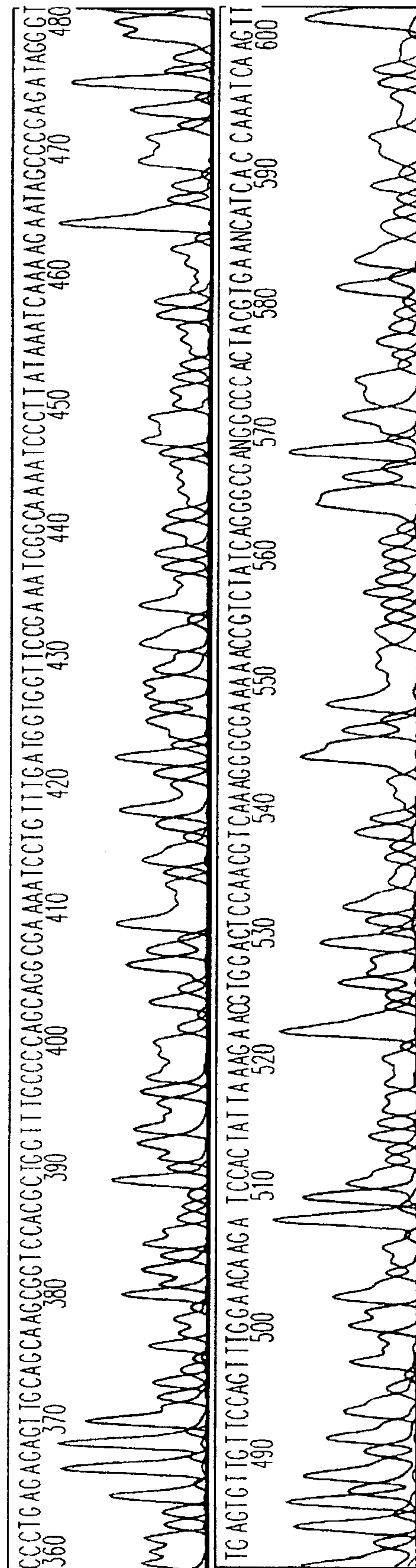


FIG. 25B

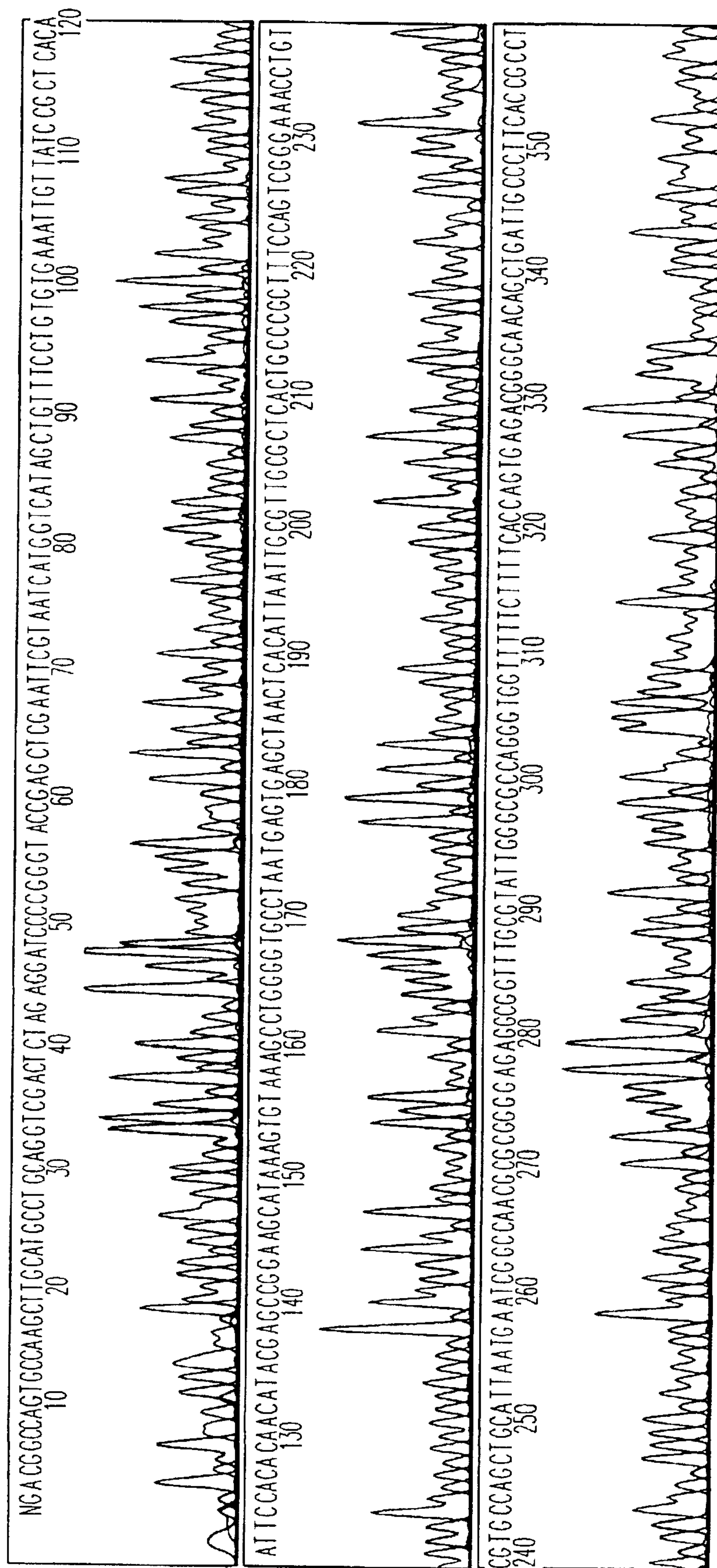


FIG. 26A

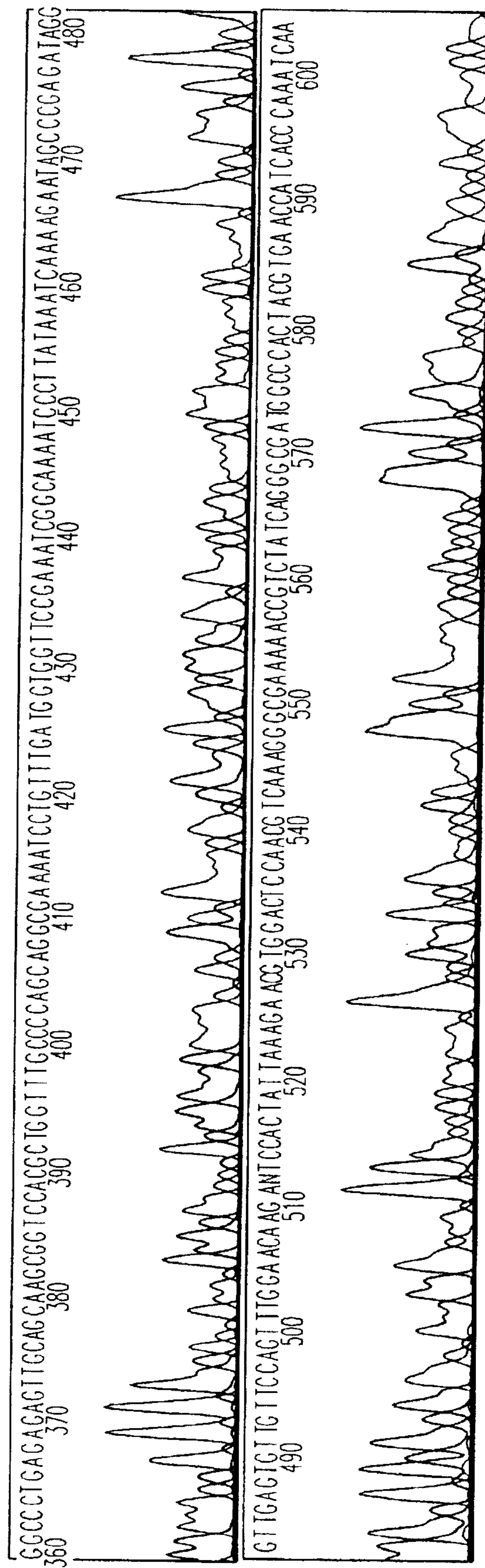


FIG. 26B

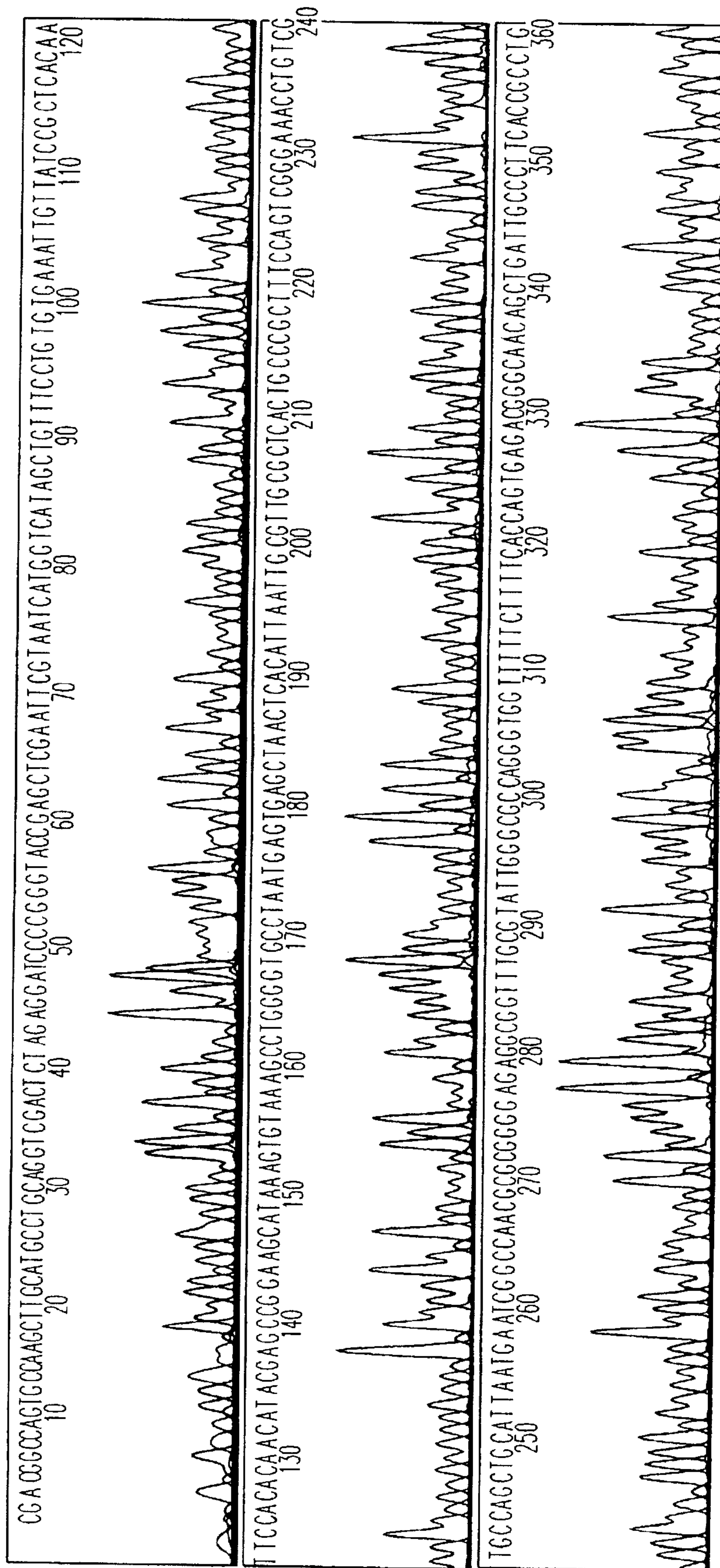


FIG.27A

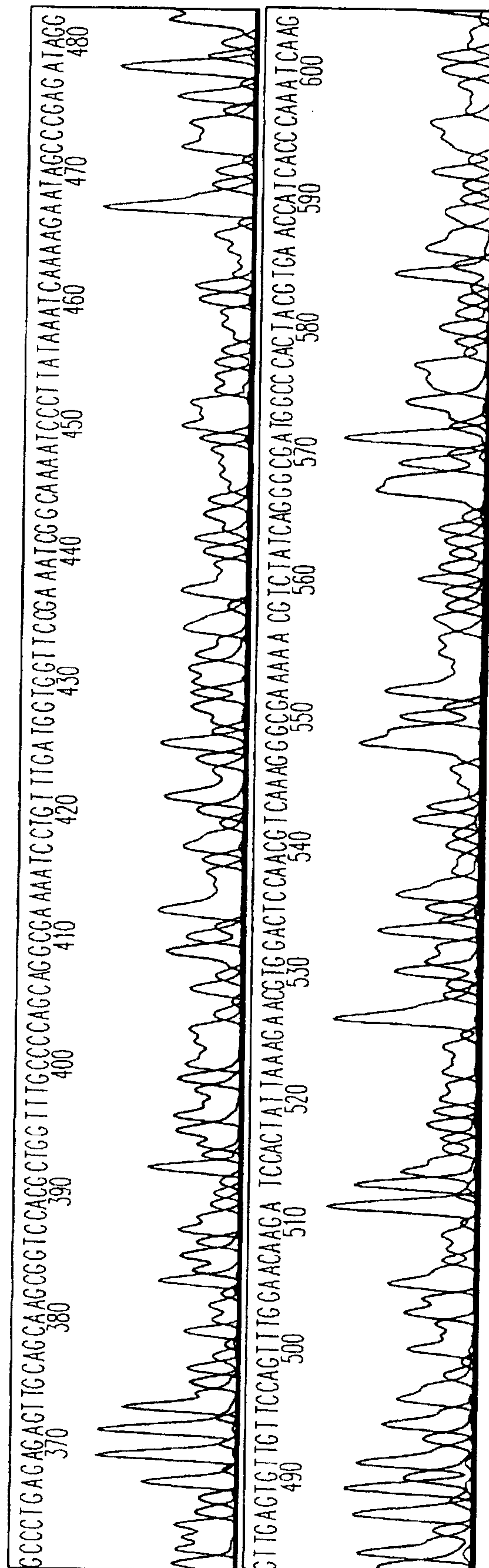


FIG. 27B