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Tsui et al.

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(54) **CYSTIC FIBROSIS GENE**

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(52) **U.S. Cl.** **530/350**
(58) **Field of Search** 530/350, 300, 530/326; 424/88

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(57) **ABSTRACT**

The cystic fibrosis gene and its gene product are described for both the normal and mutant forms. The genetic and protein information is used in developing DNA diagnosis, protein diagnosis, carrier and patient screening, drug and gene therapy, cloning of the gene and manufacture of the protein, and development of cystic fibrosis affected animals.

3 Claims, 36 Drawing Sheets

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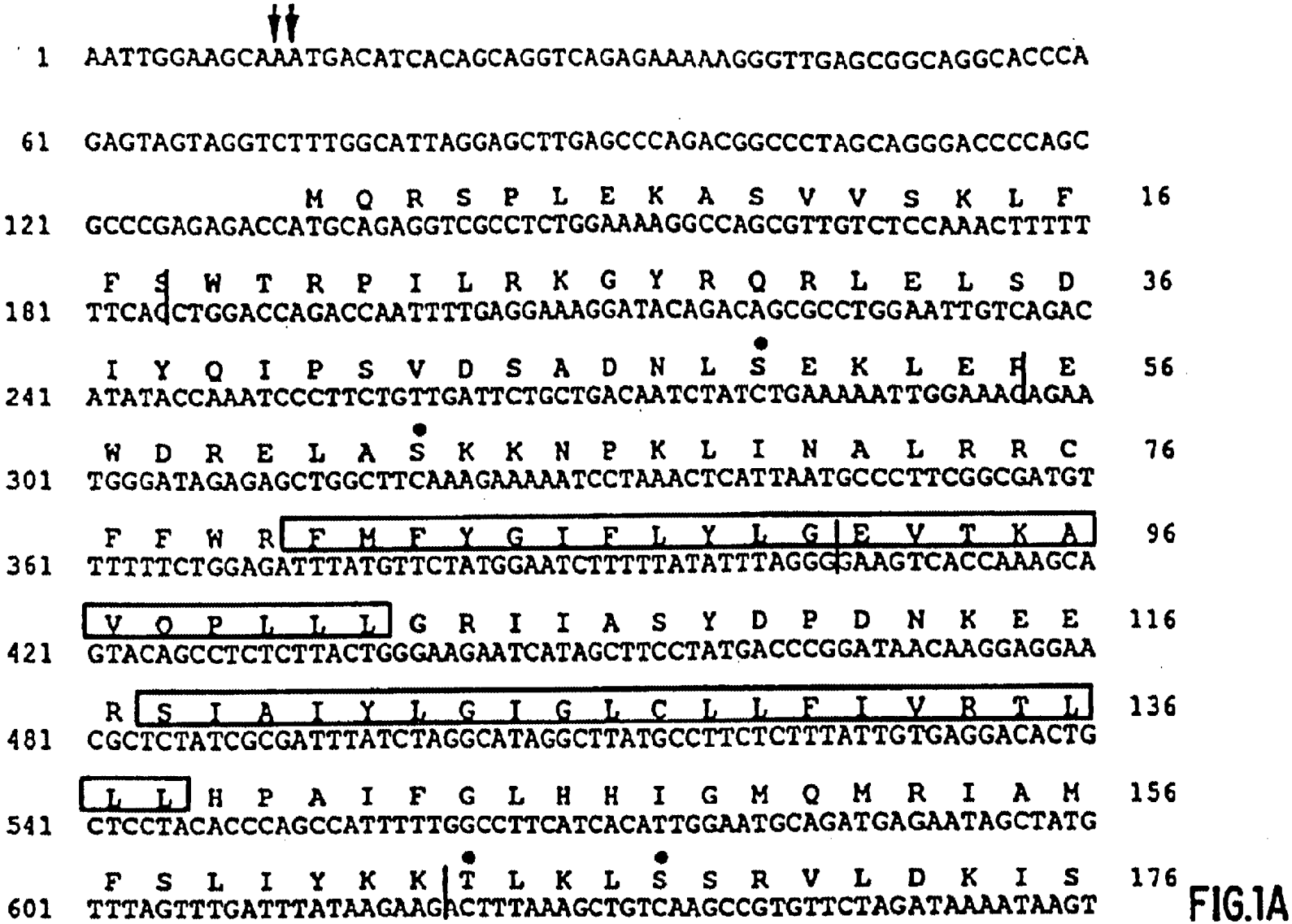
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FIG.1A

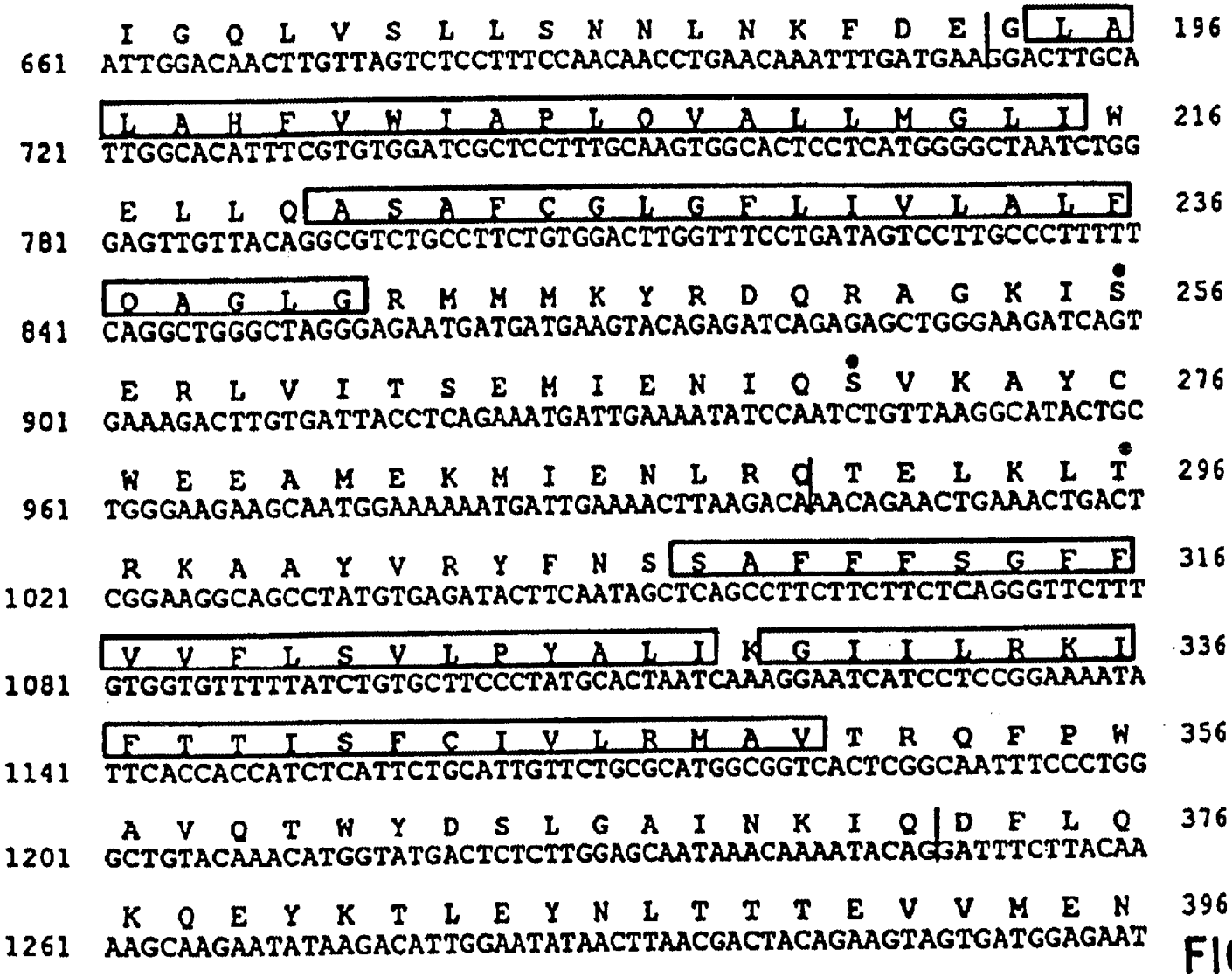
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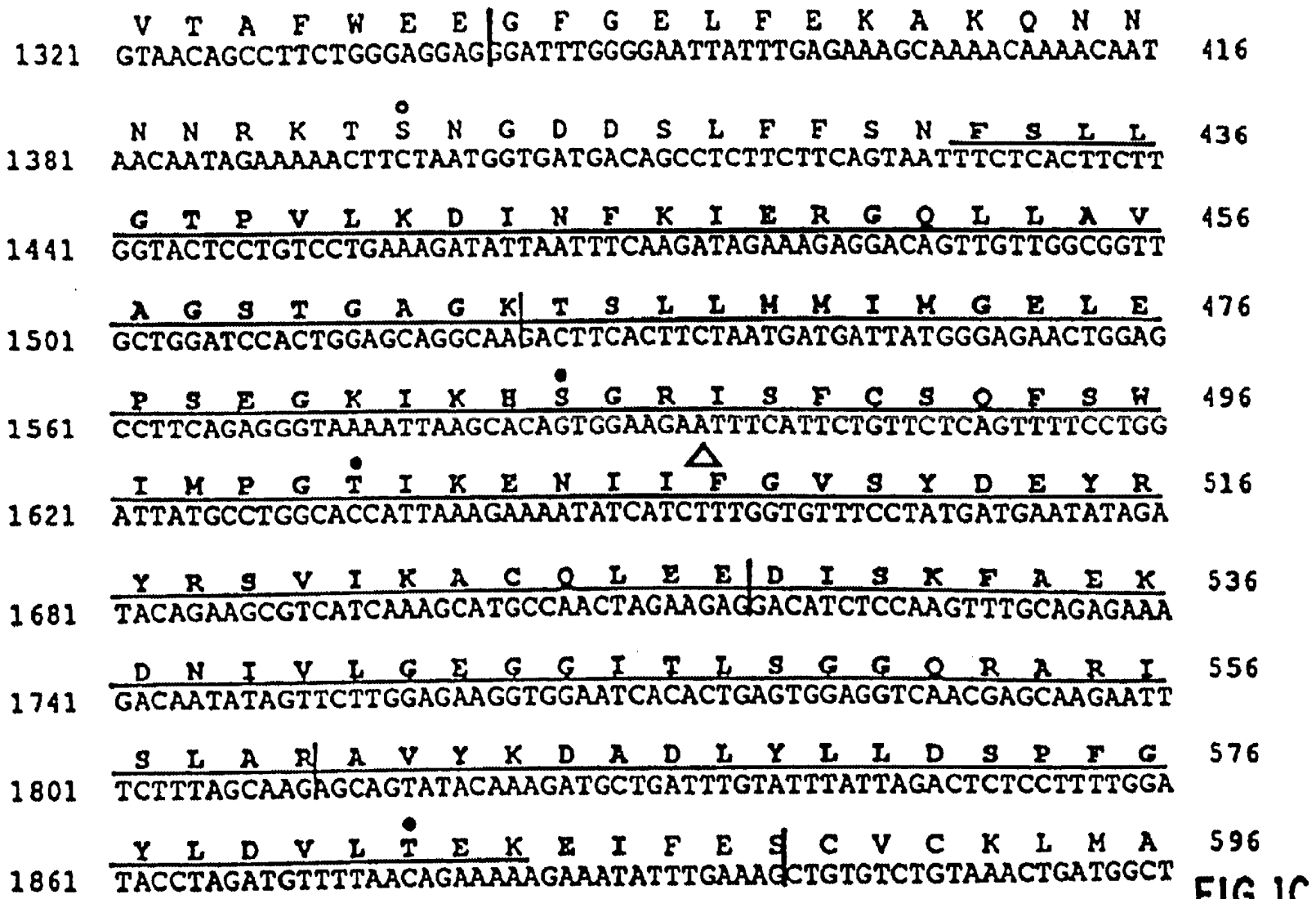
FIG. 1B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1621 ATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGA 516
Y R S V I K A C Q L E E D I S K F A E K
1681 TACAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGCAGAGAAA 536
D N I V L G E G G C I T L S G G Q R A R I
1741 GACAATATAGTTCTTGGAGAAGGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATT 556
S L A R A V Y K D A D L Y L L D S P F G
1801 TCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATTTATTAGACTCTCCTTTTGGA 576
Y L D V L T E K E I F E S C V C K L M A
1861 TACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAAACTGATGGCT 596

FIG. 1C

```
        N  K  T  R  I  L  V  T  S  K  M  E  H  L  K  K  A  D  K  I   616
1921    AACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAATA

        L  I  L  H  E  G  S  S  Y  F  Y  G  T  F  S  E  L  Q  N  L   636
1981    TTAATTTTGCATGAAGGTAGCAGCTATTTTTATGGGACATTTTCAGAACTCCAAAATCTA

        Q  P  D  F  S  S  K  L  M  G  C  D  S  F  D  Q  F  S  A  E   656
2041    CAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATTCTTTCGACCAATTTAGTGCAGAA

        R  R  N  S  I  L  T  E  T  L  H  R  F  S  L  E  G  D  A  P   676
2101    AGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCATTAGAAGGAGATGCTCCT

        V  S  W  T  E  T  K  K  Q  S  F  K  Q  T  G  E  F  G  E  K   696
2161    GTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGGAAAAA

        R  K  N  S  I  L  N  P  I  N  S  I  R  K  F  S  I  V  Q  K   716
2221    AGGAAGAATTCTATTCTCAATCCAATCAACTCTATACGAAAATTTTCCATTGTGCAAAAG

        T  P  L  Q  M  N  G  I  E  E  D  S  D  E  P  L  E  R  R  L   736
2281    ACTCCCTTACAAATGAATGGCATCGAAGAGGATTCTGATGAGCCTTTAGAGAGAAGGCTG

        S  L  V  P  D  S  E  Q  G  E  A  I  L  P  R  I  S  V  I  S   756
2341    TCCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACTGCCTCGCATCAGCGTGATCAGC

        T  G  P  T  L  Q  A  R  R  R  Q  S  V  L  N  L  M  T  H  S   776
2401    ACTGGCCCCACGCTTCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCA

        V  N  Q  G  Q  N  I  H  R  K  T  T  A  S  T  R  K  V  S  L   796
2461    GTTAACCAAGGTCAGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTG

        A  P  Q  A  N  L  T  E  L  D  I  Y  S  R  R  L  S  Q  E  T   816
2521    GCCCCTCAGGCAAACTTGACTGAACTGGATATATATTCAAGAAGGTTATCTCAAGAAACT
```

FIG.1D

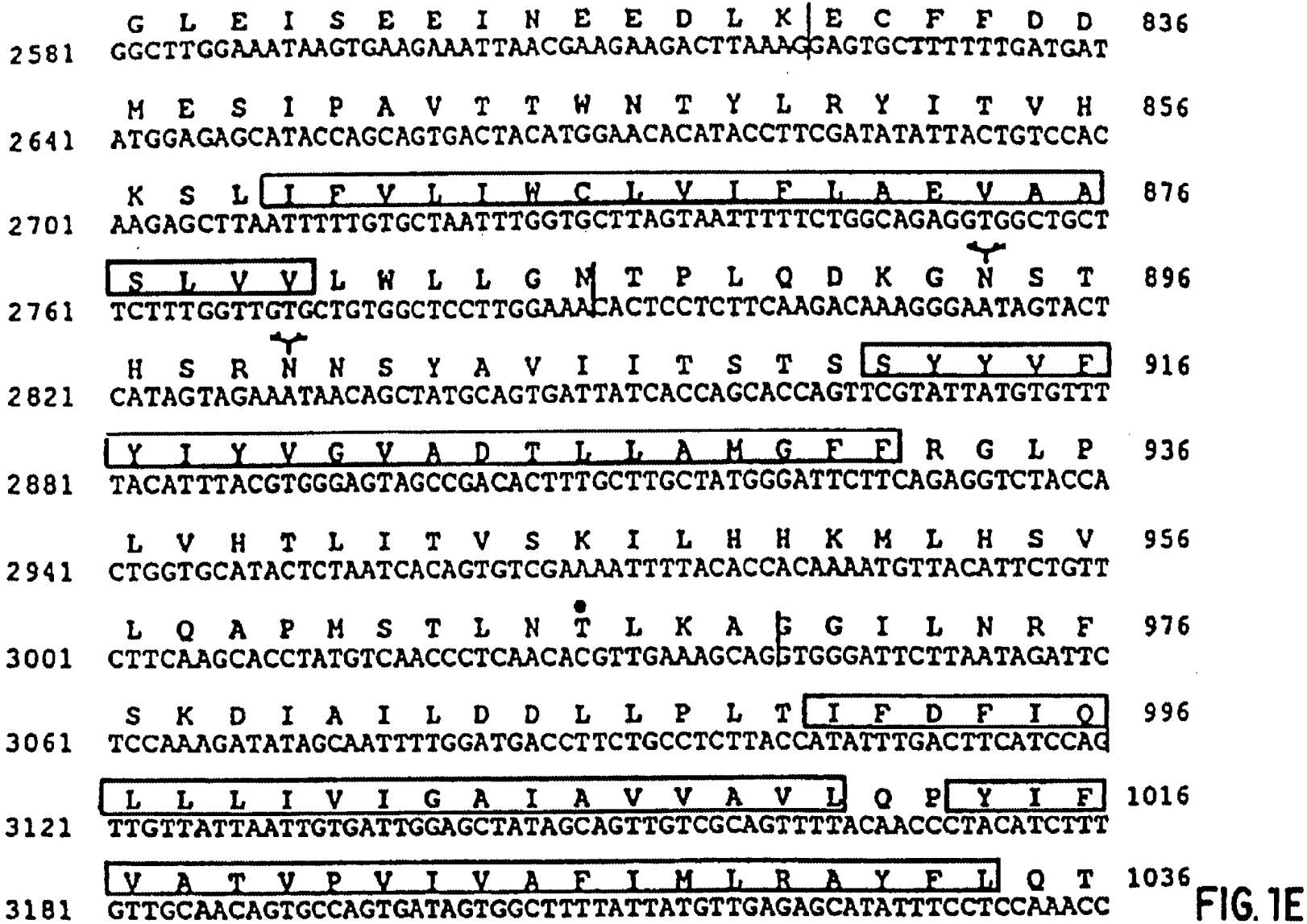

G L E I S E E I N E E D L K E C F F D D 836
2581 GGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTTTTTTGATGAT
M E S I P A V T T W N T Y L R Y I T V H 856
2641 ATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCAC
K S L I F V L I W C L V I F L A E V A A 876
2701 AAGAGCTTAATTTTTGTGCTAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCT
S L V V L W L L G M T P L Q D K G N S T 896
2761 TCTTTGGTTGTGCTGTGGCTCCTTGGAAACACTCCTCTTCAAGACAAAGGGAATAGTACT
H S R N N S Y A V I I T S T S S Y Y V F 916
2821 CATAGTAGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTCGTATTATGTGTTT
Y I Y V G V A D T L L A M G F F R G L P 936
2881 TACATTTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCA
L V H T L I T V S K I L H H K M L H S V 956
2941 CTGGTGCATACTCTAATCACAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTT
L Q A P M S T L N T L K A G G I L N R F 976
3001 CTTCAAGCACCTATGTCAACCCTCAACACGTTGAAAGCAGGTGGGATTCTTAATAGATTC
S K D I A I L D D L L P L T I F D F I Q 996
3061 TCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCATATTTGACTTCATCCAG
L L L I V I G A I A V V A V L Q P Y I F 1016
3121 TTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTT
V A T V P V I V A F I M L R A Y F L Q T 1036
3181 GTTGCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCCAAACC

FIG. 1E

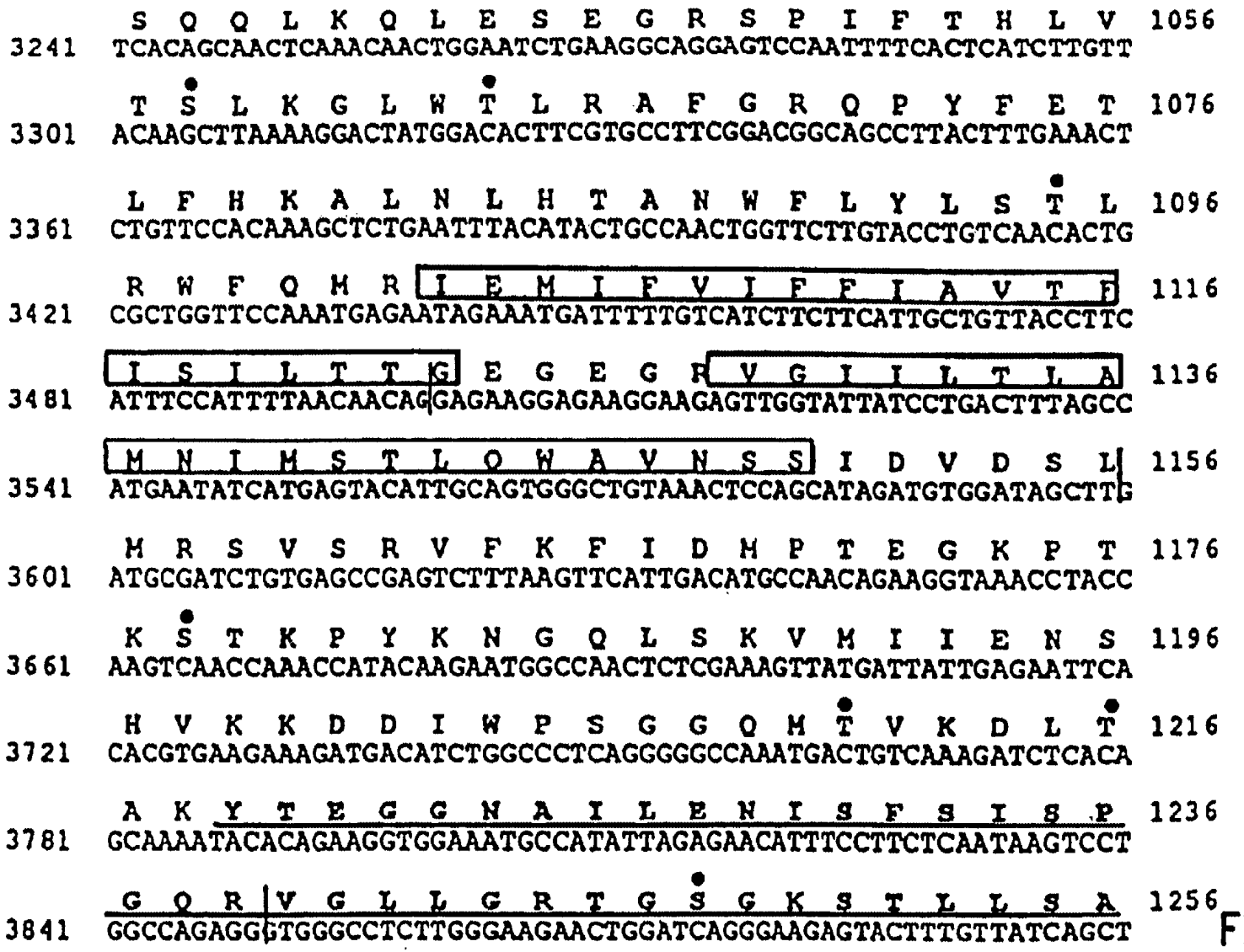

S Q Q L K Q L E S E G R S P I F T H L V 1056
3241 TCACAGCAACTCAAACAACTGGAATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTT
T S L K G L W T L R A F G R Q P Y F E T 1076
3301 ACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACGGCAGCCTTACTTTGAAACT
L F H K A L N L H T A N W F L Y L S T L 1096
3361 CTGTTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTACCTGTCAACACTG
R W F Q M R I E M I F V I F F I A V T F 1116
3421 CGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCATCTTCTTCATTGCTGTTACCTTC
I S I L T T G E G E G R V G I I L T L A 1136
3481 ATTTCCATTTTAACAACAGGAGAAGGAGAAGGAAGAGTTGGTATTATCCTGACTTTAGCC
M N I M S T L Q W A V N S S I D V D S L 1156
3541 ATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTCCAGCATAGATGTGGATAGCTTG
M R S V S R V F K F I D M P T E G K P T 1176
3601 ATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCTACC
K S T K P Y K N G Q L S K V M I I E N S 1196
3661 AAGTCAACCAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCA
H V K K D D I W P S G G Q M T V K D L T 1216
3721 CACGTGAAGAAAGATGACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACA
A K Y T E G G N A I L E N I S F S I S P 1236
3781 GCAAAATACACAGAAGGTGGAAATGCCATATTAGAGAACATTTCCTTCTCAATAAGTCCT
G Q R V G L L G R T G S G K S T L L S A 1256
3841 GGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTATCAGCT

FIG. 1F

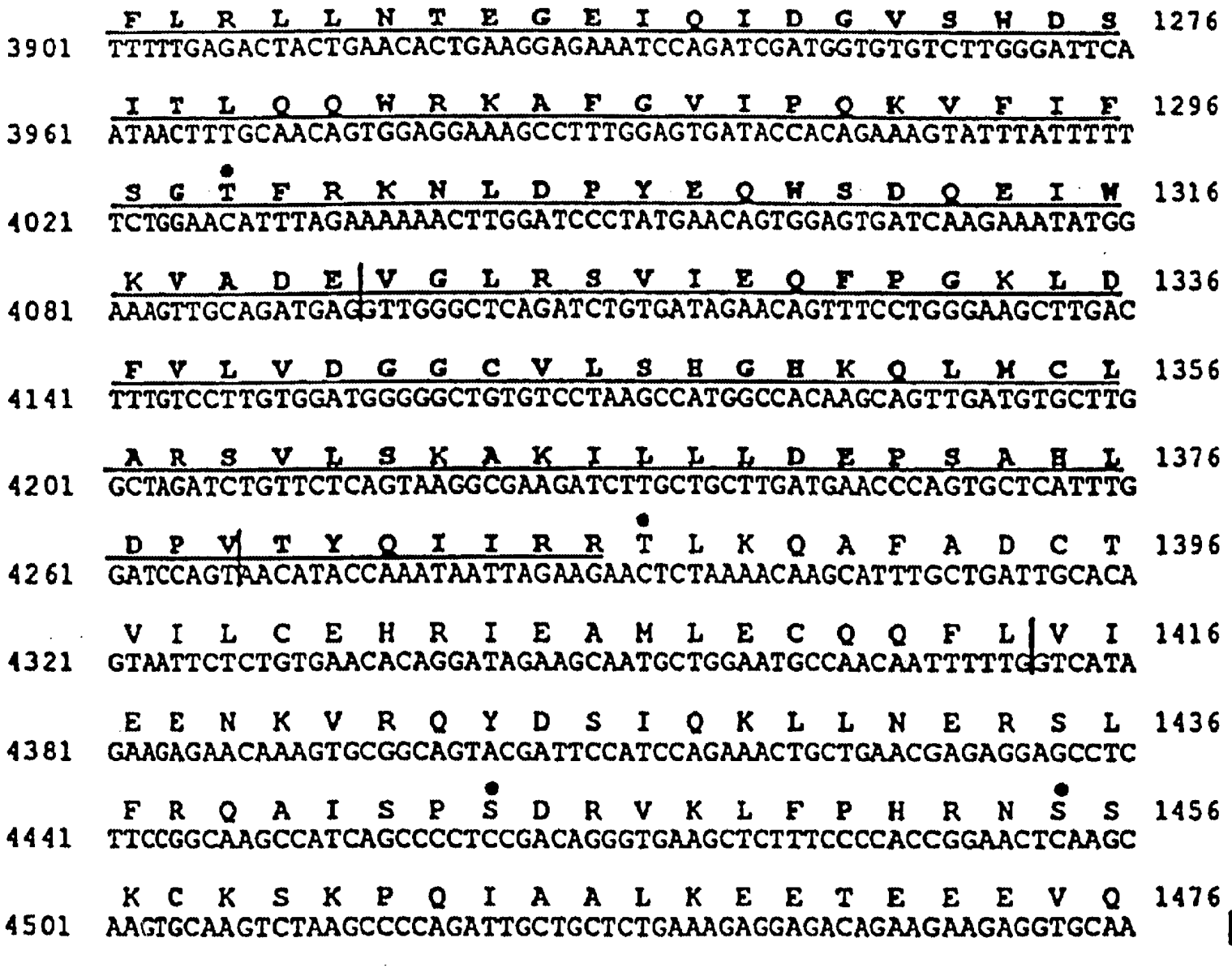

F L R L L N T E G E I Q I D G V S H D S 1276
3901 TTTTGAGACTACTGAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCA
I T L Q Q W R K A F G V I P Q K V F I F 1296
3961 ATAACTTTGCAACAGTGGAGGAAAGCCTTTGGAGTGATACCACAGAAAGTATTTATTTTT
S G T F R K N L D P Y E Q W S D Q E I W 1316
4021 TCTGGAACATTTAGAAAAAACTTGGATCCCTATGAACAGTGGAGTGATCAAGAAATATGG
K V A D E V G L R S V I E Q F P G K L D 1336
4081 AAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTTTCCTGGGAAGCTTGAC
F V L V D G G C V L S H G H K Q L M C L 1356
4141 TTTGTCCTTGTGGATGGGGGCTGTGTCCTAAGCCATGGCCACAAGCAGTTGATGTGCTTG
A R S V L S K A K I L L L D E P S A H L 1376
4201 GCTAGATCTGTTCTCAGTAAGGCGAAGATCTTGCTGCTTGATGAACCCAGTGCTCATTTG
D P V T Y Q I I R R T L K Q A F A D C T 1396
4261 GATCCAGTAACATACCAAATAATTAGAAGAACTCTAAAACAAGCATTTGCTGATTGCACA
V I L C E H R I E A M L E C Q Q F L V I 1416
4321 GTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAATTTTTGGTCATA
E E N K V R Q Y D S I Q K L L N E R S L 1436
4381 GAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAACTGCTGAACGAGAGGAGCCTC
F R Q A I S P S D R V K L F P H R N S S 1456
4441 TTCCGGCAAGCCATCAGCCCCTCCGACAGGGTGAAGCTCTTTCCCCACCGGAACTCAAGC
K C K S K P Q I A A L K E E T E E E V Q 1476
4501 AAGTGCAAGTCTAAGCCCCAGATTGCTGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAA

FIG. 1G

D T R L =

1480

```
4561  GATACAAGGCTTTAGAGAGCAGCATAAATGTTGACATGGGACATTTGCTCATGGAATTGG
4621  AGCTCGTGGGACAGTCACCTCATGGAATTGGAGCTCGTGGAACAGTTACCTCTGCCTCAG
4681  AAAACAAGGATGAATTAAGTTTTTTTTTAAAAAAGAAACATTTGGTAAGGGGAATTGAGG
4741  ACACTGATATGGGTCTTGATAAATGGCTTCCTGGCAATAGTCAAATTGTGTGAAAGGTAC
4801  TTCAAATCCTTGAAGATTTACCACTTGTGTTTTGCAAGCCAGATTTTCCTGAAAACCCTT
4861  GCCATGTGCTAGTAATTGGAAAGGCAGCTCTAAATGTCAATCAGCCTAGTTGATCAGCTT
4921  ATTGTCTAGTGAAACTCGTTAATTTGTAGTGTTGGAGAAGAACTGAAATCATACTTCTTA
4981  GGGTTATGATTAAGTAATGATAACTGGAAACTTCAGCGGTTTATATAAGCTTGTATTCCT
5041  TTTTCTCTCCTCTCCCCATGATGTTTAGAAACACAACTATATTGTTTGCTAAGCATTCCA
5101  ACTATCTCATTTCCAAGCAAGTATTAGAATACCACAGGAACCACAAGACTGCACATCAAA
5161  ATATGCCCCATTCAACATCTAGTGAGCAGTCAGGAAAGAGAACTTCCAGATCCTGGAAAT
5221  CAGGGTTAGTATTGTCCAGGTCTACCAAAAATCTCAATATTTCAGATAATCACAATACAT
5281  CCCTTACCTGGGAAAGGGCTGTTATAATCTTTCACAGGGGACAGGATGGTTCCCTTGATG
5341  AAGAAGTTGATATGCCTTTTCCCAACTCCAGAAAGTGACAAGCTCACAGACCTTTGAACT
5401  AGAGTTTAGCTGGAAAAGTATGTTAGTGCAAATTGTCACAGGACAGCCCTTCTTTCCACA
5461  GAAGCTCCAGGTAGAGGGTGTGTAAGTAGATAGGCCATGGGCACTGTGGGTAGACACACA
5521  TGAAGTCCAAGCATTTAGATGTATAGGTTGATGGTGGTATGTTTTCAGGCTAGATGTATG
5581  TACTTCATGCTGTCTACACTAAGAGAGAATGAGAGACACACTGAAGAAGCACCAATCATG
5641  AATTAGTTTTATATGCTTCTGTTTTATAATTTTGTGAAGCAAAATTTTTTCTCTAGGAAA
5701  TATTTATTTTAATAATGTTTCAAACATATATTACAATGCTGTATTTTAAAAGAATGATTA
5761  TGAATTACATTTGTATAAAATAATTTTTATATTTGAAATATTGACTTTTTATGGCACTAG
5821  TATTTTTATGAAATATTATGTTAAAACTGGGACAGGGGAGAACCTAGGGTGATATTAACC
5881  AGGGGCCATGAATCACCTTTTGGTCTGGAGGGAAGCCTTGGGGCTGATCGAGTTGTTGCC
5941  CACAGCTGTATGATTCCCAGCCAGACACAGCCTCTTAGATGCAGTTCTGAAGAAGATGGT
6001  ACCACCAGTCTGACTGTTTCCATCAAGGGTACACTGCCTTCTCAACTCCAAACTGACTCT
6061  TAAGAAGACTGCATTATATTTATTACTGTAAGAAAATATCACTTGTCAATAAAATCCATA
6121  CATTTGTGT(A)n
```

FIG. 1H

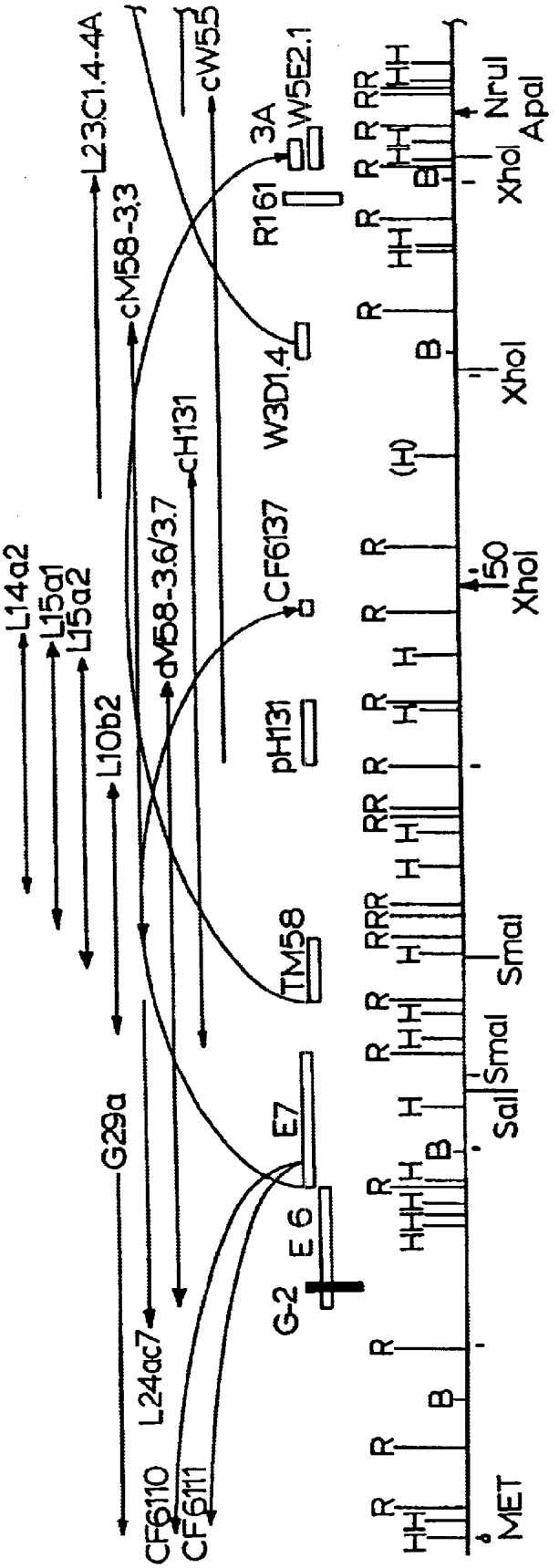

L23C1.4-4A
cW55
cM58-3.3
3A
W5E2.1
R161
W3D1.4
cH131
L14a2
L15a1
L15a2
L10b2
dM58-3.6/3.7
CF6137
pH131
TM58
G29a
E7
E6
G-2
L24ac7
CF6110
CF6111
NruI
ApaI
XhoI
XhoI
150
XhoI
SmaI
SmaI
SalI
MET

FIG.2A

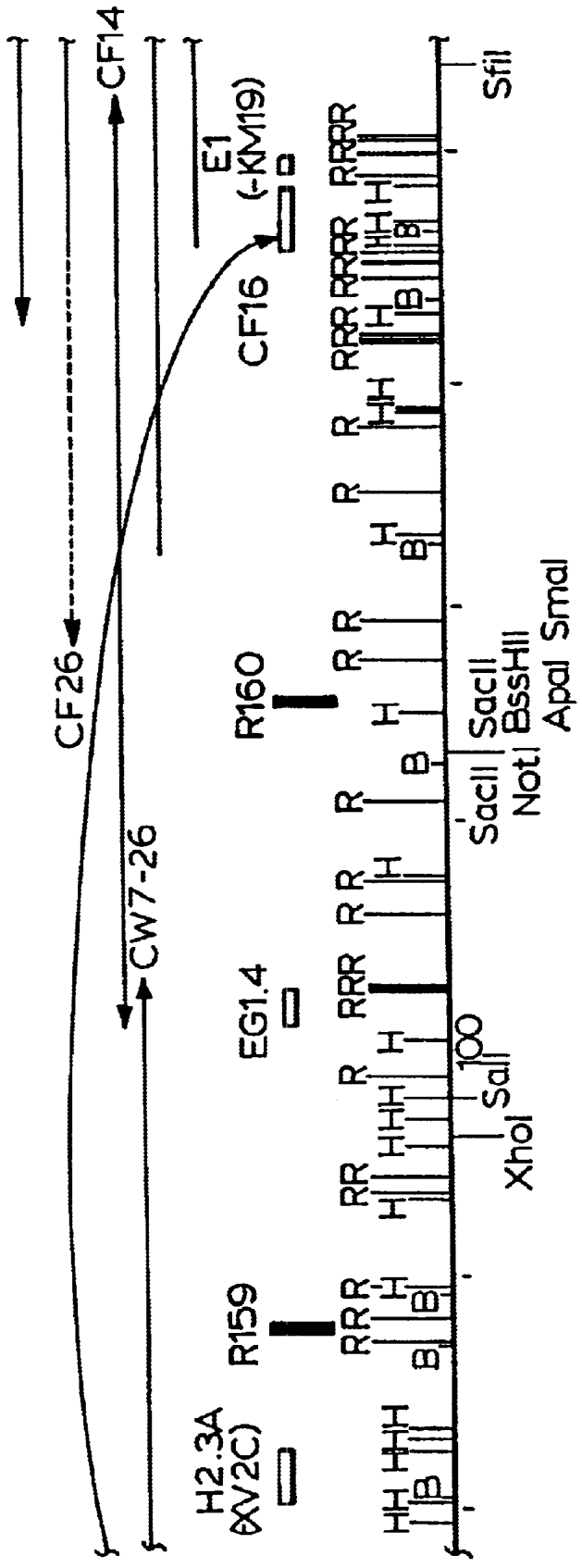

CF14
E1
(-KM19)
CF16
CF26
CW7-26
R160
EG1.4
R159
H2.3A
(V2C)
SfiI
SacII
NotI
BssHII
ApaI
SmaI
SacII
SalI
XhoI
100

FIG.2B

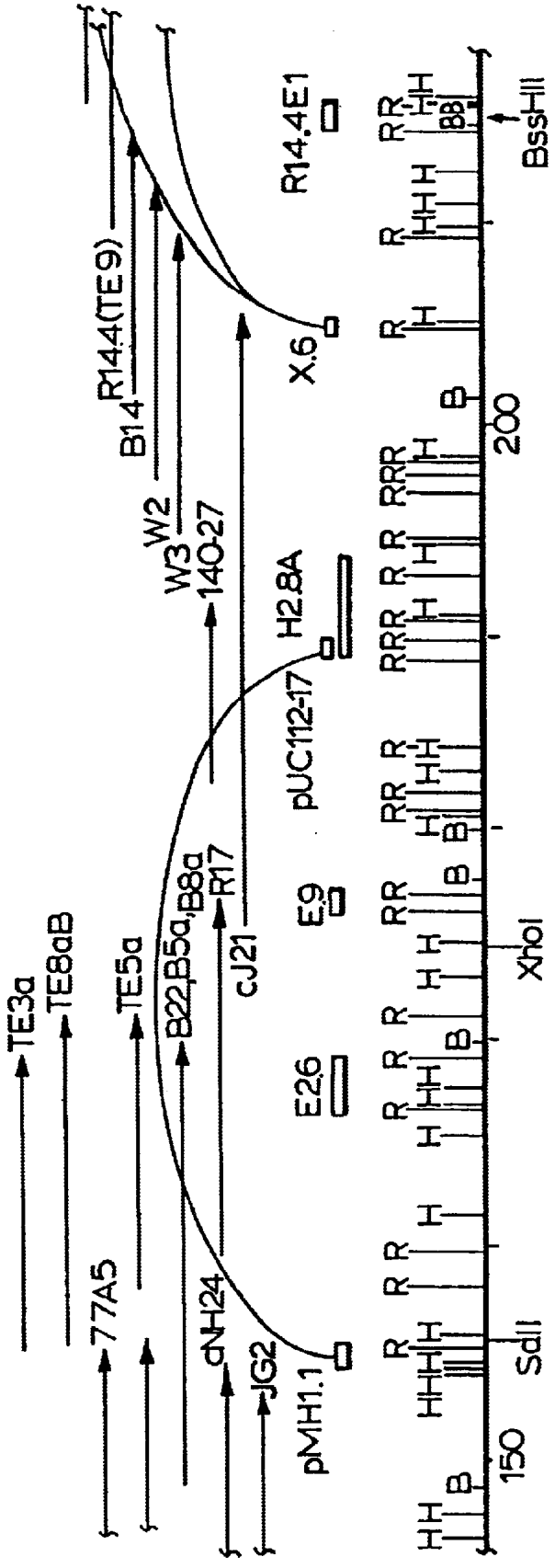

R14.4E1
R14.4(TE9)
B14
W2
W3
140-27
X.6
H2.8A
pUC112-17
E9
R17
cJ21
B22,B5a,B8a
TE5a
TE8aB
TE3a
E2.6
77A5
cNH24
JG2
pMH1.1
BssHII
XhoI
SdII
200
150

FIG. 2C

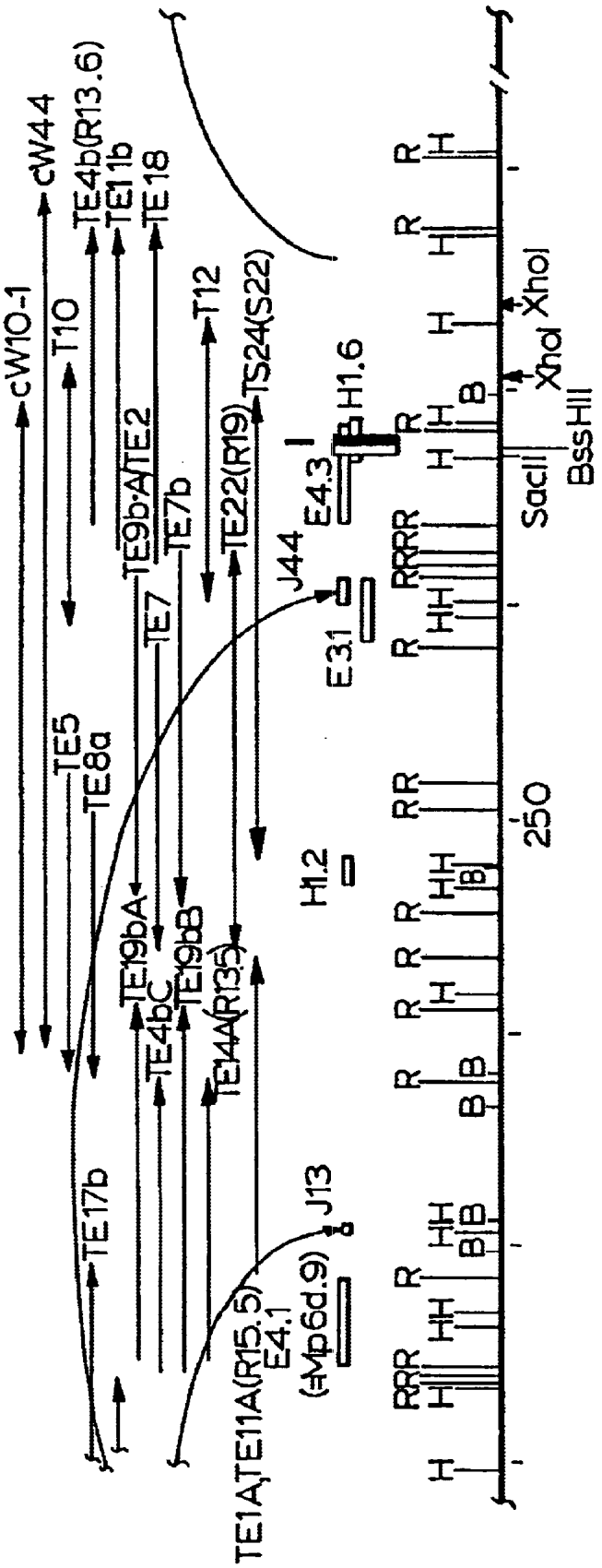

cW10-1
cW44
T10
TE4b(R13.6)
TE11b
TE18
T12
TS24(S22)
TE9b-A/TE2
TE7b
TE22(R19)
TE7
J44
E4.3
H1.6
E31
TE5
TE8a
TE19bA
TE4bC
TE19bB
TE14A(R13)
H1.2
TE17b
J13
TE1A,TE11A(R15.5)
E4.1
(=Mp6d.9)
XhoI
SacII
BssHII
250

FIG. 2D

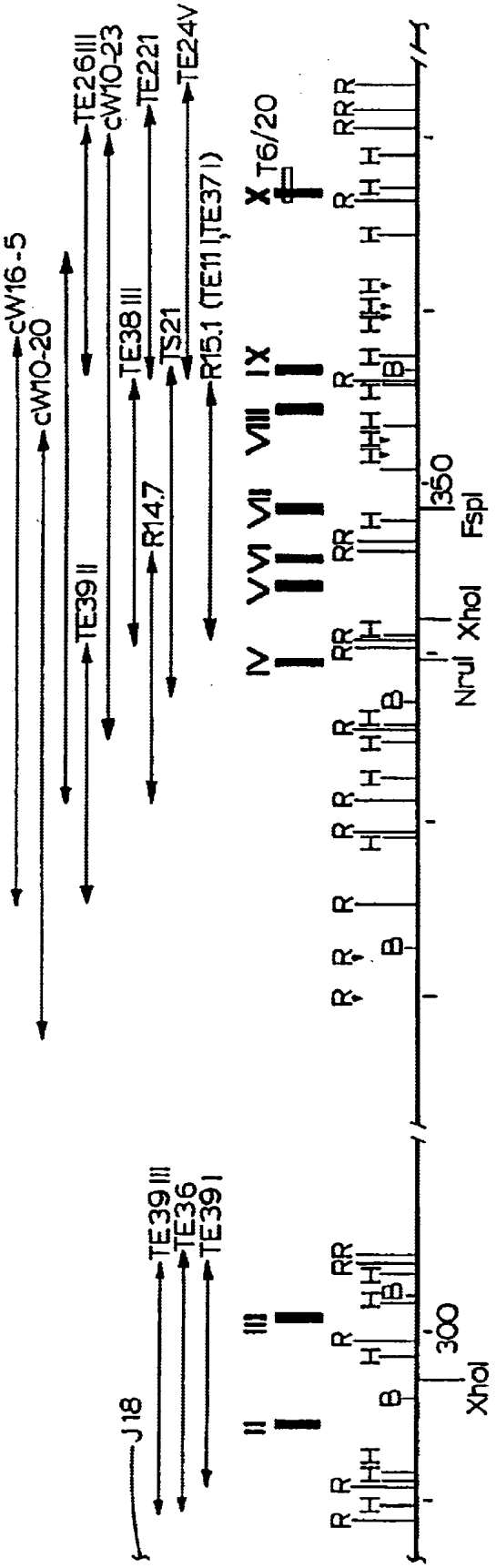

cW16-5
cW10-20
TE39II
TE26III
cW10-23
TE38III
R14.7
TE221
TS21
TE24V
R15.1 (TE11,TE37I)
X T6/20
IV V VI VII VIII IX
II III
J18
TE39III
TE36
TE39I
300
350
XhoI
NruI
FspI
R
H
B

FIG. 2E

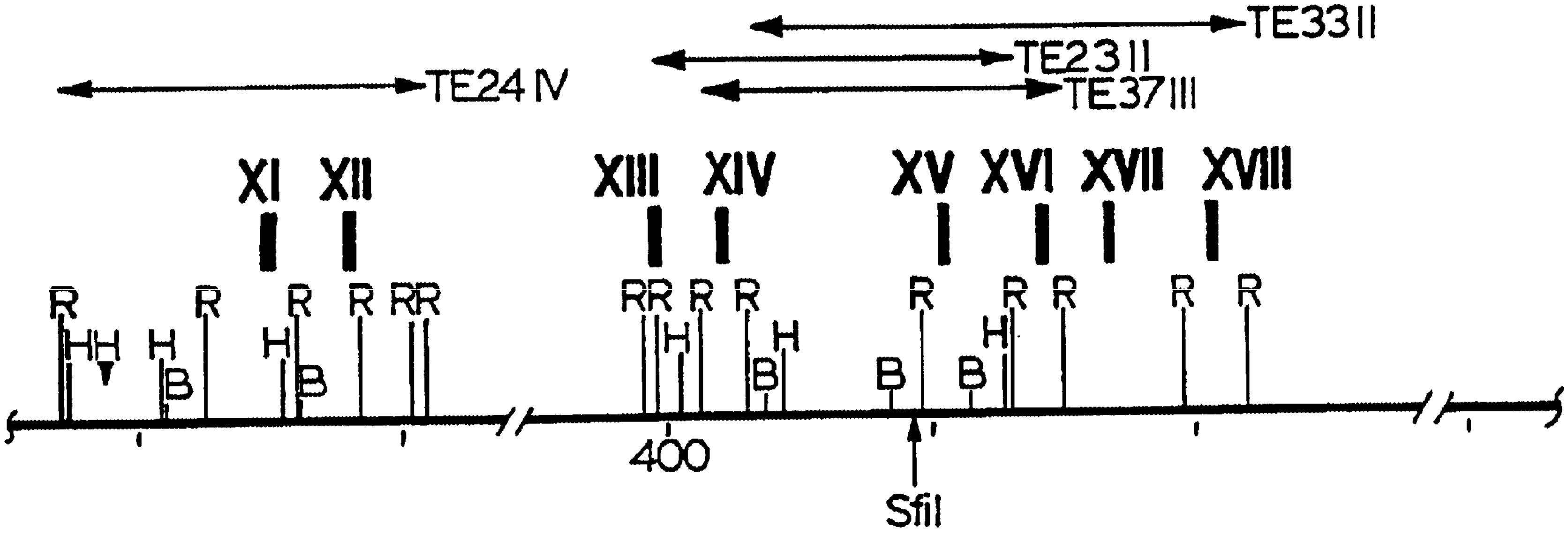

TE33 II
TE23 II
TE37 III
TE24 IV
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
R
H
B
400
SfiI

FIG. 2F

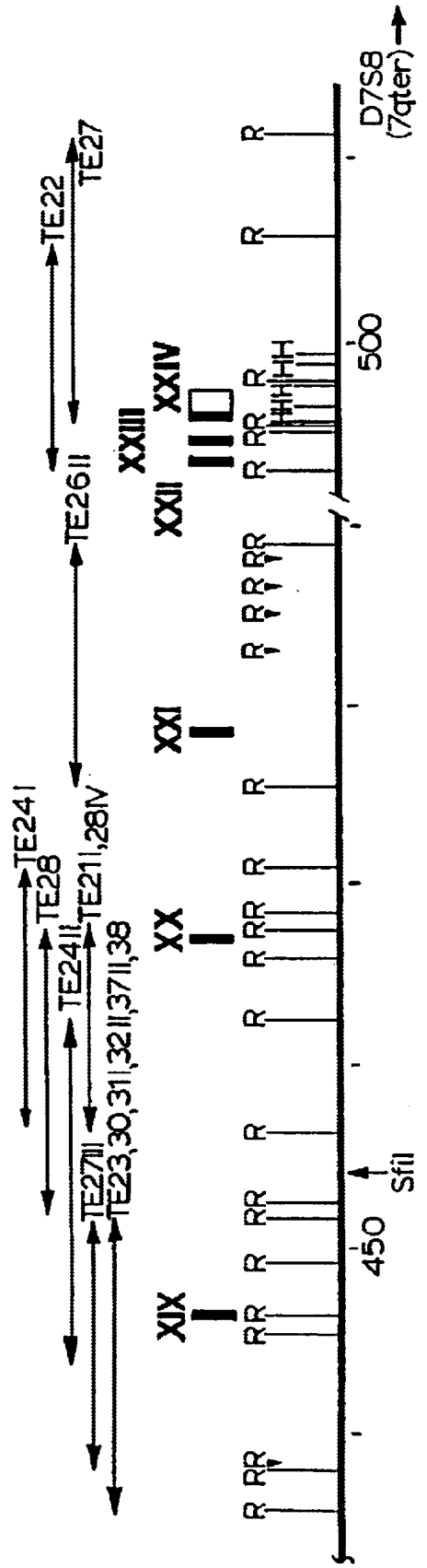
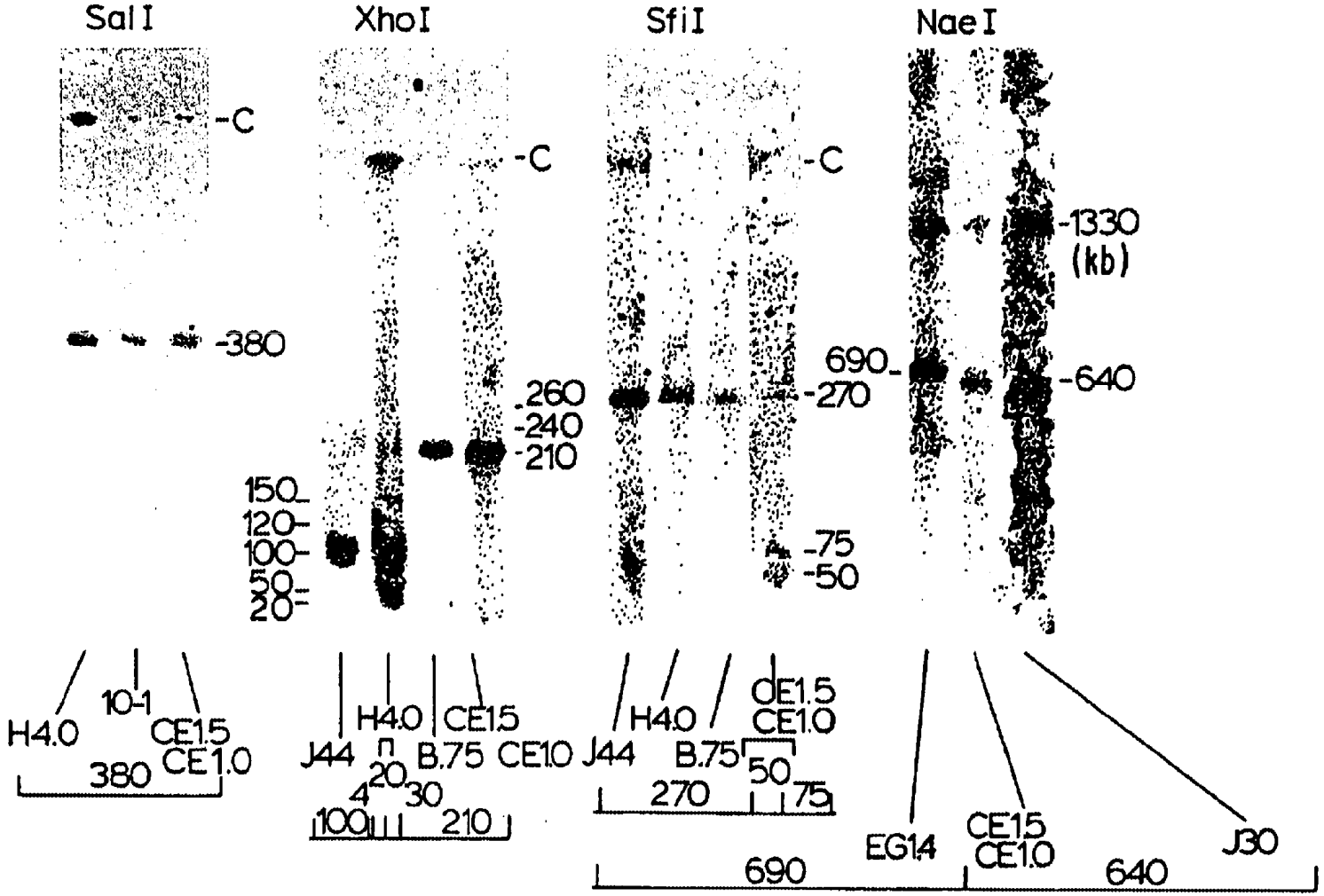

D7S8
(7qter)
TE27
TE22
TE26II
TE24I
TE28
TE24II
TE21II,28IV
TE27II
TE23,30,31I,32II,37II,38
XIX
XX
XXI
XXII
XXIII
XXIV
Sfil
500
450

FIG. 2G

FIG.3A FIG.3B FIG.3C FIG.3D



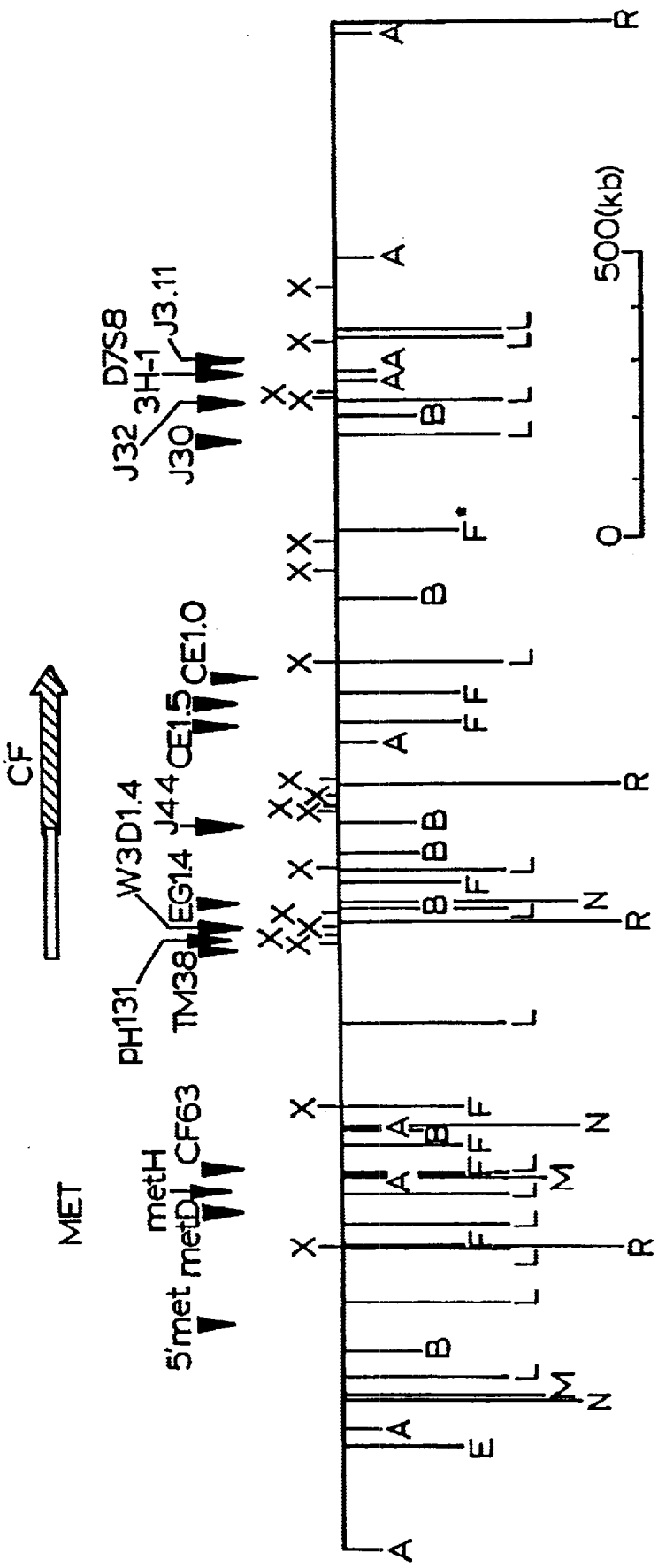

CF
MET
5'met
metH
metD1
CF63
PH131
TM38
EG14
W3D1.4
J44
CE1.5
CE1.0
J32
3H-1
D7S8
J3.11
J30
500(kb)
0

FIG. 3E

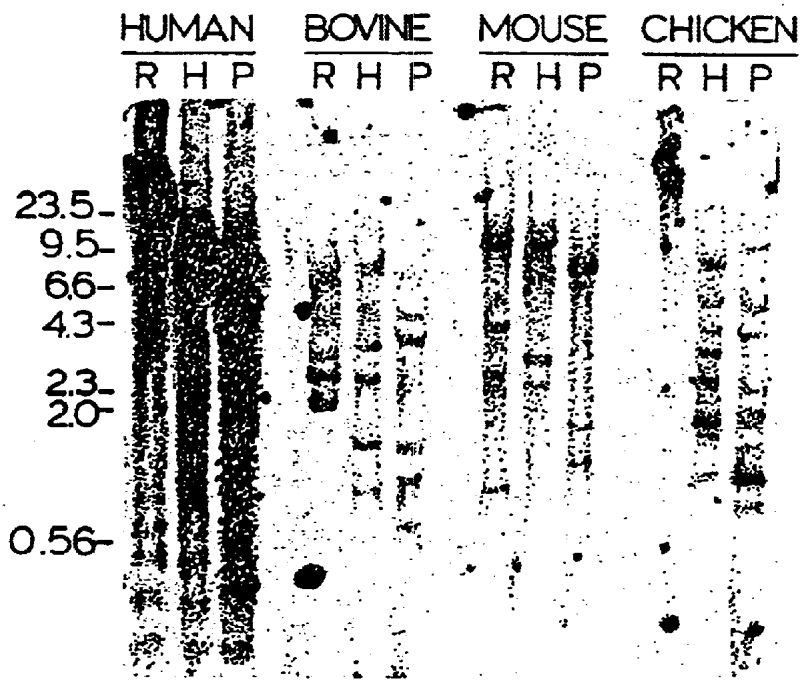

HUMAN
BOVINE
MOUSE
CHICKEN
R H P
R H P
R H P
R H P
23.5
9.5
6.6
4.3
2.3
2.0
0.56

FIG.4A

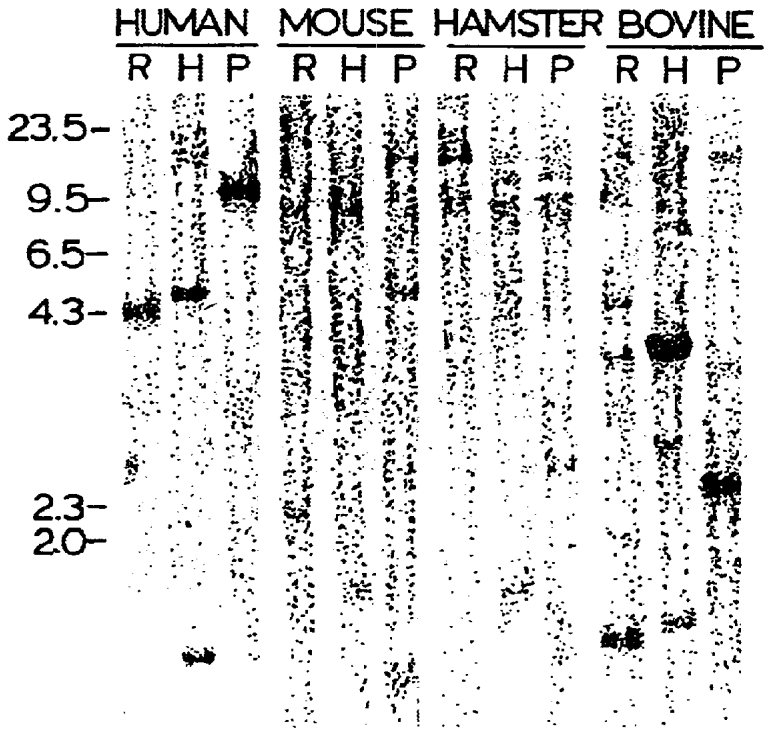

HUMAN
MOUSE
HAMSTER
BOVINE
R H P
R H P
R H P
R H P
23.5
9.5
6.5
4.3
2.3
2.0

FIG.4B

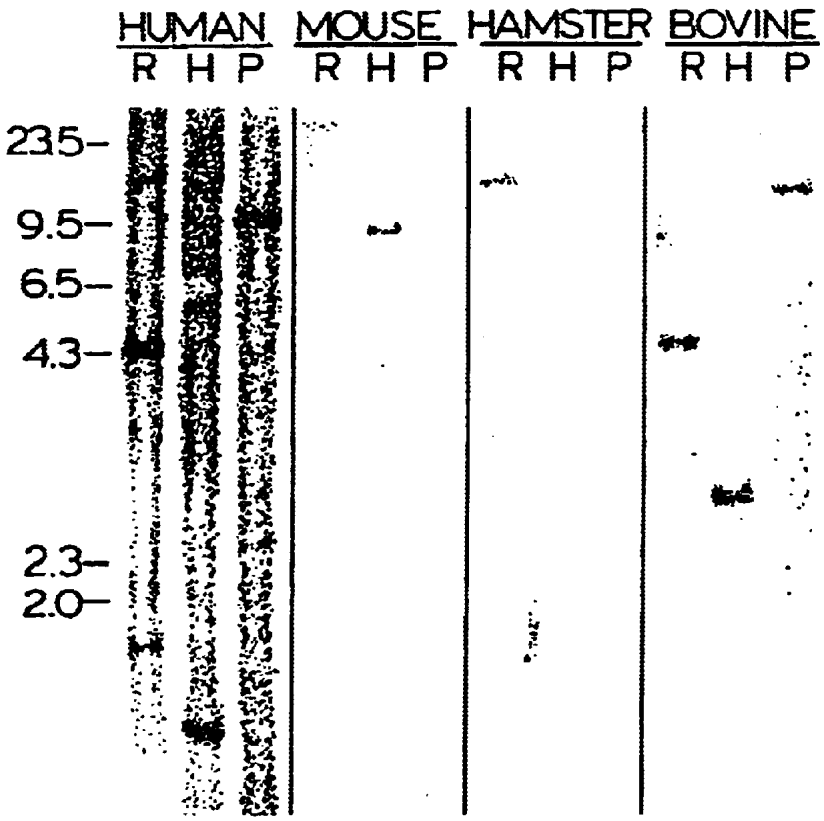

HUMAN
MOUSE
HAMSTER
BOVINE
R H P R H P R H P R H P
23.5–
9.5–
6.5–
4.3–
2.3–
2.0–

FIG.4C

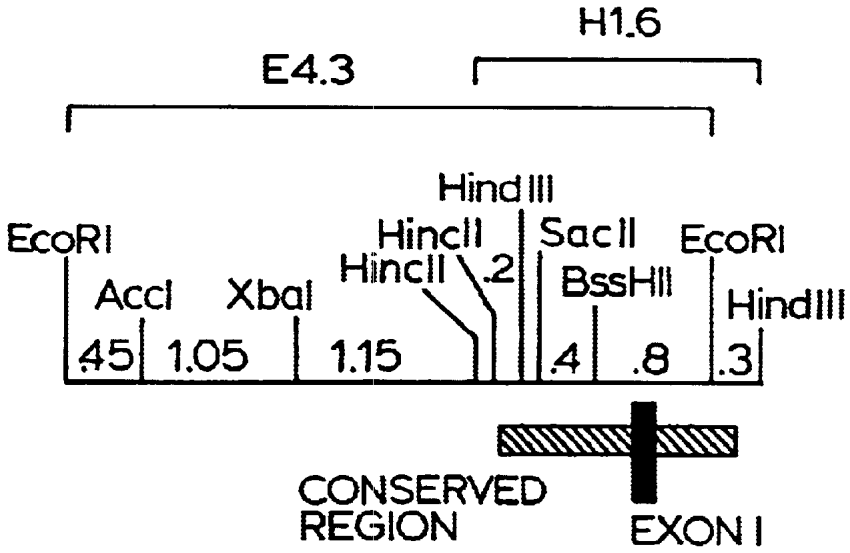

H1.6
E4.3
HindIII
EcoRI
HincII
HincII
.2
SacII
EcoRI
AccI
XbaI
BssHII
HindIII
.45
1.05
1.15
.4
.8
.3
CONSERVED
REGION
EXON I

FIG.4D

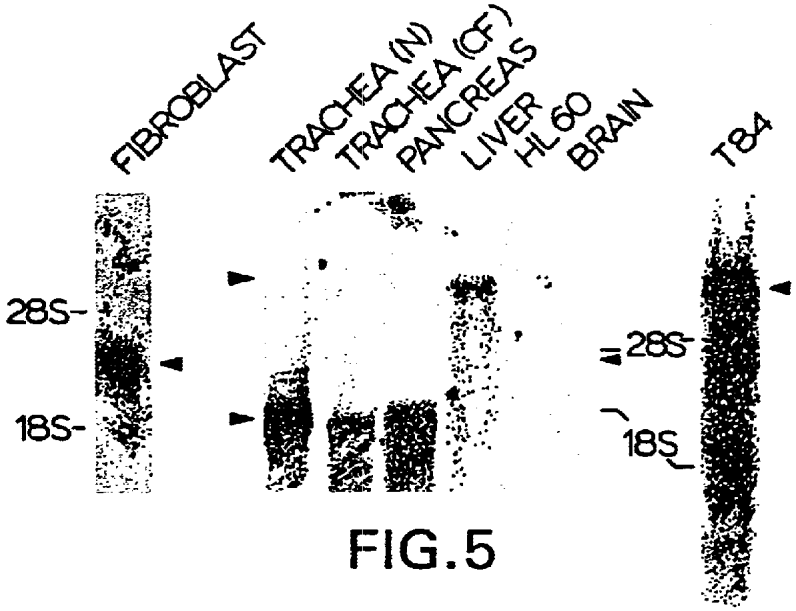

FIBROBLAST
TRACHEA (N)
TRACHEA (CF)
PANCREAS
LIVER
HL 60
BRAIN
T84
28S
18S
28S
18S

FIG. 5

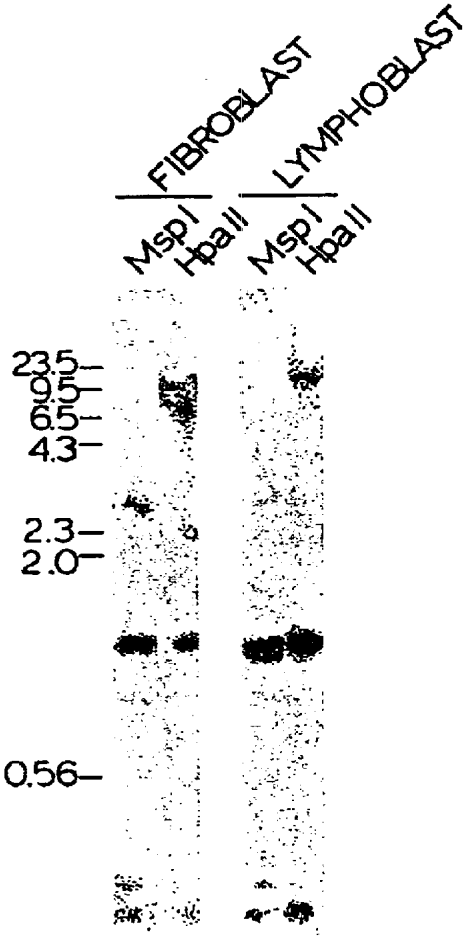

FIBROBLAST
LYMPHOBLAST
MspI
HpaII
MspI
HpaII
23.5
9.5
6.5
4.3
2.3
2.0
0.56

FIG. 6

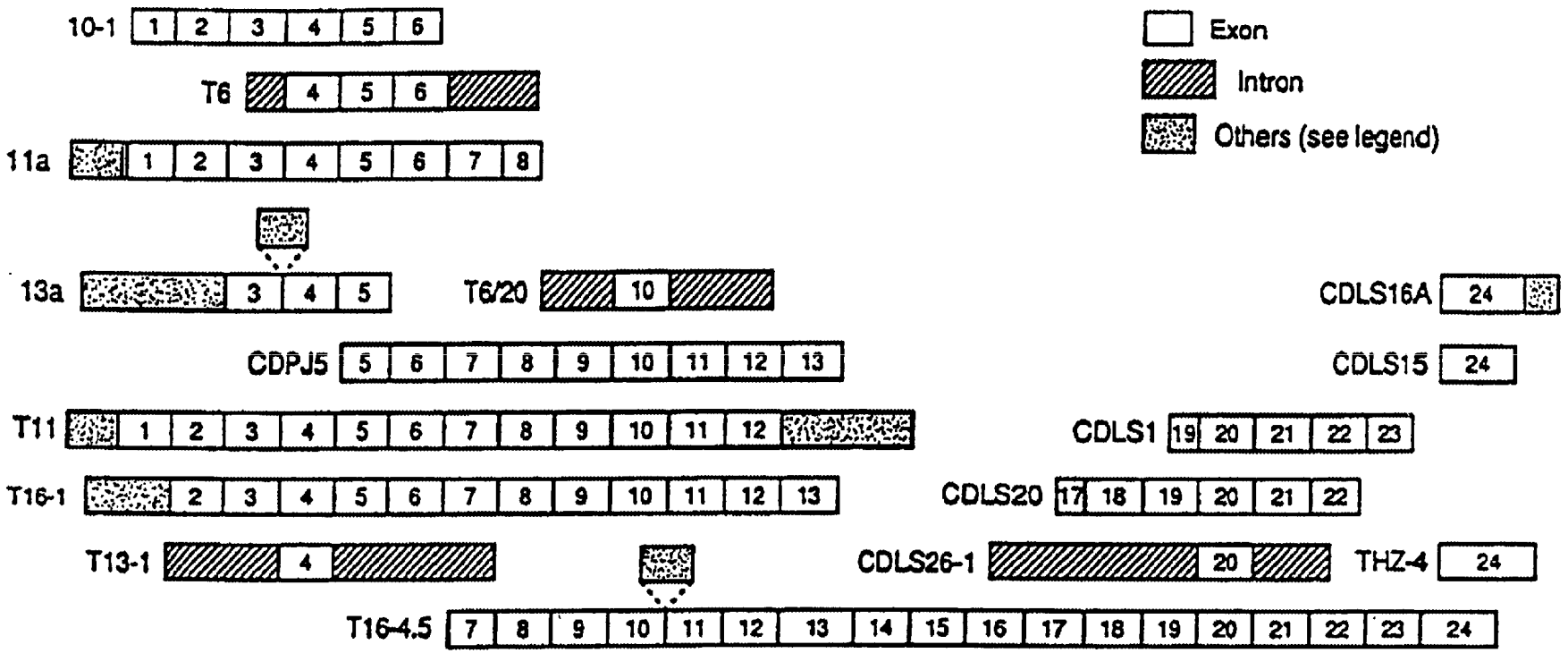

10-1
1 2 3 4 5 6
T6
4 5 6
11a
1 2 3 4 5 6 7 8
13a
3 4 5
T6/20
10
CDPJ5
5 6 7 8 9 10 11 12 13
T11
1 2 3 4 5 6 7 8 9 10 11 12
T16-1
2 3 4 5 6 7 8 9 10 11 12 13
T13-1
4
T16-4.5
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Exon
Intron
Others (see legend)
CDLS16A
24
CDLS15
24
CDLS1
19 20 21 22 23
CDLS20
17 18 19 20 21 22
CDLS26-1
20
THZ-4
24

FIG. 7A

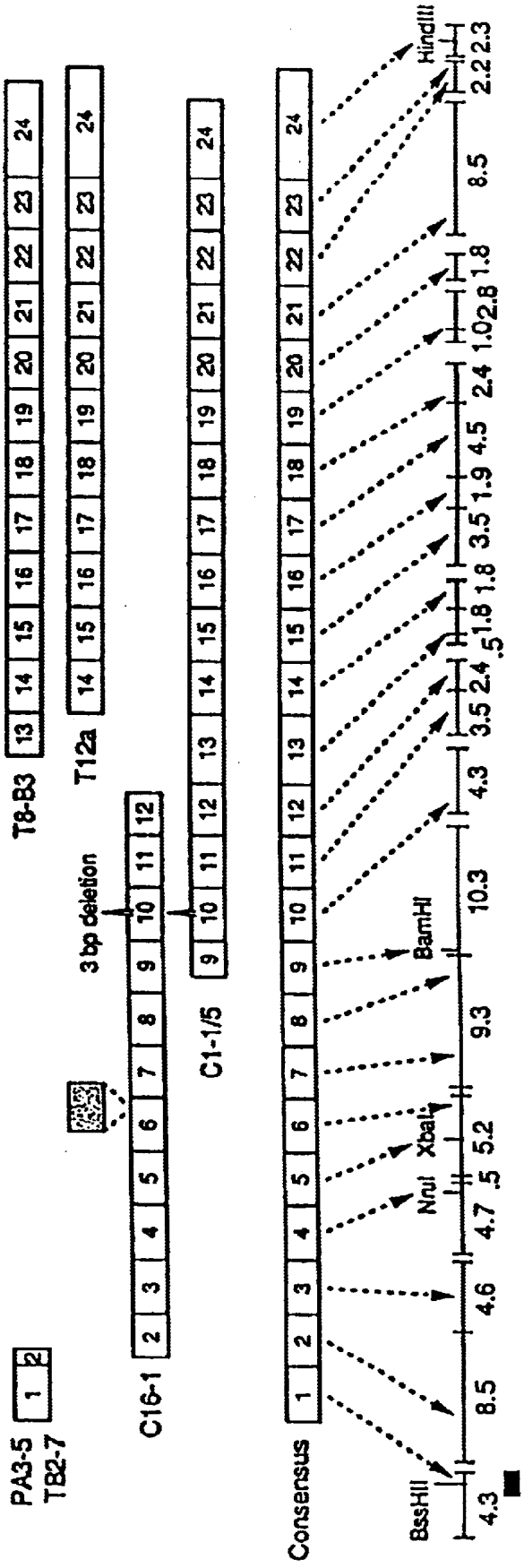

PA3-5
TB2-7
C16-1
3 bp deletion
T8-B3
T12a
C1-1/5
Consensus
BssHII
NruI
XbaI
BamHI
HindIII
4.3
8.5
4.6
4.7
.5
5.2
9.3
10.3
4.3
3.5
2.4
.5
1.8
1.8
3.5
1.9
4.5
2.4
1.0
2.8
1.8
8.5
2.2
2.3
H1.6

FIG. 7B

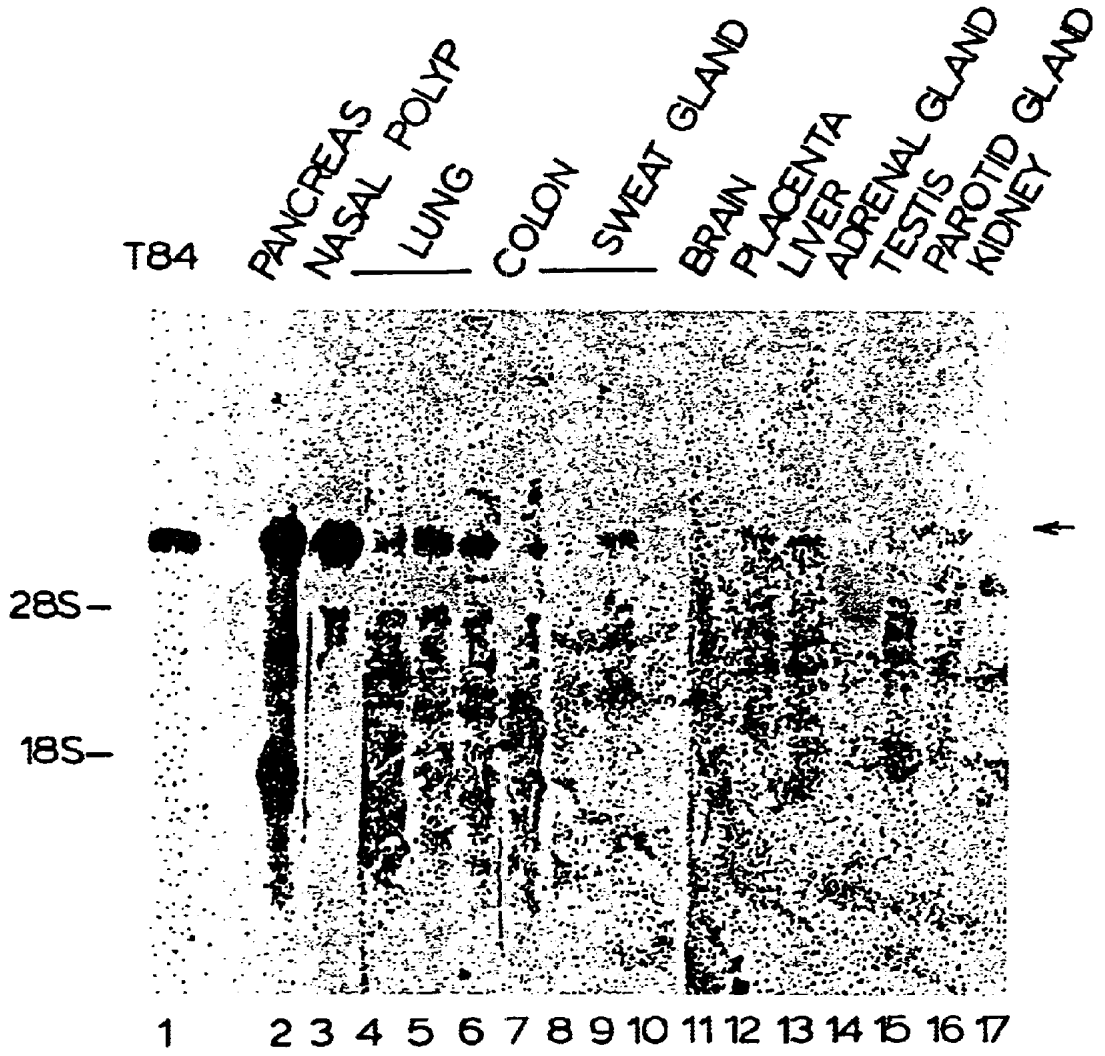

T84
PANCREAS
NASAL POLYP
LUNG
COLON
SWEAT GLAND
BRAIN
PLACENTA
LIVER
ADRENAL GLAND
TESTIS
PAROTID GLAND
KIDNEY
28S–
18S–
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

FIG.8

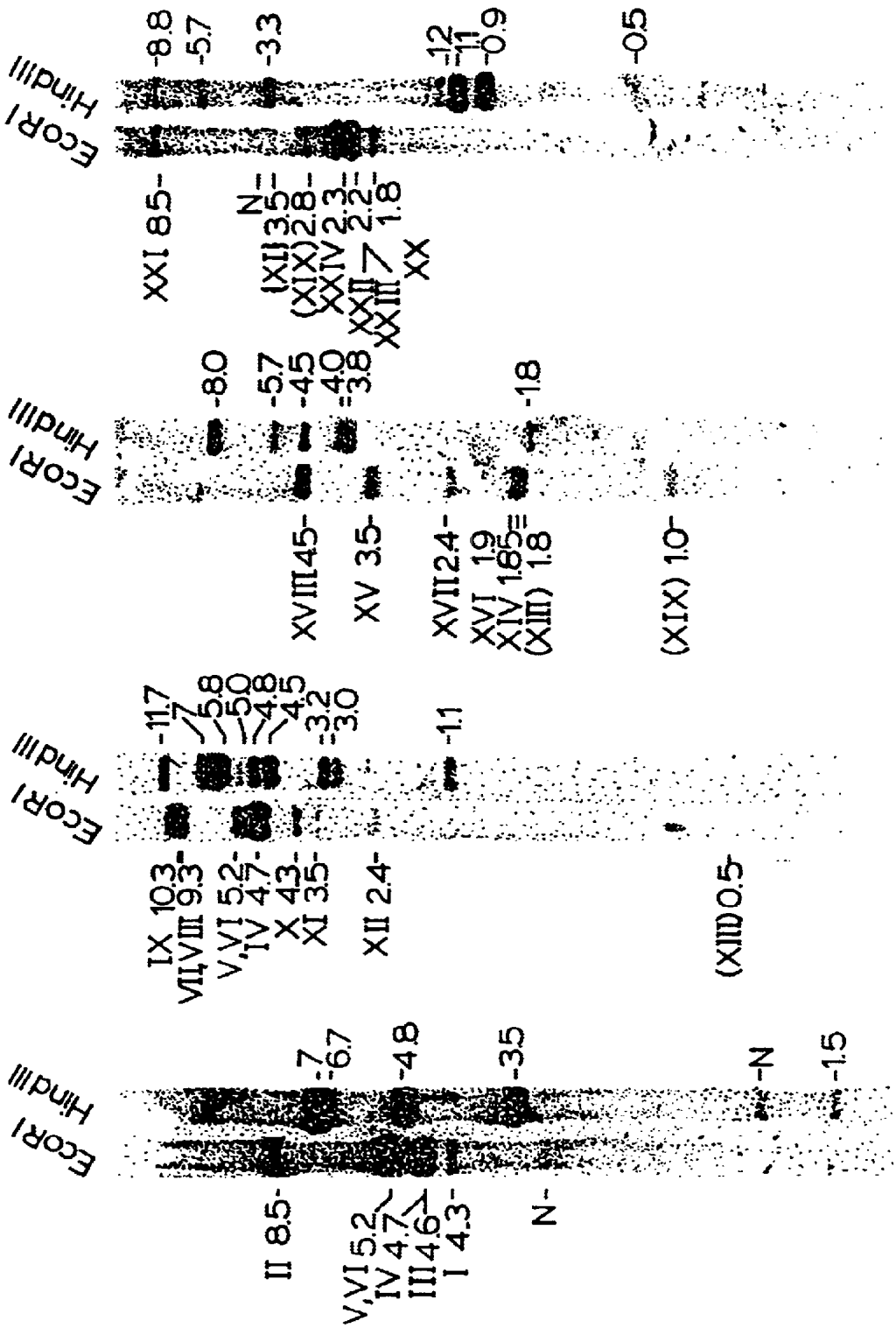


FIG.9A FIG.9B FIG.9C FIG.9D

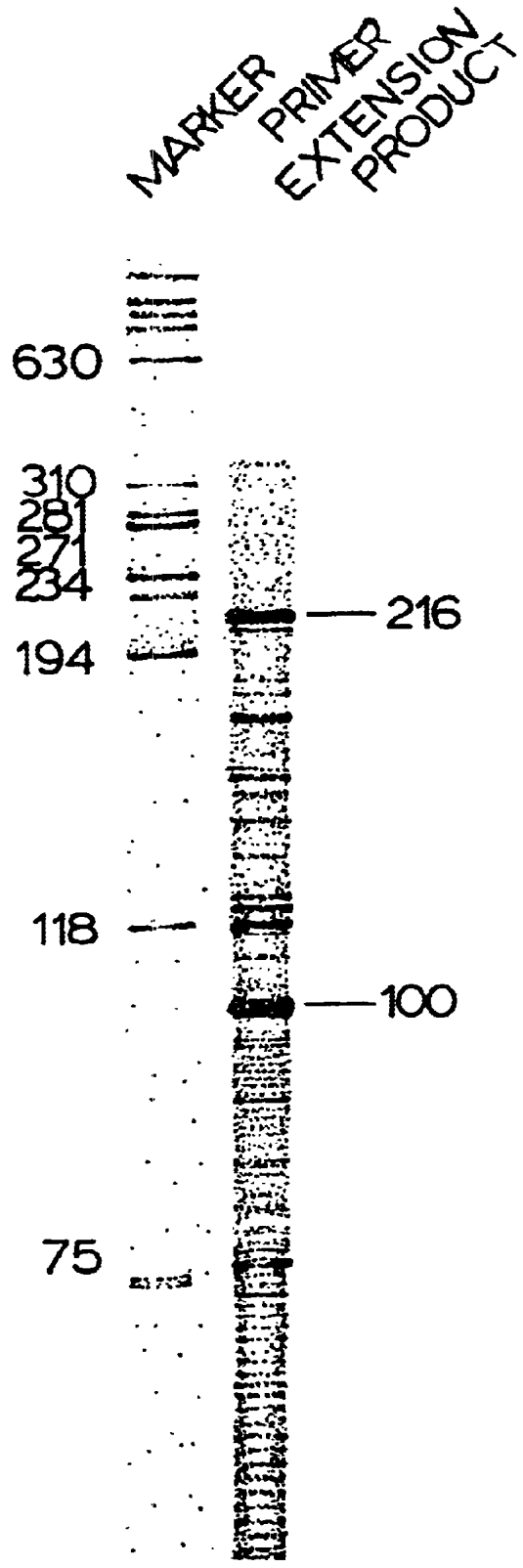

MARKER
PRIMER EXTENSION PRODUCT
630
310
281
271
234
194
118
75
216
100

FIG. 10A

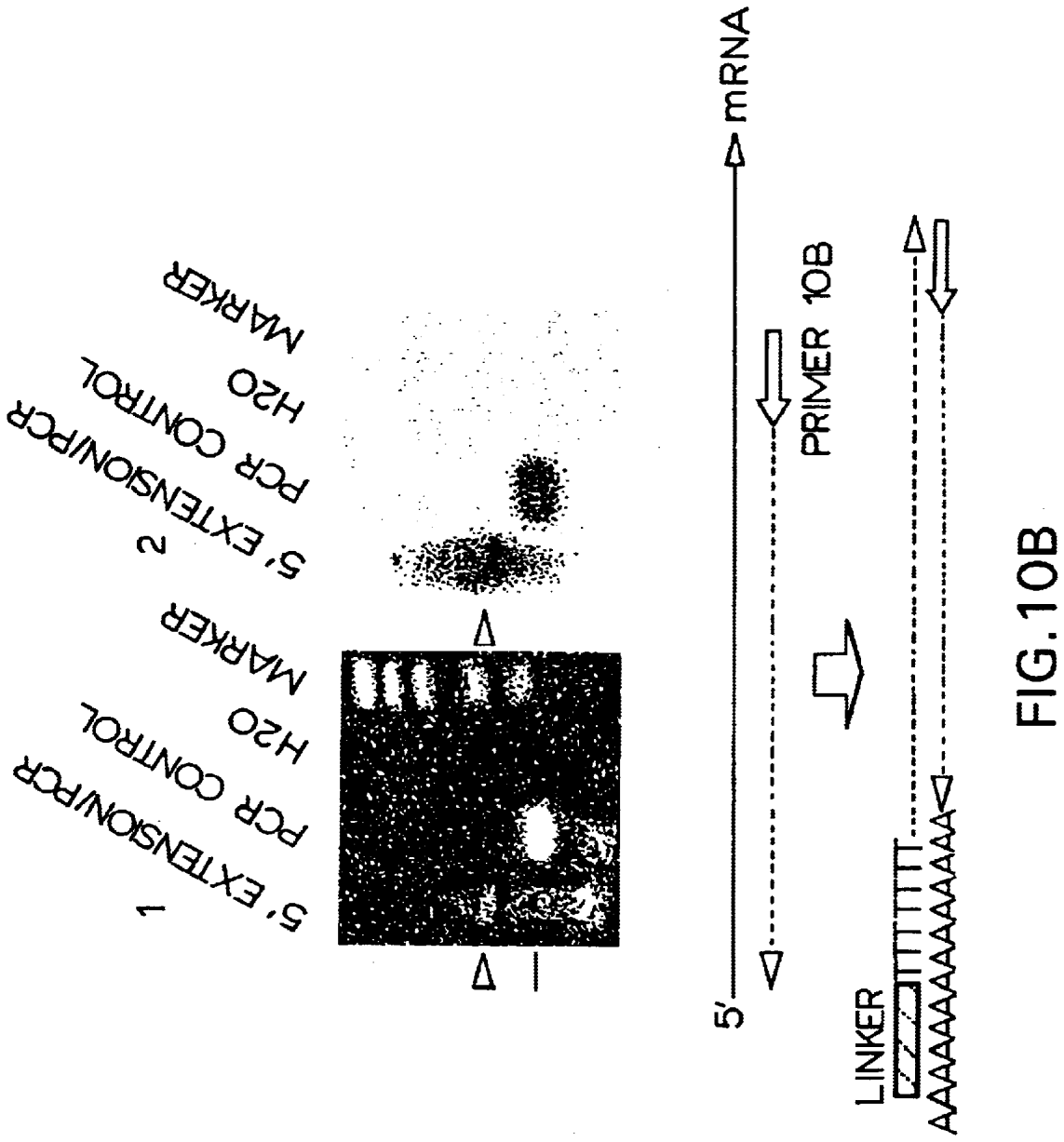

mRNA
PRIMER 10B
5'
LINKER
AAAAAAAAAAAAAA
5' EXTENSION/PCR
1
PCR CONTROL
H2O
MARKER
5' EXTENSION/PCR
2
PCR CONTROL
H2O
MARKER

FIG. 10B

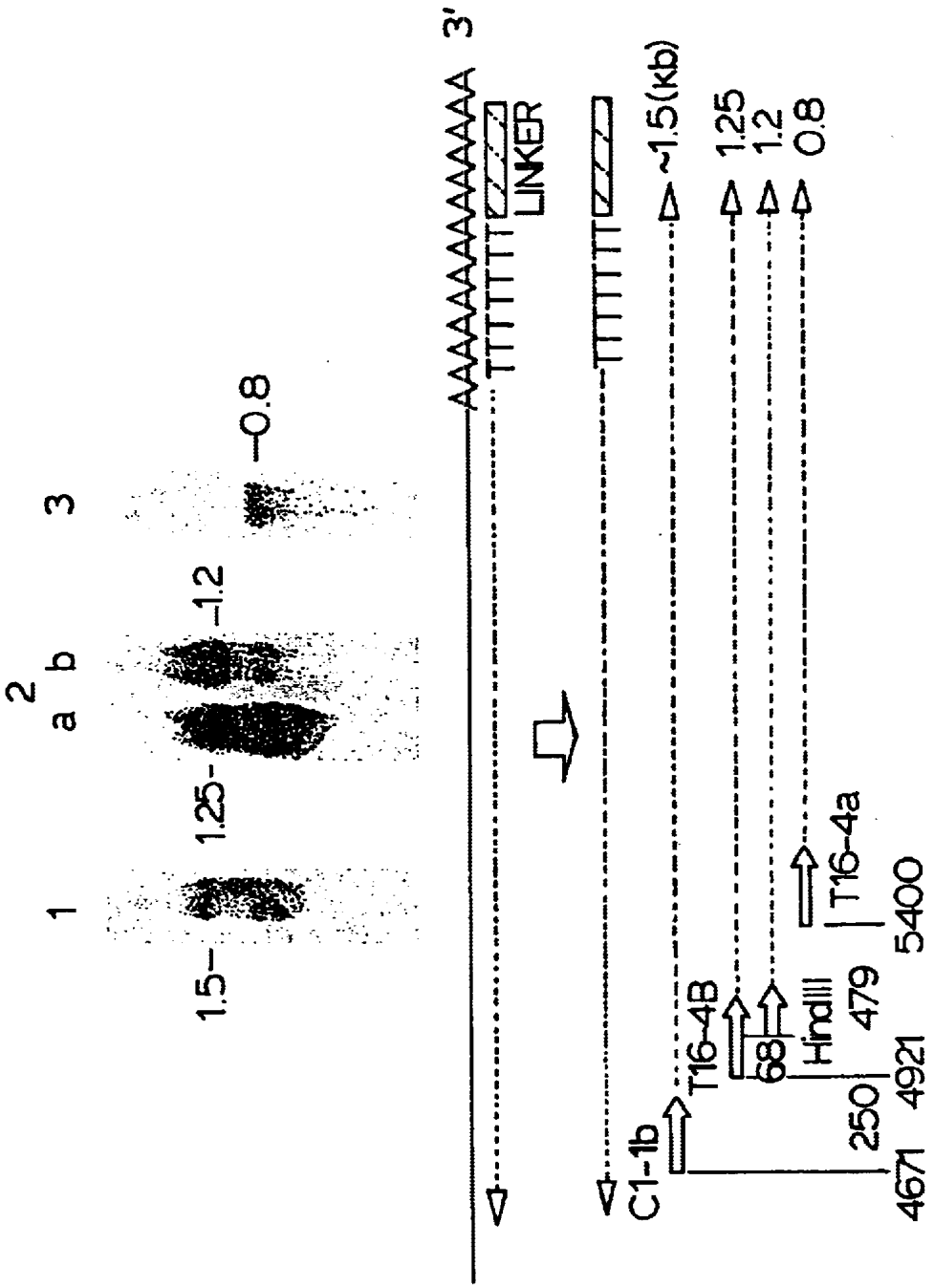

1
2
a b
3
1.5–
1.25–
–1.2
–0.8
3'
AAAAAAAAAAAAA
TTTTTTT
LINKER
TTTTTTT
~1.5(kb)
1.25
1.2
0.8
C1-1b
T16-4B
68
HindIII
479
T16-4a
250
4921
5400
4671

FIG.10C

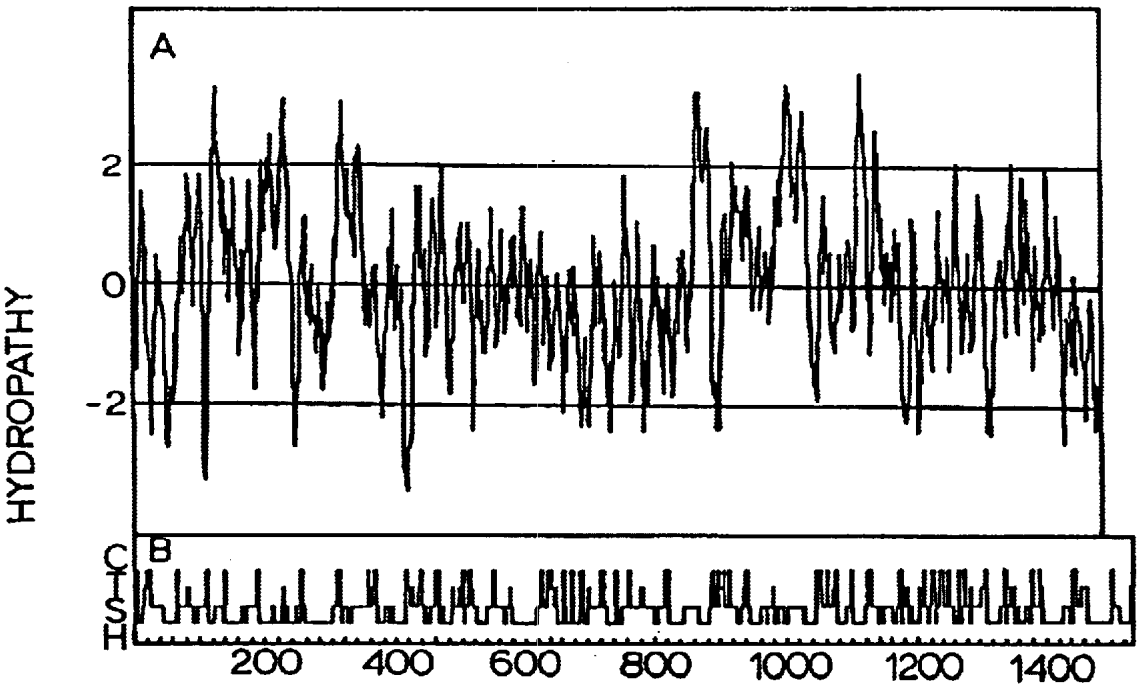

A
2
0
-2
HYDROPATHY
B
C
H
S
H
200
400
600
800
1000
1200
1400

FIG. 11

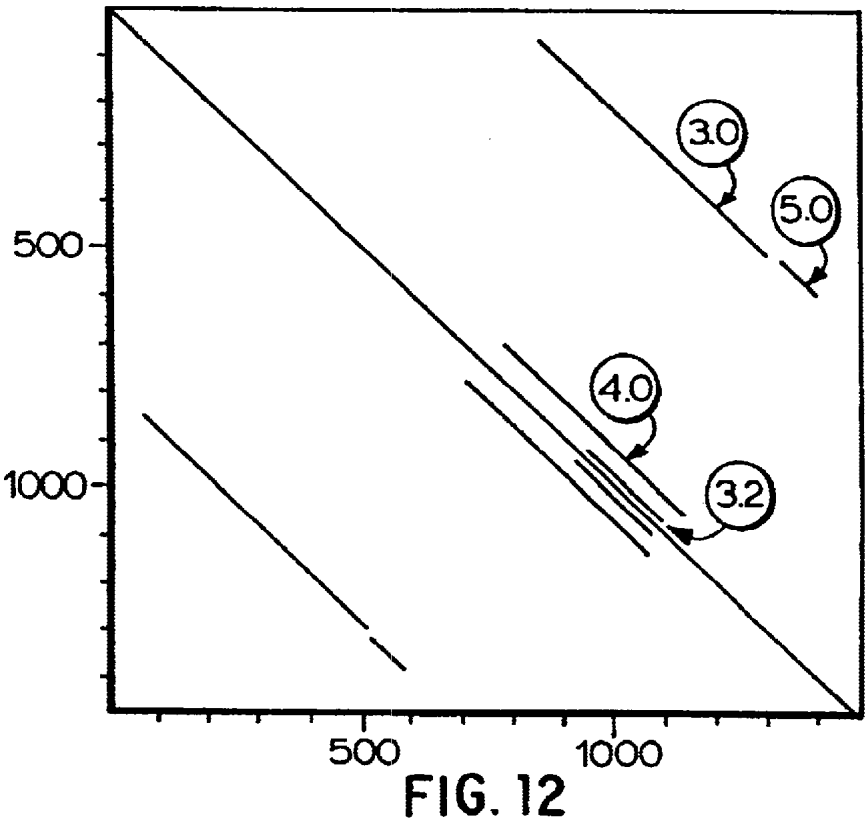

3.0
5.0
4.0
3.2
500
1000
500
1000

FIG. 12

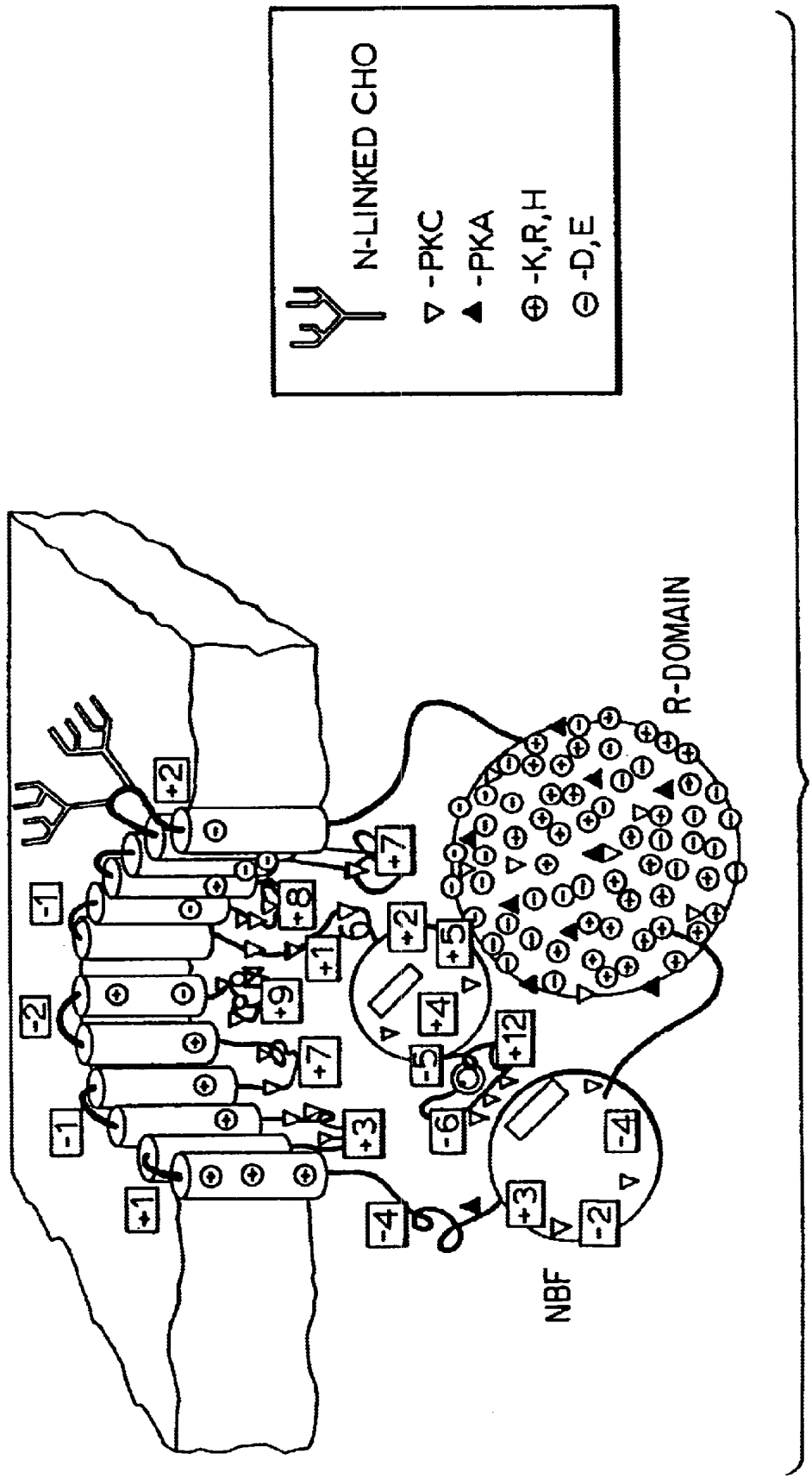


FIG. 13

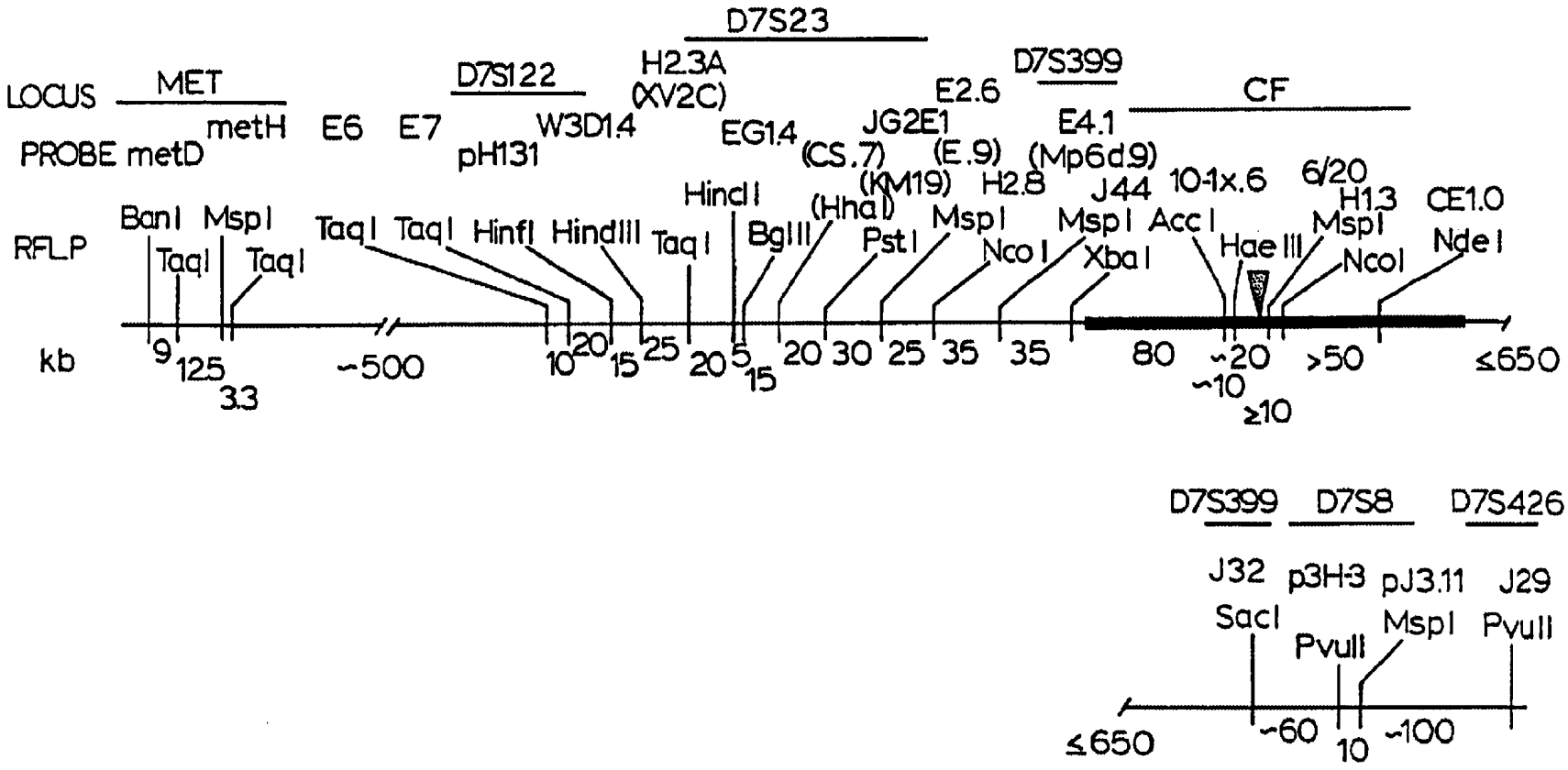

D7S23
LOCUS
MET
D7S122
H2.3A
(XV2C)
D7S399
CF
E2.6
PROBE metD
metH
E6
E7
W3D1.4
pH131
EG1.4
JG2E1
(CS.7)
(E.9)
E4.1
(Mp6d.9)
(KM19)
H2.8
J44
10-1x.6
6/20
H1.3
CE1.0
RFLP
Ban I
Msp I
Taq I
Taq I
Taq I
Taq I
Hinf I
Hind III
Taq I
Hind I
Bgl II
(Hha I)
Pst I
Msp I
Nco I
Msp I
Xba I
Acc I
Hae III
Msp I
Nco I
Nde I
kb
9
125
3.3
~500
10
20
15
25
20
5
15
20
30
25
35
35
80
~20
~10
≥10
>50
≤650
D7S399
D7S8
D7S426
J32
p3H-3
pJ3.11
J29
Sac I
Pvu II
Msp I
Pvu II
≤650
~60
10
~100

FIG. 14

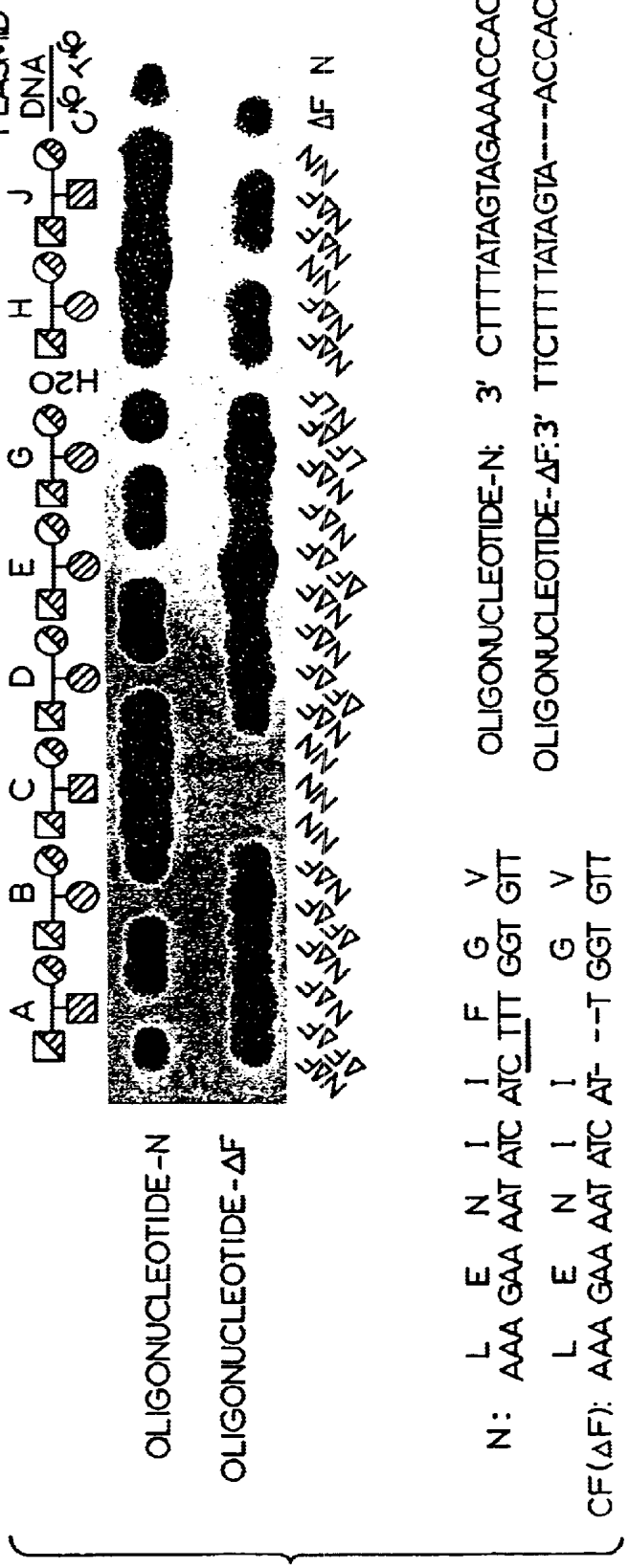

PLASMID DNA
A
B
C
D
E
G
H2O
H
J
OLIGONUCLEOTIDE-N
OLIGONUCLEOTIDE-ΔF
N: L E N I I F G V AAA GAA AAT ATC ATC TTT GGT GTT
CF(ΔF): L E N I I G V AAA GAA AAT ATC AT- --T GGT GTT
OLIGONUCLEOTIDE-N: 3' CTTTTATAGTAGAAACCAC 5'
OLIGONUCLEOTIDE-ΔF:3' TTCTTTTATAGTA---ACCACAA 5'

FIG. 15


CFTR (N) FSLLGTPVLKDINFKIERGQLLAVAGSTGAGKTSLLMMIMG ISFCSQFSWIMPGTIK-ENIIFGVSYD
CFTR (C) YTEGGNAILENISFSISPGQRVGLLGRTGSGKSTLLSAFLR DSITLQQWRKAFGVIPQKVFIFSGTFR
hmdr1 (N) PSRKEVKILKGLNLKVQSGQTVALVGNSGCGKSTTVQLMQR IGVVSQEPVLFATTI-AENIRYGRENV
hmdr1 (C) PTRPDIPVLQGLSLEVKKGQTLALVGSSGCGKSTVVQLLER LGIVSQEPILFDCSI-AENIAYGDNSR
mmdr1 (N) PSRSEVQILKGLNLKVKSGQTVALVGNSGCGKSTTVQLMQR IGVVSQEPVLFATTI-AENIRYGREDV
mmdr1 (C) PTRPNIPVLQGLSLEVKKGQTLALVGSSGCGKSTVVQLLER LGEVSQEPILFDCSI-AENIAYGDNSR
mmdr2 (N) PSRANIKILKGLNLKVKSGQTVALVGNSGCGKSTTVQLLQR IGVVSQEPVLSFTTI-AENIRYGRGNV
mmdr2 (C) PTRANVPVLQGLSLEVKKGQTLALVGSSGCGKSTVVQLLER LGIVSQEPILFDCSI-AENIAYGDNSR
pfmdr (N) DTRKDVEIYKDLSFTLLKEGKTYAFVGESGCGKSTILKLIE IGVVSQDPLLFSNSI-KNNIKYSLYSL
pfmdr (C) ISRPNVPIYKNLSFTCDSKKTTAIVGETGSGKSTFMNLLLR FSIVSQEPMLFNMSI-YENIKFGREDA
STE6 (N) PSRPSEAVLKNVSLNFSAGQFTFIVGKSGSGKSTLSNLLLR ITVVEQRCTLFNDTL-RKNILLGSTDS
STE6 (C) PSAPTAFVYKNMNFDMFCGQTLGIIGESGTGKSTLVLLLTK ISVVEQKPLLFNGTI-RDNLTYGLQDE
hlyB YKPDSPVILDNINISIKQGEVIGIVGRSGSGKSTLIKLIQR VGVVLQDNVLLNRSI-IDNISLAPGMS
White IPAPRKHLLKNVCGVAYPGELLAVMGSSGAGKTTLLNALAF RCAYVQQDDLFIGLIAREHLIFQAMVR
MbpX KSLGNLKILDRVSLYVPKFSLIALLGPSGSGKSSLLRILAG MSFVFQHYALFKHMTVYENISFGLRLR
BtuD QDVAESTRLGPLSGEVRAGRILHLVGPNGAGKSTLLARIAG YLSQQQTPPFATPVWHYLTLHQHDKTR
PstB FYYGKFHALKNINLDTAKNQVTAFIGPSGCGKSTLLRTFNK VGMVFQKPTPFPMSI-YDNIAFGVRLF
hisP RRYGGHEVLKGVSLQARAGDVISIIGSSGSGKSTFLRCINF GIMVFQHFNLWSHMTVLENVMEAPIQV
malK KAWGEVVVSKDINIDIHEGEFVVFVGPSGCGKSTLLRMIAG VGMVFQSYALYPHLSVAENMSFGLKPA
oppD TPDGDVTAVNDLNFTLRAGETLGIVGESGSGKSQTAFALMG ISMIFQDPMTSLNPYMRVGEQLMEVLM
oppF QPPKTLKAVDGVTLRLYEGETLGVVGESGCGKSTFARAIIG IQMIFQDPLASLNPRMTIGEIIAEPLR
RbsA (N) KAVPGVKALSGAALNVYPGRVMALVGENGAGKSTMMKVLTG AGIIHQELNLIPQLTIAENIFLGREFV
RbsA (C) VDNLCGPGVNDVSFTLRKGEILGVSGLMGAGRTELMKVLYG ISEDRKRDGLVLGMSVKENMSLTALRY
UvrA LTGARGNNLKDVTLTLPVGLFTCITGVSGSGKSTLINDTLF TYTGVFTPVRELFAGVPESRARGYTPG
NodI KSYGGKIVVNDLSFTIAAGECFGLLGPNGAGKSTIIRMILG IGIVSQEDNLDLEFTVRENLLVYGRYF
FtsE AYLGGRQALQGVTFHMQPGEMAFLTGHSGAGKSTLLKLICG IGMIFQDHHLLMDRTVYDNVAIPLIIA

FIG. 16A

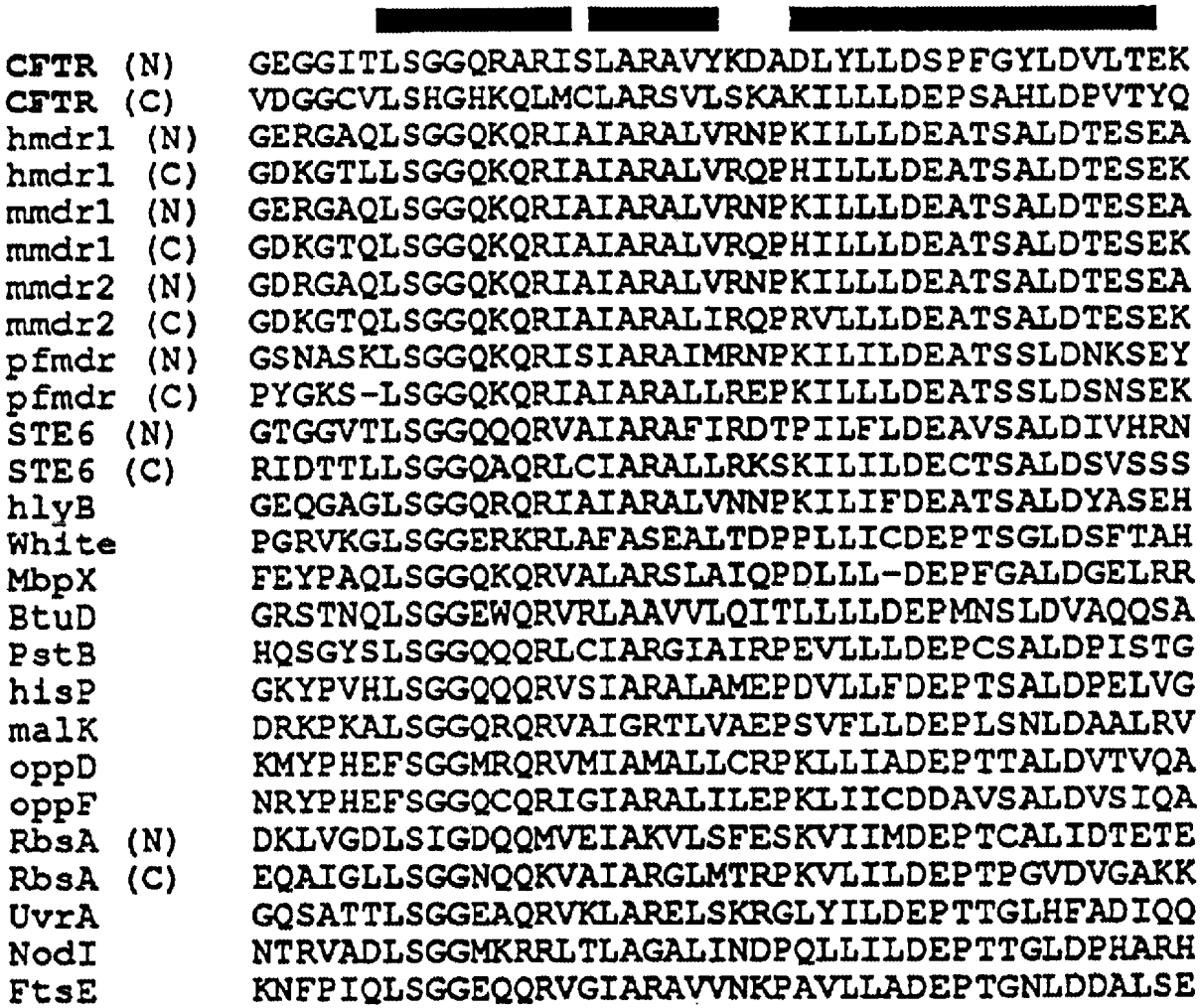

CFTR (N) GEGGITLSGGQRARISLARAVYKDADLYLLDSPFGYLDVLTEK
CFTR (C) VDGGCVLSHGHKQLMCLARSVLSKAKILLLDEPSAHLDPVTYQ
hmdr1 (N) GERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEA
hmdr1 (C) GDKGTLLSGGQKQRIAIARALVRQPHILLLDEATSALDTESEK
mmdr1 (N) GERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEA
mmdr1 (C) GDKGTQLSGGQKQRIAIARALVRQPHILLLDEATSALDTESEK
mmdr2 (N) GDRGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEA
mmdr2 (C) GDKGTQLSGGQKQRIAIARALIRQPRVLLLDEATSALDTESEK
pfmdr (N) GSNASKLSGGQKQRISIARAIMRNPKILILDEATSSLDNKSEY
pfmdr (C) PYGKS-LSGGQKQRIAIARALLREPKILLLDEATSSLDSNSEK
STE6 (N) GTGGVTLSGGQQQRVAIARAFIRDTPILFLDEAVSALDIVHRN
STE6 (C) RIDTTLLSGGQAQRLCIARALLRKSKILILDECTSALDSVSSS
hlyB GEQGAGLSGGQRQRIAIARALVNNPKILIFDEATSALDYASEH
White PGRVKGLSGGERKRLAFASEALTDPPLLICDEPTSGLDSFTAH
MbpX FEYPAQLSGGQKQRVALARSLAIQPDLLL-DEPFGALDGELRR
BtuD GRSTNQLSGGEWQRVRLAAVVLQITLLLLDEPMNSLDVAQQSA
PstB HQSGYSLSGGQQQRLCIARGIAIRPEVLLLDEPCSALDPISTG
hisP GKYPVHLSGGQQQRVSIARALAMEPDVLLFDEPTSALDPELVG
malK DRKPKALSGGQRQRVAIGRTLVAEPSVFLLDEPLSNLDAALRV
oppD KMYPHEFSGGMRQRVMIAMALLCRPKLLIADEPTTALDVTVQA
oppF NRYPHEFSGGQCQRIGIARALILEPKLIICDDAVSALDVSIQA
RbsA (N) DKLVGDLSIGDQQMVEIAKVLSFESKVIIMDEPTCALIDTETE
RbsA (C) EQAIGLLSGGNQQKVAIARGLMTRPKVLILDEPTPGVDVGAKK
UvrA GQSATTLSGGEAQRVKLARELSKRGLYILDEPTTGLHFADIQQ
NodI NTRVADLSGGMKRRLTLAGALINDPQLLILDEPTTGLDPHARH
FtsE KNFPIQLSGGEQQRVGIARAVVNKPAVLLADEPTGNLDDALSE

FIG.16B

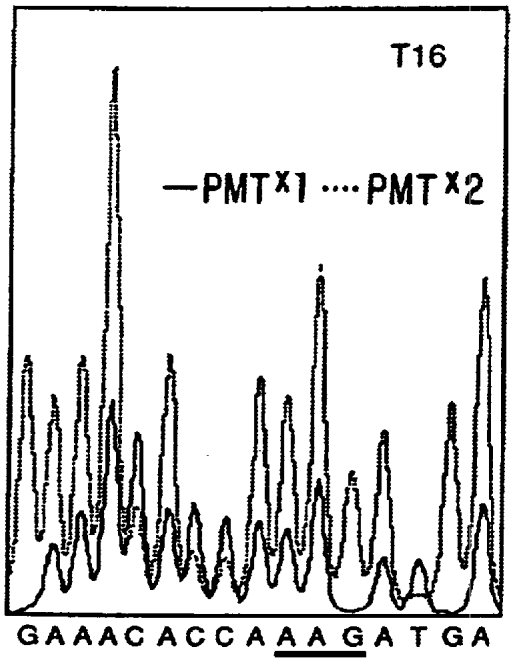


FIG.17A

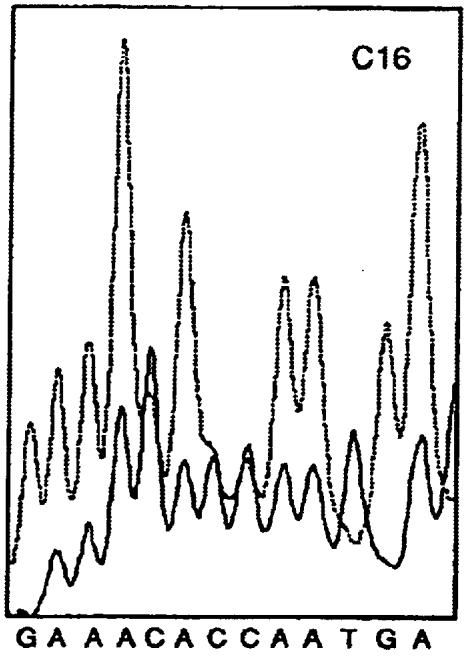


FIG.17B

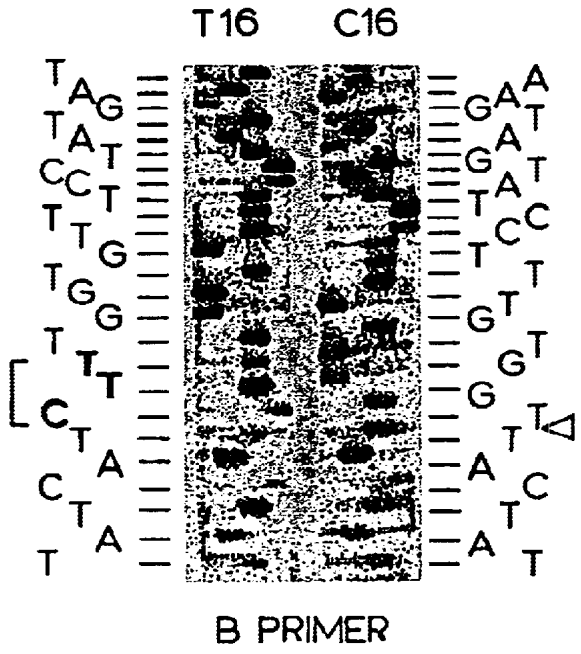


FIG.18A

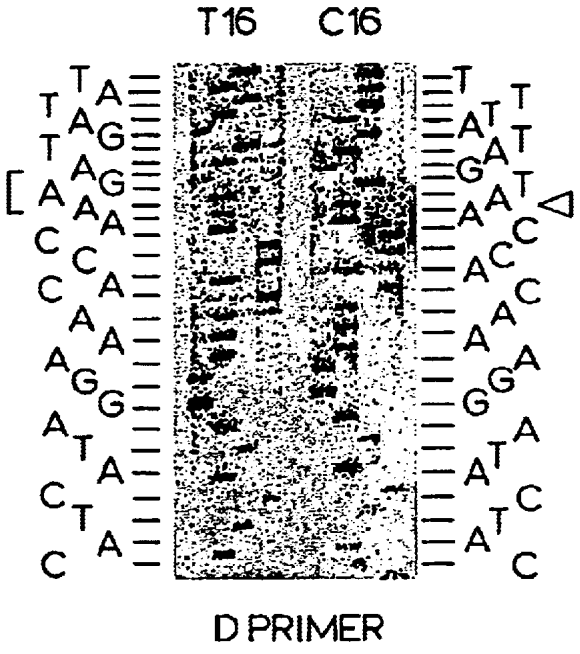


FIG.18B

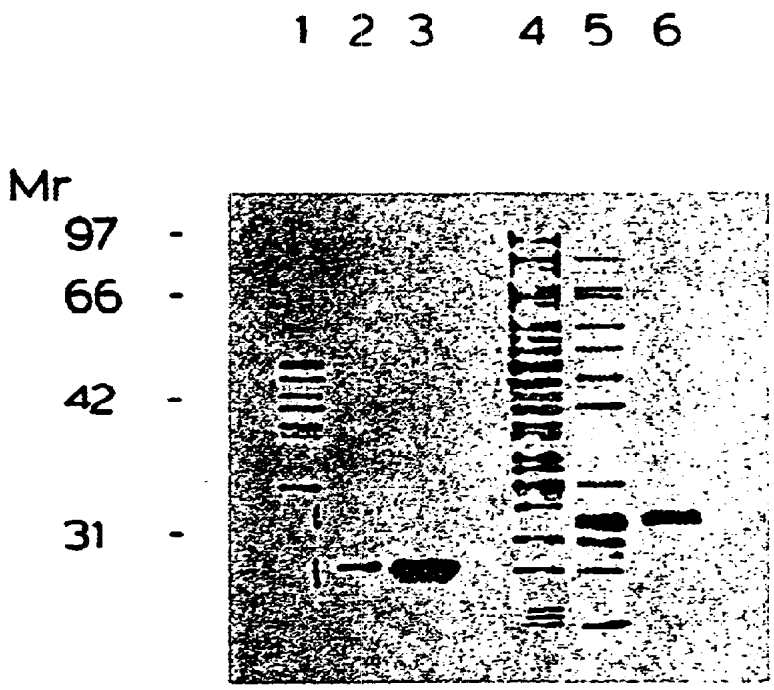


FIG. 19A

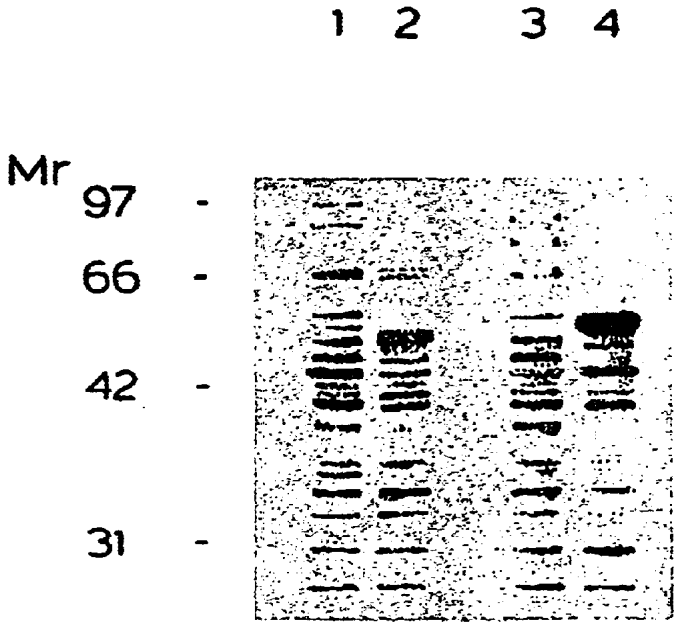


FIG. 19B

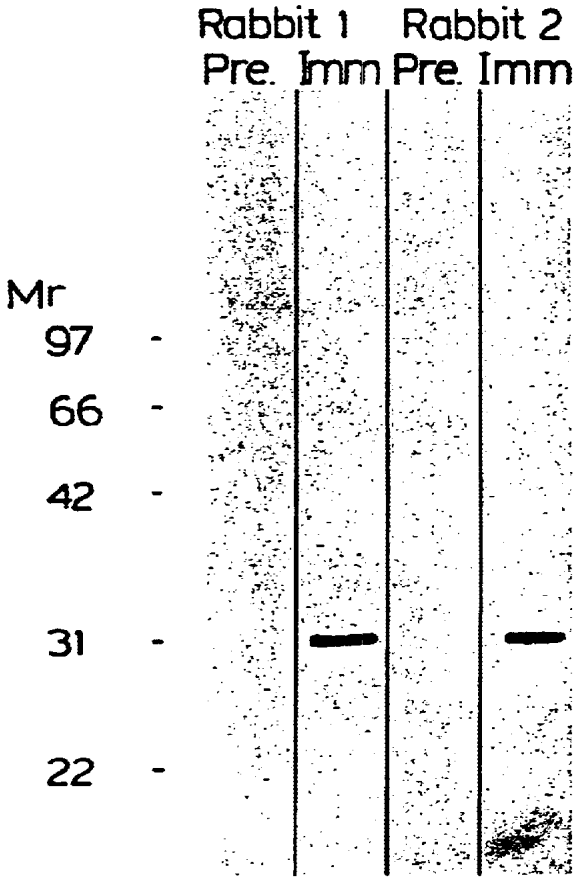


FIG.20

FIG.21

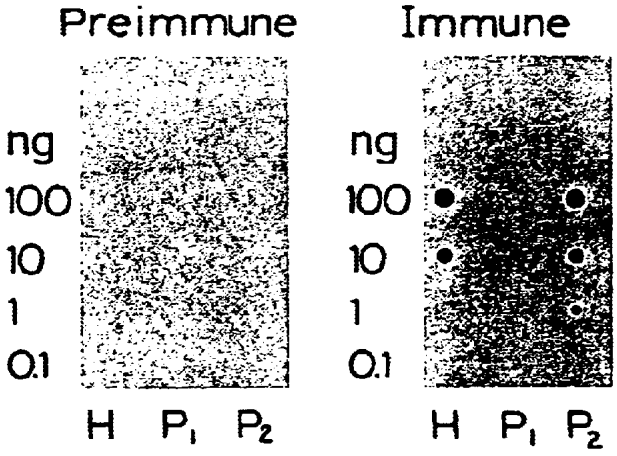


FIG.22

CYSTIC FIBROSIS GENE

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. application Ser. No. 08/252,778, filed Jun. 2, 1994, which is a divisional of U.S. application Ser. No. 08/123,864, filed Sep. 20, 1993. This application is also a continuation of U.S. application Ser. No. 08/123,864, filed Sep. 20, 1993, which is a continuation of U.S. application Ser. No. 07/401,609, filed Aug. 31, 1989 (now abandoned), which is a continuation-in-part (CIP) of U.S. application Ser. No. 07/399,945, filed Aug. 24, 1989 (now abandoned), which is a CIP of U.S. application Ser. No. 07/396,894, filed Aug. 22, 1989 (now abandoned).

This invention was made with government support under Grants R01 DK39690-02 and DK34944 awarded by the United States National Institutes of Health. The United States government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates generally to the cystic fibrosis (CF) gene, and, more particularly to the identification, isolation and cloning of the DNA sequence corresponding to the normal and mutant CF genes, as well as their transcripts and gene products. The present invention also relates to methods of screening for and detection of CF carriers, CF diagnosis, prenatal CF screening and diagnosis, and gene therapy utilizing recombinant technologies and drug therapy using the information derived from the DNA, protein, and the metabolic function of the cystic fibrosis transmembrane inductance regulator protein (CFTR).

BACKGROUND OF THE INVENTION

CF is the most common severe autosomal recessive genetic disorder in the Caucasian population. It affects approximately 1 in 2000 live births in North America (Boat et al, *The Metabolic Basis of Inherited Disease,* 6th ed, pp 2649–2680, McGraw Hill, NY (1989)). Approximately 1 in 20 persons are carriers of the disease.

Although the disease was first described in the late 1930's, the basic defect remains unknown. The major symptoms of cystic fibrosis include chronic pulmonary disease, pancreatic exocrine insufficiency, and elevated sweat electrolyte levels. The symptoms are consistent with cystic fibrosis being an exocrine disorder. Although recent advances have been made in the analysis of ion transport across the apical membrane of the epithelium of CF patient cells, it is not clear that the abnormal regulation of chloride channels represents the primary defect in the disease. Given the lack of understanding of the molecular mechanism of the disease, an alternative approach has therefore been taken in an attempt to understand the nature of the molecular defect through direct cloning of the responsible gene on the basis of its chromosomal location.

However, there is no clear phenotype that directs an approach to the exact nature of the genetic basis of the disease, or that allows for an identification of the cystic fibrosis gene. The nature of the CF defect in relation to the population genetics data has not been readily apparent. Both the prevalence of the disease and the clinical heterogeneity have been explained by several different mechanisms: high mutation rate, heterozygote advantage, genetic drift, multiple loci, and reproductive compensation.

Many of the hypotheses can not be tested due to the lack of knowledge of the basic defect. Therefore, alternative approaches to the determination and characterization of the CF gene have focussed on an attempt to identity the location of the gene by genetic analysis.

Linkage analysis of the CF gene to antigenic and protein markers was attempted in the 1950's, but no positive results were obtained (Steinberg et al *Am. J. Hum. Genet.* 8: 162–176, (1956); Steinberg and Morton *Am. J. Hum. Genet* 8: 177–189, (1956); Goodchild et al *J. Med. Genet.* 7: 417–419, 1976).

More recently, it has become possible to use RFLP's to facilitate linkage analysis. The first linkage of an RFLP marker to the CF gene was disclosed in 1985 (Tsui et al. *Science* 230: 1054–1057, 1985) in which linkage was found between the CF gene and an uncharacterized marker DOCRI-917. The association was found in an analysis of 39 families with affected CF children. This showed that although the chromosomal location had not been established, the location of the disease gene had been narrowed to about 1% of the human genome, or about 30 million nucleotide base pairs.

The chromosomal location of the D0CRI-917 probe was established using rodent-human hybrid cell lines containing different human chromosome complements. It was shown that DOCR1-917 (and therefore the CF gene) maps to human chromosome 7.

Further physical and genetic linkage studies were pursued in an attempt to pinpoint the location of the CF gene. Zengerling et al (*Am. J. Hum. Genet.* 40: 228–236 (1987) describe the use of human-mouse somatic cell hybrids to obtain a more detailed physical relationship between the CF gene and the markers known to be linked with it. This publication shows that the CF gene can be assigned to either the distal region of band q22 or the proximal region of band q31 on chromosome 7.

Rommens et al (*Am. J. Hum. Genet.* 43: 645–663, (1988) give a detailed discussion of the isolation of many new 7q31 probes. The approach outlined led to the isolation of two new probes, D7S122 and D7S340, which are close to each other. Pulsed field gel electrophoresis mapping indicates that these two RFLP markers are between two markers known to flank the CF gene, MET (White, R., Woodward S., Leppert M., et al. *Nature* 318: 382–384, (1985)) and D7S8 [Wainwright, B. J., Scambler, P. J., and J. Schmidtke, *Nature* 318: 384–385 (1985)), therefore in the CF gene region. The discovery of these markers provides a starting point for chromosome walking and jumping.

Estivill et al, (*Nature* 326: 840–845(1987)) disclose that a candidate cDNA gene was located and partially characterized. This however, does not teach the correct location of the CF gene. The reference discloses a candidate cDNA gene downstream of a CpG island, which are undermethylated GC nucleotide-rich regions upstream of many vertebrate genes. The chromosomal localization of the candidate locus is identified as the XV2C region. This region is described in European Patent Application 88303645.1. However, that actual region does not include the CF gene.

A major difficulty in identifying the CF gene has been the lack of cytologically detectable chromosome rearrangements or deletions, which greatly facilitated all previous successes in the cloning of human disease genes by knowledge of map position.

Such rearrangements and deletions could be observed cytologically and as a result, a physical location on a particular chromosome could be correlated with the particular disease. Further, this cytological location could be correlated with a molecular location based on known relation-

ship between publicly available DNA probes and cytologically visible alterations in the chromosomes. Knowledge of the molecular location of the gene for a particular disease would allow cloning and sequencing of that gene by routine procedures, particularly when the gene product is known and cloning success can be confirmed by immunoassay of expression products of the cloned genes.

In contrast, neither the cytological location nor the gene product of the gene for cystic fibrosis was known in the prior art. With the recent identification of MET and D7S8, markers which flanked the CF gene but did not pinpoint its molecular location, the present inventors devised various novel gene cloning strategies to approach the CF gene in accordance with the present invention. The methods employed in these strategies include chromosome jumping from the flanking markers, cloning of DNA fragments from a defined physical region with the use of pulsed field gel electrophoresis, a combination of somatic cell hybrid and molecular cloning techniques designed to isolate DNA fragments from undermethylated CpG islands near CF, chromosome microdissection and cloning, and saturation cloning of a large number of DNA markers from the 7q31 region. By means of these novel strategies, the present inventors were able to identify the gene responsible for cystic fibrosis where the prior art was uncertain or, even in one case, wrong.

The application of these genetic and molecular cloning strategies has allowed the isolation and cDNA cloning of the cystic fibrosis gene on the basis of its chromosomal location, without the benefit of genomic rearrangements to point the way. The identification of the normal and mutant forms of the CF gene and gene products has allowed for the development of screening and diagnostic tests for CF utilizing nucleic acid probes and antibodies to the gene product. Through interaction with the defective gene product and the pathway in which this gene product is involved, therapy through normal gene product supplementation and gene manipulation and delivery are now made possible.

SUMMARY OF THE INVENTION

The gene involved in the cystic fibrosis disease process, hereinafter the "CF gene" and its functional equivalents, has been identified, isolated and cDNA cloned, and its transcripts and gene products identified and sequenced. A three base pair deletion leading to the omission of a phenylalanine residue in the gene product has been determined to correspond to the mutations of the CF gene in approximately 70% of the patients affected with CF, with different mutations involved in most if not all the remaining cases.

With the identification and sequencing of the gene and its gene product, nucleic acid probes and antibodies raised to the gene product can be used in a variety of hybridization and immunological assays to screen for and detect the presence of either a normal or a defective CF gene or gene product. Assay kits for such screening and diagnosis can also be provided.

Patient therapy through supplementation with the normal gene product, whose production can be amplified using genetic and recombinant techniques, or its functional equivalent, is now also possible. Correction or modification of the defective gene product through drug treatment means is now possible. In addition, cystic fibrosis can be cured or controlled through gene therapy by correcting the gene defect in situ or using recombinant or other vehicles to deliver a DNA sequence capable of expression of the normal gene product to the cells of the patient.

According to an aspect of the invention, a DNA molecule comprises a DNA sequence selected from the group consisting of:

(a) DNA sequences which correspond to the DNA sequence as set forth in the following FIGS. **1A–1H** from amino acid residue position 1 to position 1480;

(b) DNA sequences encoding normal CFTR polypeptide having the sequence according to the following FIGS. **1A–1H** for amino acid residue positions from 1 to 1480;

(c) DNA sequences which correspond to a fragment of the sequence of the following FIGS. **1A–1H** including at least 16 sequential nucleotides between amino acid residue positions 1 and 1480;

(d) DNA sequences which comprise at least 16 nucleotides and encode a fragment of the amino acid sequence of the following FIGS. **1A–1H**; and

(e) DNA sequences encoding an epitope encoded by at least 18 sequential nucleotides in the sequence of the following FIGS. **1A–1H** between amino acid residue positions 1 and 1480.

According to another aspect of the invention, a purified mutant CF gene comprises a DNA sequence encoding an amino acid sequence for a protein where the protein, when expressed in cells of the human body, is associated with altered cell function which correlates with the genetic disease cystic fibrosis.

According to another aspect of the invention, a purified RNA molecule comprises an RNA sequence corresponding to the above DNA sequence.

According to another aspect of the invention, a DNA molecule comprises a cDNA molecule corresponding to the above DNA sequence.

According to another aspect of the invention, a purified nucleic acid probe comprises a DNA or RNA nucleotide sequence corresponding to the above noted selected DNA sequences of groups (a) to (e).

According to another aspect of the invention, a DNA molecule comprises a DNA sequence encoding mutant CFTR polypeptide having the sequence according to the following FIGS. **1A–1H** for amino acid residue positions 1 to 1480. The sequence is further characterized by a three base pair mutation which results in the deletion of phenylalanine from amino acid residue position 508.

According to another aspect of the invention, a DNA molecule comprises a cDNA molecule corresponding to the above DNA sequence.

According to another aspect of the invention, the cDNA molecule comprises a DNA sequence selected from the group consisting of:

(a) DNA sequences which correspond to the mutant DNA sequence and which encode, on expression, for mutant CFTR polypeptide;

(b) DNA sequences which correspond to a fragment of the mutant DNA sequences, including at least twenty nucleotides;

(c) DNA sequences which comprise at least twenty nucleotides and encode a fragment of the mutant CFTR protein amino acid sequence; and

(d) DNA sequences encoding an epitope encoded by at least eighteen sequential nucleotides in the mutant DNA sequence.

According to another aspect of the invention, purified RNA molecule comprising RNA sequence corresponds to the mutant DNA sequence.

A purified nucleic acid probe comprising a DNA or RNA nucleotide sequence corresponding to the mutant sequences as recited above.

According to another aspect of the invention, a recombinant cloning vector comprising the DNA sequences of the

normal or mutant DNA and fragments thereof is provided. The vector, according to an aspect of this invention, is operatively linked to an expression control sequence in the recombinant DNA molecule so that the normal CFTR protein can be expressed, or alternatively with the other selected mutant DNA sequence the mutant CFTR polypeptide can be expressed. The expression control sequence is selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof.

According to another aspect of the invention, a method for producing normal CFTR polypeptide comprises the steps of:

(a) culturing a host cell transtected with the recombinant vector for the normal DNA sequence in a medium and under conditions favorable for expression of the normal CFTR polypeptide; and

(b) isolating the expressed normal CFTR polypeptide.

According to another aspect of the invention, a method for producing a mutant CFTR polypeptide comprises the steps of:

(a) culturing a host cell transfected with the recombinant vector for the mutant DNA sequence in a medium and under conditions favorable for expression of the mutant CFTR polypeptide; and

(b) isolating the expressed mutant CFTR polypeptide.

According to another aspect of the invention, a purified protein of human cell membrane origin comprises an amino sequence encoded by the mutant DNA sequence where the protein, when present in human cell membrane, is associated with cell function which causes the genetic disease cystic fibrosis.

According to another aspect of the invention, the CFTR polypeptide is characterized by a molecular weight of about 170,000 daltons and an epithelial cell transmembrane ion conductance affecting activity.

According to another aspect of the invention, a substantially pure CFTR protein normally expressed in human epithelial cells and characterized by being capable of participating in regulation and in control of ion transport through epithelial cells by binding to epithelial cell membrane to modulate ion movement through channels formed in the epithelial cell membrane.

According to another aspect of the invention, a process for isolating the CFTR protein comprises:

(a) extracting peripheral proteins from membranes of epithelial cells to provide membrane material having integral proteins including said CFTR protein;

(b) solubilizing said integral proteins of said membrane material to form a solution of said integral proteins;

(c) separating said CFTR protein to remove any remaining other proteins of mammalian origin.

According to another aspect of the invention, a method is provided for screening a subject to determine if the subject is a CF carrier or a CF patient comprising the steps of providing a biological sample of the subject to be screened and providing an assay for detecting in the biological sample, the presence of at least a member from the group consisting of the normal CF gene, normal CF gene products, a mutant CF gene, mutant CF gene products and mixtures thereof.

According to another aspect of the invention, an immunologically active anti-CFTR polyclonal or monoclonal antibody specific for CFTR polypeptide is provided.

According to another aspect of the invention, a kit for assaying for the presence of a CF gene by immunoassay techniques comprises:

(a) an antibody which specifically binds to a gene product of the CF gene;

(b) reagent means for detecting the binding of the antibody to the gene product; and

(c) the antibody and reagent means each being present in amounts effective to perform the immunoassay.

According to another aspect of the invention, a kit for assaying for the presence of a CF gene by hybridization technique comprises:

(a) an oligonucleotide probe which specifically binds to the CF gene;

(b) reagent means for detecting the hybridization of the oligonucleotide probe to the CF gene; and

(c) the probe and reagent means each being present in amounts effective to perform the hybridization assay.

According to another aspect of the invention, a method is provided for treatment for cystic fibrosis in a patient. The treatment comprises the step of administering to the patient a therapeutically effective amount of the normal CFTM protein.

According to another aspect of the invention, a method of gene therapy for cystic fibrosis comprises the step of delivery of a DNA molecule which includes a sequence corresponding to the normal DNA sequence encoding for normal CFTR protein.

According to another aspect of the invention, an animal comprises an heterologous cell system. The cell system includes a recombinant cloning vector which includes the recombinant DNA sequence corresponding to the mutant DNA sequence which induces cystic fibrosis symptoms in the animal.

According to another aspect of the invention, a transgenic mouse exhibits cystic fibrosis symptoms.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1A–1H** is the nucleotide sequence of the CF gene and the amino acid sequence of the CFTR protein.

FIGS. **2A–2G** is a restriction map of the CF gene and the schematic strategy used to chromosome walk and jump to the gene.

FIGS. **3A–3E** is a pulsed-field-gel electrophoresis map of the region including and surrounding the CF gene.

FIGS. **4A**, **4B** and **4C** show the detection of conserved nucleotide sequences by cross-species hybridization.

FIG. **4D** is a restriction map of overlapping segments of probes E4.3 and H1.6.

FIG. **5** is an RNA blot hybridization analysis, using genomic and cDNA probes. Hybridization to fibroblast, trachea (normal and CF), pancreas, liver, HL60, T84, and brain RNA is shown.

FIG. **6** is the methylation status of the E4.3 cloned region at the 5' end of the CF gene.

FIGS. **7A–7B** is a restriction map of the CFTR cDNA showing alignment of the cDNA to the genomic DNA fragments.

FIG. **8** is an RNA gel blot analysis depicting hybridization by a portion of the CFTR cDNA (clone 10-1) to a 6.5 kb mRNA transcript in various human tissues.

FIGS. **9A–9D** are a DNA blot hybridization analysis depicting hybridization by the CFTR cDNA clones to genomic DNA digested with EcoRI and Hind III.

FIGS. **10A–10C** are a primer extension experiment characterizing the 5' and 3' ends of the CFTR cDNA.

FIG. **11** is a hydropathy profile and shows predicted secondary structures of CFTR.

FIG. **12** is a dot matrix analysis of internal homologies in the predicted CFTR polypeptide.

FIG. **13** is a schematic model of the predicted CFTR protein.

FIG. **14** is a schematic diagram of the restriction fragment length polymorphisms (RFLP's) closely linked to the CF gene where the inverted triangle indicates the location of the F508 3 base pair deletion.

FIG. **15** represents the detection of the F508 mutation by oligonucleotide hybridization with Probe N detecting the normal sequence and Probe F detecting the CF mutant sequence.

FIGS. **16A–16B** represent alignment of the most conserved segments of the extended NBFs of CFTR with comparable regions of other proteins.

FIGS. **17A–17B** are the DNA sequence around the F508 deletion.

FIGS. **18A–18B** are a representation of the nucleotide sequencing gel showing the DNA sequence at the F508 deletion.

FIGS. **19A** and **19B** are Coomassie Blue stained polyacrylamide gels following electrophoresis of protein from bacterial lysates (JX 101) which bacteria was transformed with the pGEX plasmids.

FIG. **20** is immunoblots of bacterial lysates containing fusion protein #1 (on Table 8) with preimmune and immune sera from two different rabbits.

FIG. **21** is an immunoblot of T-84 membranes using immune serum from rabbit #1 of FIG. **20**.

FIG. **22** is immunodot blots probed with preimmune and immune sera from a rabbit immunized with the KLH conjugate of peptide #2 of Table 8.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

1. Definitions

In order to facilitate review of the various embodiments of the invention and an understanding of various elements and constituents used in making the invention and using same, the following definition of terms used in the invention description is as follows:

CF—cystic fibrosis

CF carrier—a person in apparent health whose chromosomes contain a mutant CF gene that may be transmitted to that person's offspring.

CF patient—a person who carries a mutant CF gene on each chromosome, such that they exhibit the clinical symptoms of cystic fibrosis.

CF gene—the gene whose mutant forms are associated with the disease cystic fibrosis. This definition is understood to include the various sequence polymorphisms that exist, wherein nucleotide substitutions in the gene sequence do not affect the essential function of the gene product. This term primarily relates to an isolated coding sequence, but can also include some or all of the flanking regulatory elements and/or introns.

CF—PI—cystic fibrosis pancreatic insufficient, the major clinical subgroup of cystic fibrosis patients, characterized by insufficient pancreatic exocrine function.

CF—PS—cystic fibrosis pancreatic sufficient, a clinical subgroup of cystic fibrosis patients with sufficient pancreatic exocrine function for normal digestion of food.

CF—cystic fibrosis transmembrane conductance regulator protein, encoded by the CF gene. This definition includes the protein as isolated from human or animal sources, as produced by recombinant organisms, and as chemically or enzymatically synthesized. This definition is understood to include the various polymorphic forms of the protein wherein amino acid substitutions in the variable regions of the sequence does not affect the essential functioning of the protein, or its hydropathic profile or secondary or tertiary structure.

DNA—standard nomenclature is used to identify the bases.

Intronless DNA—a piece of DNA lacking internal noncoding segments, for example, cDNA.

IRP locus sequence—(protooncogene int-1 related), a gene located near the CF gene.

Mutant CFTR—a protein that is highly analagous to CFTR in terms of primary, secondary, and tertiary structure, but wherein a small numer of amino acid substitutions and/or deletions and/or insertions result in impairment of its essential function, so that organisms whose epithelial cells express mutant CFTR rather than CFTR demonstrate the symptoms of cystic fibrosis.

mCF—a mouse gene orthologous to the human CF gene

NBFs—nucleotide (ATF) binding folds

ORF—open reading frame

PCR—polymerase chain reaction

Protein—standard single letter nomenclature is used to identify the amino acids

R-domain—a highly charged cytoplasmic domain of the CFTR protein

RSV—Rous Sarcoma Virus

SAP—surfactant protein

RFLP—restriction fragment length polymorphism

2. Isolating the CF Gene

Using chromosome walking, jumping, and cDNA hybridization, DNA sequences encompassing >500 kilobase pairs (kb) have been isolated from a region on the long arm of human chromosome 7 containing the cystic fibrosis (CF) gene. Several transcribed sequences and conserved segments have been identified in this region. one of these corresponds to the CF gene and spans approximately 250 kb of genomic DNA. Overlapping complementary DNA (cDNA) clones have been isolated from epithelial cell libraries with a genomic DNA segment containing a portion of the cystic fibrosis gene. The nucleotide sequence of the isolated cDNA is shown in FIGS. **1A–1H**. In each row of the respective sequences the lower row is a list by standard nomenclature of the nucleotide sequence. The upper row in each respective row of sequences is standard single letter nomenclature for the amino acid corresponding to the respective codon.

Accordingly, the invention provides a cDNA molecule comprising a DNA sequence selected from the group consisting of:

(a) DNA sequences which correspond to the DNA sequence of FIGS. **1A–1H** from amino acid residue position 1 to position 1480;

(b) DNA sequences encoding normal CFTR polypeptide having the sequence according to FIGS. **1A–1H** for amino acid residue positions from 1 to 1480;

(c) DNA sequences which correspond to a fragment of the sequence of FIGS. **1A–1H** including at least 16 sequential nucleotides between amino acid residue positions 1 and 1480;

(d) DNA sequences which comprise at least 16 nucleotides and encode a fragement of the amino acid sequence of FIGS. **1A–1H**; and

(e) DNA sequences encoding an epitope encoded by at least 18 sequential nucleotides in the sequence of FIGS. **1A–1H** between amino acid residue positions 1 and 1480.

The invention also provides a cDNA molecule comprising a DNA sequence selected from the group consisting of:

a) DNA sequences which correspond to the DNA sequence encoding mutant CFTR polypeptide characterized by cystic fibrosis-associated activity in human epithelial cells, or the DNA sequence of FIGS. **1A–1H** for the amino acid residue positions 1 to 1480 yet further characterized by a three base pair mutation which results in the deletion of phenylalanine from amino acid residue position 508;

b) DNA sequences which correspond to fragments of the sequences of paragraph a) and which include at least sixteen nucleotides;

c) DNA sequences which comprise at least sixteen nucleotides and encode a fragment of the amino acid sequence encoded for by the DNA sequences of paragraph a); and

d) DNA sequences encoding an epitope encoded by at least 18 sequential nucleotides in the sequence of the DNA of paragraph a).

Transcripts of approximately 6,500 nucleotides in size are detectable in tissues affected in patients with CF. Based upon the isolated nucleotide sequence, the predicted protein consists of two similar regions, each containing a first domain having properties consistent with membrane association and a second domain believed to be involved in ATP binding.

A 3 bp deletion which results in the omission of a phenylalanine residue at the center of the first predicted nucleotide binding domain (amino acid position 508 of the CF gene product) has been detected in CF patients. This mutation in the normal DNA sequence of FIGS. **1A–1H** corresponds to approximately 70% of the mutations in cystic fibrosis patients. Extended haplotype data based on DNA markers closely linked to the putative disease gene suggest that the remainder of the CF mutant gene pool consists of multiple, different mutations. A small set of these latter mutant alleles (approximately 8%) may confer residual pancreatic exocrine function in a subgroup of patients who are pancreatic sufficient.

2.1 Chromosome Walking and Jumping

Large amounts of the DNA surrounding the D7S122 and D75340 linkage regions of Rommens et al supra were searched for candidate gene sequences. In addition to conventional chromosome walking methods, chromosome jumping techniques were employed to accelerate the search process. From each jump endpoint a new bidirectional walk could be initiated. Sequential walks halted by "unclonable" regions often encountered in the mammalian genome could be circumvented by chromosome jumping.

The chromosome jumping library used has been described previously (Collins et al, *Science* 235, 1046 (1987); Ianuzzi at al, *Am. J. Hum. Genet.* 44, 695 (1989)). The original library was prepared from a preparative pulsed field gel, and was intended to contain partial EcoR1 fragments of 70–130 kb; subsequent experience with this library indicates that smaller fragments were also represented, and jumpsizes of 25–110 kb have been found. The library was plated on sup^- host MC1061 and screened by standard techniques, (Maniatis et al). Positive clones were subcloned into pBRΔ23Ava and the beginning and end of the jump identified by EcoR1 and Ava 1 digestion, as described in Collins, *Genome analysis: A practical approach* (IRL, London, 1988), pp. 73–94. For each clone, a fragment from the end of the jump was checked to confirm its location on chromosome 7. The contiguous chromosome region covered by chromosome walking and jumping was about 250 kb. Direction of the jumps was biased by careful choice of probes, as described by Collins et al and Ianuzzi et al, supra. The entire region cloned, including the sequences isolated with the use of the CF gene cDNA, is approximately 500 kb.

The schematic representation of the chromosome walking and jumping strategy is illustrated in FIGS. **2A–2G**. CF gene exons are indicated by Roman numerals in these figures. Horizontal lines above the map indicate walk steps whereas the arcs above the map indicate jump steps. The Figure proceeds from left to right in each of six tiers with the direction of ends toward 7cen and 7qter as indicated. The restriction map for the enzymes EcoRI, HindIII, and BamHI is shown above the solid line, spanning the entire cloned region. Restriction sites indicated with arrows rather than vertical lines indicate sites which have not been unequivocally positioned. Additional restriction sites for other enzymes are shown below the line. Gaps in the cloned region are indicated by ||. These occur only in the portion detected by cDNA clones of the CF transcript. These gaps are unlikely to be large based on pulsed field mapping of the region. The walking clones, as indicated by horizontal arrows above the map, have the direction of the arrow indicating the walking progress obtained with each clone. Cosmid clones begin with the letter c; all other clones are phage. Cosmid CF26 proved to be a chimera; the dashed portion is derived from a different genomic fragment on another chromosome. Roman numerals I through XXIV indicate the location of exons of the CF gene. The horizontal boxes shown above the line are probes used during the experiments. Three of the probes represent independent subcloning of fragments previously identified to detect polymorphisms in this region: H2.3A corresponds to probe XV2C (X. Estivill et al, *Nature,* 326: 840 (1987)), probe E1 corresponds to KM19 (Estivill, supra), and probe E4.1 corresponds to Mp6d.9 (X. Estivill et al. *Am. J. Hum. Genet.* 44, 704 (1989)). G-2 is a subfragment of E6 which detects a transcribed sequence. R161, R159, and R160 are synthetic oligonucleotides constructed from parts of the IRP locus sequence (B. J. Wainwright et al, *EMBO J.,* 7: 1743 (1988)), indicating the location of this transcript on the genomic map.

As the two independently isolated DNA markers, D7S122 (pH131) and D7S340 (TM58), were only approximately 10 kb apart (FIGS. **2A–2G**), the walks and jumps were essentially initiated from a single point. The direction of walking and jumping with respect to MET and D7S8 was then established with the crossing of several rare-cutting restriction endonuclease recognition sites (such as those for Xho I, Nru I and Not I, see FIGS. **2A–2G**) and with reference to the long range physical map of J. M. Rommens et al. *Am. J. Hum. Genet.,* in press; A. M. Poustka, et al, *Genomics* 2, 337 (1988); M. L. Drumm et al. *Genomics* 2, 346 (1988). The pulsed field mapping data also revealed that the Not I site identified by the inventors of the present invention (see FIG. **2B**, position 113 kb) corresponded to the one previously found associated with the IRP locus (Estivill et al 1987, supra). Since subsequent genetic studies showed that CF was most likely located between IRP and D7S8 (M. Farrall et al, *Am. J. Hum. Genet.* 43, 471 (1988), B.-S. Kerem et al. *Am. J. Hum. Genet.* 44, 827 (1989)), the walking and

jumping effort was continued exclusively towards cloning of this interval. It is appreciated, however, that other coding regions, as identified in FIGS. **2**A–**2**G, for example, G-2, CF14 and CF16, were located and extensively investigated. Such extensive investigations of these other regions revealed that they were not the CF gene based on genetic data and sequence analysis. Given the lack of knowledge of the location of the CF gene and its characteristics, the extensive and time consuming examination of the nearby presumptive coding regions did not advance the direction of search for the CF gene. However, these investigations were necessary in order to rule out the possibility of the CF gene being in those regions.

Three regions in the 280 kb segment were found not to be readily recoverable in the amplified genomic libraries initially used. These less clonable regions were located near the DNA segments H2.3A and X.6, and just beyond cosmid cW44, at positions 75–100 kb, 205–225 kb, and 275–285 kb in FIGS. **2**A–**2**G, respectively. The recombinant clones near H2.3A were found to be very unstable with dramatic rearrangements after only a few passages of bacterial culture. To fill in the resulting gaps, primary walking libraries were constructed using special host-vector systems which have been reported to allow propagation of unstable sequences (A. R. Wyman, L. B. Wolfe, D. Botstein, *Proc. Nat. Acad. Sci. U.S.A.* 82, 2880 (1985); K. F. Wertman, A. R. Wyman, D. Botstein, *Gene* 49, 253 (1986); A. R. Wyman, K. F. Wertman, D. Barker, C. Helms, W. H. Petri, *Gene,* 49, 263 (1986)). Although the region near cosmid cW44 remains to be recovered, the region near X.6 was successfully rescued with these libraries.

2.2 Construction of Genomic Libraries

Genomic libraries were constructed after procedures described in MANIATIS, et al, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982) and are listed in Table 1. This includes eight phage libraries, one of which was provided by T. Maniatis (Fritsch et al, *Cell,* 19:959 (1980)); the rest were constructed as part of this work according to procedures described in Maniatis et al, supra. Four phage libraries were cloned in λDASH (commercially available from Stratagene) and three in λFIX (commercially available from Stratagene), with vector arms provided by the manufacturer. One λDASH library was constructed from Sau 3A-partially digested DNA from a human-hamster hybrid containing human chromosome 7 (4AF/102/K015) (Rommens et al *Am. J. Hum. Genet* 43, 4 (1988)), and other libraries from partial Sau3A, total BamHI, or total EcoRI digestion of human peripheral blood or lymphoblastoid DNA. To avoid loss of unstable sequences, five of the phage libraries were propagated on the recombination-deficient hosts DB1316 ($recD^-$), CES 200 ($recBC^-$) (Wyman et al, supra, Wertman et al supra, Wyman et al supra); or TAP90 (Patterson et al *Nucleic Acids Res.* 15:6298 (1987)). Three cosmid libraries were then constructed. In one the vector pCV108, (Lau et al *Proc. Natl. Acad. Sci USA* 80:5225 (1983)) was used to clone partially digested (Sau 3A) DNA from 4AF/102/K015 (Rommens et al *Am.J. Hum. Genet.* 43:4 (1968)). A second cosmid library was prepared by cloning partially digested (Mbo I) human lymphoblastoid DNA into the vector pWE-IL2R, prepared by inserting the RSV (Rous Sarcoma Virus) promoter-driven cDNA for the interleukin-2 receptor α-chain (supplied by M. Fordis and B. Howard) in place of the neo-resistance gene of pWE15 (Wahl et al *Proc. Natl. Acad. Sci. USA* 84:2160 (1987)). An additional partial Mbo I cosmid library was prepared in the vector pWE-IL2-Sal, created by inserting a Sal I linker into the Bam HI cloning site of pWE-EL2R (M. Drumm, unpublished data); this allows the use at the partial fill-in technique to ligate Sal I and Mbo I ends, preventing tandem insertions (Zabarovsky et al *Gene* 42:19 (1986)). Cosmid libraries were propagated in *E. coli* host strains DH1 or 490A (M. Steinmetz, A. Winoto, K. Minard, L. Hood, *Cell* 28, 489(1982)).

TABLE 1

GENOMIC LIBRARIES

Vector	Source of human DNA	Host	Complexity	Ref
λ Charon 4A	HaeII/AluI-partially digested total human liver DNA	LE392	1×10^6 (amplified)	Lawn et al 1980
pCV108	Sau3a-partially digested DNA from 4AF/KO15	DK1	3×10^6 (amplified)	
λdash	Sau3a-partially digested DNA from 4AF/KO15	LE392	1×10^6 (amplified)	
λdash	Sau3a-partially digested total human peripheral blood DNA	DB1316	1.5×10^6	
λdash	BamHI-digested total human peripheral blood DNA	DB1316	1.5×10^6	
λdash	EcoRI-partially digested total human peripheral blood DNA	DB1316	8×10^6	
λFIX	MboI-partially digested human lymphoblastoid DNA	LE392	1.5×10^6	
λFIX	MboI-partially digested human lymphoblastoid DNA	CE200	1.2×10^6	
λFIX	MboI-partially digested human lymphoblastoid DNA	TAP90	1.3×10^6	
pWE-IL2R	MboI-partially digested human lymphoblastoid DNA	490A	5×10^5	
pWE-IL2R-Sal	MboI-partially digested human lymphoblastoid DNA	490A	1.2×10^6	
λCh3A Δlac (jumping)	EcoRI-partially digested (24-110 kb) human lymphoblastoid DNA	MC1061	3×10^6	Collins et al supra and Iannuzzi et al supra

Three of the phage libraries wore propagated and amplified in *E. coli* bacterial strain LE392. Four subsequent libraries were plated on the recombination-deficient hosts DB1316 ($recD^-$) or CES200 ($rec BC^-$) (Wyman 1985, supra; Wertman 1986, supra; and Wyman 1986, supra) or in one case TAP90 (T. A. Patterson and M. Dean, *Nucleic Acids Research* 15, 6298 (1987)).

Single copy DNA segments (free of repetitive elements) near the ends of each phage or cosmid insert were purified and used as probes for library screening to isolate overlapping DNA fragments by standard procedures. (Maniatis, et al, supra).

$1–2 \times 10^6$ phage clones were plated on 25–30 150 mm petri dishes with the appropriate indicator bacterial host and incubated at 37° C. for 10–16 hr. Duplicate "lifts" were prepared for each plate with nitrocellulose or nylon membranes, prehybridized and hybridized under conditions described (Rommens et al, 1988, supra). Probes were labelled with ^{32}P to a specific activity of $>5 \times 10^8$ cpm/μg using the random priming procedure (A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* 132, 6 (1983)). The cosmid library was spread on ampicillin-containing plates and screened in a similar manner.

DNA probes which gave high background signals could often be used more successfully by preannealing the boiled probe with 250 μg/ml sheared denatured placental DNA for 60 minutes prior to adding the probe to the hybridization bag.

For each walk step, the identity of the cloned DNA fragment was determined by hybridization with a somatic cell hybrid panel to confirm its chromosomal location, and by restriction mapping and southern blot analysis to confirm its colinearity with the genome.

The total combined cloned region of the genomic DNA sequences was isolated and the overlapping cDNA clones, extended >500 kb. To ensure that the DNA segments isolated by the chromosome walking and jumping procedures were colinear with the genomic sequence, each segment was examined by:

(a) hybridization analysis with human-rodent somatic hybrid cell lines to confirm chromosome 7 localization,

(b) pulsed field gel electrophoresis, and

(c) comparison of the restriction map of the cloned DNA to that of the genomic DNA.

Accordingly, single copy human DNA sequences were isolated from each recombinant phage and cosmid clone and used as probes in each of these hybridization analyses as performed by the procedure of Maniatis, et al supra.

While the majority of phage and cosmid isolates represented correct walk and jump clones, a few resulted from cloning artifacts or cross-hybridizing sequences from other regions in the human genome, or from the hamster genome in cases where the libraries were derived from a human-hamster hybrid cell line. Confirmation of correct localization was particularly important for clones isolated by chromosome jumping. Many jump clones were considered and resulted in non-conclusive information leading the direction of investigation away from the gene.

2.3 Confirmation of the Restriction Map

Further confirmation of the overall physical map of the overlapping clones was obtained by long range restriction mapping analysis with the use of pulsed field gel electrophoresis (J. M. Rommens, et al. *Am. J. Hum. Genet,* in press, A. M. Poustka et al, 1988, supra M. L. Drumm et al, 1988 supra).

FIGS. **3**A to **3**E illustrate the findings of the long range restriction mapping study, where a schematic representation of the region is given in FIG. **3**E. DNA from the human-hamster cell line 4AF/102/K015 was digested with the enzymes (A) Sa1 I, (B) Xho I, (C) Sfi I and (D) Nae I, separated by pulsed field gel electrophoresis, and transferred to Zetaprobe™ (BioRad). For each enzyme a single blot was sequentially hybridized with the probes indicated below each of the panels of FIGS. **3**A–**3**D, with stripping of the blot between hybridizations. The symbols for each enzyme of FIG. **3**E are: A, Nae I; B, Bss HII; F. Sfi I; L, Sa1 I; M, M1u I; N, Not I; R, Nru I; and X, Xho l. C corresponds to the compression zone region of the gel. DNA preparations, restriction digestion, and crossed field gel electrophoresis methods have been described (Rommens et al, in press, supra). The gels in FIGS. **3**A–**3**D were run in 0.5×TBE at 7 volts/cm for 20 hours with switching linearly ramped from 10–40 seconds for FIGS. **3**A–**3**C, and at 8 volts/cm for 20 hours with switching ramped linearly from 50–150 seconds for FIG. **3**D, Schematic interpretations of the hybridization pattern are given below each panel. Fragment lengths are in kilobases and were sized by comparison to oligomerized bacteriophage λDNA and *Saccharomyces cerevisiae* chromosomes.

H4.0, J44, EG1.4 are genomic probes generated from the walking and jumping experiments (see FIGS. **2**A–**2**G). J30 has been isolated by four consecutive jumps from D7S8 (Collins et al. 1987, supra; Ianuzzi et al, 1989, supra; M. Dean, et al, submitted for publication). 10-1, B.75, and CE1.5/1.0 are cDNA probes which cover different regions of the CF transcript: 10-1 contains exons I–VI, B.75 contains exons V–XII, and CE1.5/1.0 contains exons XII–XXIV. Shown in FIG. **3**E is a composite map of the entire MET-D7S8 interval. The boxed region indicates the segment cloned by walking and jumping, and the slashed portion indicates the region covered by the CF transcript. The CpG-rich region associated with the D7S23 locus (Estivill et al, 1987, supra) is at the Not I site shown in parentheses. This and other sites shown in parentheses or square brackets do not cut in 4AF/102/K015, but have been observed in human lymphoblast cell lines.

2.4 Identification of CF Gene

Based on the findings of long range restriction mapping detailed above it was determined that the entire CF gene is contained on a 380 kb Sal I fragment. Alignment of the restriction sites derived from pulsed field gel analysis to those identified in the partially overlapping genomic DNA clones revealed that the size of the CF gene was approximately 250 kb.

The most informative restriction enzyme that served to align the map of the cloned DNA fragments and the long range restriction map was Xho I; all or the 9 Xho 1 sites identified with the recombinant DNA clones appeared to be susceptible to at least partial cleavage in genomic DNA (compare maps in FIGS. **1**A–**1**H and **2**A–**2**G). Furthermore, hybridization analysis with probes derived from the 3' end of the CF gene identified 2 SfiI sites and confirmed the position of an anticipated Nae I site.

These findings further supported the conclusion that the DNA segments isolated by the chromosome walking and jumping procedures were colinear with the genuine sequence.

2.5 Criteria for Identification

A positive result based on one or more of the following criteria suggested that a cloned DNA segment may contain candidate gene sequences:

(a) detection of cross-hybridizing sequences in other species (as many genes show evolutionary conservation),

(b) identification of CpG islands, which often mark the 5' end of vertebrate genes (A. P. Bird, *Nature,* 321, 209 (1986); M. Gardiner-Garden and M. Frommer, *J. Mol. Biol.* 196, 261 (1987)),

(c) examination of possible mRNA transcripts in tissues affected in CF patients,

(d) isolation of corresponding cDNA sequences,

(e) identification of open reading frames by direct sequencing of cloned DNA segments.

Cross-species hybridization showed strong sequence conservation between human and bovine DNA when CF14, E4.3 and H1.6 were used as probes, the results of which are shown in FIGS. **4**A, **4**B and **4**C.

Human, bovine, mouse, hamster, and chicken genomic DNAs were digested with Eco RI (R), Hind III (H), and Pst I (P), electrophoresed, and blotted to Zetabind™ (BioRad). The hybridization procedures of Rommens et al, 1988, supra, were used with the most stringent wash at 55° C.,

0.2×SSC, and 0.1% SDS. The probes used for hybridization, in FIGS. **4A–4C**, included: (A) entire cosmid CF14, (B) E4.3, (C) H1.6. In the schematic of FIG. **4D**, the shaded region indicates the area of cross-species conservation.

The fact that different subsets of bands were detected in bovine DNA with these two overlapping DNA segments (H1.6 and E4.3) suggested that the conserved sequences were located at the boundaries of the overlapped region (FIG. **4D**). When these DNA segments were used to detect RNA transcripts from a variety of tissues, no hybridization signal was detected. In an attempt to understand the cross-hybridizing region and to identify possible open reading frames, the DNA sequences of the entire H1.6 and part of the E4.3 fragment were determined. The results showed that, except for a long stretch of CG-rich sequence containing the recognition sites for two restriction enzymes (Bss HII and Sac II), often found associated with undermethylated CpG islands, there were only short open reading frames which could not easily explain the strong cross-species hybridization signals.

To examine the methylation status of this highly CpG-rich region revealed by sequencing, genomic DNA samples prepared from fibroblasts and lymphoblasts were digested with the restriction enzymes Hpa II and Msp I and analyzed by gel blot hybridization. The enzyme Hpa II cuts the DNA sequence 5'-CCGG-3' only when the second cytosine is unmethylated, whereas Msp I cuts this sequence regardless of the state of methylation. Small DNA fragments were generated by both enzymes, indicating that this CpG-rich region is indeed undermethylated in genomic DNA. The gel-blot hybridization with the E4.3 segment (FIG. **6**) reveals very small hybridizing fragments with both enzymes, indicating the presence of a hypomethylated CpG island.

The above results strongly suggest the presence of a coding region at this locus. Two DNA segments (E4.3 and H1.6) which detected cross-species hybridization signals from this area were used as probes to screen cDNA libraries made from several tissues and cell types.

cDNA libraries from cultured epithelial cells were prepared as follows. Sweat gland cells derived from a non-CF individual and from a CF patient were grown to first passage as described (G. Collie et al, *In Vitro Cell. Dev. Biol.* 21, 592,1985). The presence of outwardly rectifying channels was confirmed in these cells (J. A. Tabcharani, T. J. Jensen, J. R. Riordan, J. W. Hanrahan, *J. Memb. Biol.,* in press) but the CF cells were insensitive to activation by cyclic AMP (T. J. Jensen, J. W. Hanrahan, J. A. Tabcharanit, M. Buchwald and J. R. Riordan, *Pediatric Pulmonology,* Supplement 2, 100, 1988). RNA was isolated from then by the method of J. M. Chirgwin et al (*Biochemistry* 18, 5294, 1979). Poly A+RNA was selected (H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. USA* 69, 1408, 1972) and used as template for the synthesis of cDNA with oligo (dT) 12–18 as a primer. The second strand was synthesized according to Gubler and Hoffman (*Gene* 25, 263, 1983). This was methylated with Eco RI methylase and ends were made flush with T4 DNA polymerase. Phosphorylated Eco RI linkers were ligated to the cDNA and restricted with Eco RI. Removal of excess linkers and partial size fractionation was achieved by Biogel A-50 chromatography. The cDNAs were then ligated into the Eco RI site of the commercialy available lamdba ZAP. Recombinants were packaged and propagated in *E. coli* BB4. Portions of the packaging mixes were amplified and the remainder retained for screening prior to amplification. The same procedures were used to construct a library from RNA isolated from preconfluent cultures of the T-84 colonic carcinoma cell line (Dharmsathaphorn, K. et al. *Am. J. Physiol.* 246, G204,1984). The numbers of independent recombinants in the three libraries were: 2×10^6 for the non-CF sweat gland cells, 4.5×10^6 for the CF sweat gland cells and 3.2×10^6 from T-84 cells. These phages were plated at 50,000 per 15 cm plate and plaque lifts made using nylon membranes (Biodyne) and probed with DNA fragments labelled with ^{32}P using DNA polymerase I and a random mixture of oligonucleotides as primer. Hybridization conditions were according to G. M. Wahl and S. L. Berger (*Meth. Enzymol.* 152,415, 1987). Bluescript™ plasmids were rescued from plaque purified clones by excision with M13 helper phage. The lung and pancreas libraries were purchased from Clontech Lab Inc. with reported sizes of 1.4×10^6 and 1.7×10^6 independent clones.

After screening 7 different libraries each containing 1×10^5–5×10^6 independent clones, 1 single clone (identified as 10-1) was isolated with H1.6 from a cDNA library made from the cultured sweat gland epithelial cells of an unaffected (non-CF) individual.

DNA sequencing analysis showed that 10-1 contained an insert of 920 bp in size and one potential, long open reading frame (ORF). Since one end of the sequence shared perfect sequence identity with H1.6, it was concluded that the cDNA clone was probably derived from this region. The DNA sequence in common was, however, only 113 bp long (see FIGS. **1A–1H** and **7A–7B**). As detailed below, this sequence in fact corresponded to the 5'-most exon of the putative CF gene. The short sequence overlap thus explained the weak hybridization signals in library screening and inability to detect transcripts in RNA gel-blot analysis. Tn addition, the orientation of the transcription unit was tentatively established on the basis of alignment of the genomic DNA sequence with the presumptive ORF of 10-1.

Since the corresponding transcript was estimated to be approximately 6500 nucleotides in length by RNA gel-blot hybridization experiments, further cDNA library screening was required in order to clone the remainder of the coding region. As a result of several successive screenings with cDNA libraries generated from the colonic carcinoma cell line T84, normal and CF sweat gland cells, pancreas and adult lungs, 18 additional clones were isolated (FIGS. **7A–7B**, as subsequently discussed in greater detail). DNA sequence analysis revealed that none of these cDNA clones corresponded to the length of the observed transcript, but it was possible to derive a consensus sequence based on overlapping regions. Additional cDNA clones corresponding to the 5' and 3' ends of the transcript were derived from 5' and 3' primer-extension experiments. Together, these clones span a total of about 6.1 kb and contain an ORF capable of encoding a polypeptide of 1480 amino acid residues (FIGS. **1A–1H**).

It was unusual to observe that most of the cDNA clones isolated here contained sequence insertions at various locations of the restriction map of FIGS. **7A–7B**. The map details the genomic structure of the CF gene. Exon/intron boundaries are given where all cDNA clones isolated are schematically represented on the upper half of the figure. Many of these extra sequences clearly corresponded to intron regions reversely transcribed during the construction of the cDNA, as revealed upon alignment with genomic DNA sequences.

Since the number of recombinant cDNA clones for the CF gene detected in the library screening was much less than would have been expected from the abundance of transcript estimated from RNA hybridization experiments, it seemed probable that the clones that contained aberrant structures

were preferentially retained while the proper clones were lost during propagation. Consistent with this interpretation, poor growth was observed for the majority of the recombinant clones isolated in this study, regardless of the vector used.

The procedures used to obtain the 5' and 3' ends of the cDNA were similar to those described (M. Frohman et al, *Proc. Nat. Acad. Sci,* USA, 85, 8998–9002, 1988). For the 5' end clones, total pancreas and T84 poly A+RNA samples were reverse transcribed using a primer, (10b), which is specific to exon 2 similarly as has been described for the primer extension reaction except that radioactive tracer was included in the reaction. The fractions collected from an agarose bead column of the first strand synthesis were assayed by polymerase chain reaction (PCR) of eluted fractions. The oligonucleotides used were within the 10-1 sequence (145 nucleotides apart) just 5' of the extension primer. The earliest fractions yielding PCR product were pooled and concentrated by evaporation and subsequently tailed with terminal deoxynucleotidyl transferase (BRL Labs.) and dATP as recommended by the supplier (BRL Labs). A second strand synthesis was then carried out with Taq Polymerase (Cetus, AmpliTaq™) using an oligonucleotide containing a tailed linker sequence 5'CGGAATTCTCGAGATC$(T)_{12}$3' (SEQ ID NO:1).

Amplification by an anchored (PCR) experiment using the linker sequence and a primer just internal to the extension primer which possessed the Eco RI restriction site at its 5' end was then carried out. Following restriction with the enzymes Eco RI and Bgl II and agarose gel purification size selected products were cloned into the plasmid Bluescript KS available from Stratagene by standard procedures (Maniatis et al, supra). Essentially all of the recovered clones contained inserts of less than 350 nucleotides. To obtain the 3' end clones, first strand cDNA was prepared with reverse transcription of 2 μg T84 poly A+RNA using the tailed linker oligonucleotide previously described with conditions similar to those of the primer extension. Amplification by PCR was then carried out with the linker oligonucleotide and three different oligonucleotides corresponding to known sequences of clone T16-4.5. A preparative scale reaction (2×100 ul) was carried out with one of these oligonucleotides with the sequence 5'ATGAAGTCCAAGGATTTAG3'(SEQ ID NO:2).

This oligonucleotide is approximately 70 nucleotides upstream of a Hind III site within the known sequence of T16-4.5. Restriction of the PCR product with Hind III and Xho 1 was followed by agarose gel purification to size select a band at 1.0–1.4 kb. This product was then cloned into the plasmid Bluescript KS available from Stratagene. Approximately 20% of the obtained clones hybridized to the 3' end portion of T16-4.5. 10/10 of plasmids isolated from these clones had identical restriction maps with insert sizes of approx. 1.2 kb. All of the PCR reactions were carried out for 30 cycles in buffer suggested by an enzyme supplier.

An extension primer positioned 157 nt from the 5'end of 10-1 clone was used to identify the start point of the putative CF transcript. The primer was end labeled with $\gamma(^{32}P)$ATP at 5000 Curies/mmole and T4 polynucleotide kinase and purified by spun column gel filtration. The radiolabeled primer was then annealed with 4–5 ug poly A+RNA prepared from T-84 colonic carcinoma cells in 2× reverse transcriptase buffer for 2 hrs. at 60° C. Following dilution and addition of AMV reverse transcriptase (Life Sciences, Inc.) incubation at 41° C. proceeded for 1 hour. The sample was then adjusted to 0.4M NaOR and 20 mM EDTA, and finally neutralized, with NH_4OAc, pH 4.6, phenol extracted, ethanol precipitated, redissolved in buffer with formamide, and analyzed on a polyacrylamide sequencing gel. Details of these methods have been described (*Meth. Enzymol.* 152, 1987, Ed. S. L. Berger, A. R. Kimmel, Academic Press, N.Y.).

Results of the primer extension experiment using an extension oligonucleotide primer starting 157 nucleotides from the 5' end of 10-1 is shown in FIG. **10**A. End labeled φX174 bacteriophage digested with Hae III (BRL Labs) is used as size marker. Two major products are observed at 216 and 100 nucleotides. The sequence corresponding to 100 nucleotides in 10-1 corresponds to a very GC rich sequence (11/12) suggesting that this could be a reverse transcriptase pause site. The 5' anchored PCR results are shown in FIG. **10**B. The 1.4% agarose gel shown on the left was blotted and transferred to Zetaprobe™ membrane (Bio-Rad Lab). DNA gel blot hybridization with radiolabeled 10-1 is shown on the right. The 5' extension products are seen to vary in size from 170–280 nt with the major product at about 200 nucleotides. The PCR control lane shows a fragment of 145 nucleotides. It was obtained by using the test oligomers within the 10-1 sequence. The size markers shown correspond to sizes of 154, 220/210, 298, 344, 394 nucleotides (1 kb ladder purchased from BRL Lab).

The schematic shown below in FIG. **10**B outlines the procedure to obtain double stranded cDNA used for the amplification and cloning to generate the clones PA3-5 and TB2-7 shown in FIGS. **7**A–**7**B. The anchored PCR experiments to characterize the 3' end are shown in FIG. **10**C. As depicted in FIG. **10**C, three primers whose relative position to each other were known were used for amplification with reversed transcribed T84 RNA as described. These products were separated on a 1% agarose gel and blotted onto nylon membrane as described above. DNA-blot hybridization with the 3' portion of the T16-4.5 clone yielded bands of sizes that corresponded to the distance between the specific oligomer used and the 3'end of the transcript. These bands in lanes 1, 2a and 3 are shown schematically in FIG. **10**C. The band in lane 3 is weak as only 60 nucleotides of this segment overlaps with the probe used. Also indicated in the schematic and as shown in the lane 2b is the product generated by restriction of the anchored PCR product to facilitate cloning to generate the THZ-4 clone shown in FIGS. **7**A–**7**B.

DNA-blot hybridization analysis of genomic DNA digested with EcoRI and HindIII enzymes probed with portions of cDNAs spanning the entire transcript suggest that the gene contains at least 24 exons numbered as Roman numerals I through XX1V (see FIGS. **9**A–**9**D). These correspond to the numbers 1 through 24 shown in FIGS. **7**A–**7**B. The size of each band is given in kb.

In FIGS. **7**A–**7**B, open boxes indicate approximate positions of the 24 exons which have been identified by the isolation of >22 clones from the screening of cDNA libraries and from anchored PCR experiments designed to clone the 5' and 3' ends. The lengths in kb of the Eco RI genomic fragments detected by each exon is also indicated. The hatched boxes in FIGS. **7**A–**7**B indicate the presence of intron sequences and the stippled boxes indicate other sequences. Depicted in the lower left by the closed box is the relative position of the clone H1.6 used to detect the first cDNA clone 10-1 from among 10^6 phage of the normal sweat gland library. As shown in FIGS. **4**D and **7**A–**7**B, the genomic clone H1.6 partially overlaps with an EcoRI fragment of 4.3 kb. All or the cDNA clones shown were hybridized to genomic DNA and/or were fine restriction mapped. Examples of the restriction sites occurring within the cDNAs and in the corresponding genomic fragments are indicated.

With reference to FIGS. 9A–9D, the hybridization analysis includes probes; i.e., cDNA clones 10-1 for FIG. 9A, T16-1 (3' portion) for FIG. 9B, T16-4.5 (central portion) for FIG. 9C and T16-4.5 (3' end portion) for FIG. 9D. In panel A of FIG. 9A, the cDNA probe 10-1 detects the genomic bands for exons I through VI. The 3' portion of T16-1 generated by NruI restriction detects exons IV through XIII as shown in FIG. 9B. This probe partially overlaps with 10-1. FIGS. 9A–9C, respectively, show genomic bands detected by the central and 3' end EcoRI fragments of the clone T16-4.5. Two EcoRI sites occur within the cDNA sequence and split exons XIII and XIX. As indicated by the exons in parentheses, two genomic EcoRI bands correspond to each of these exons. Cross hybridization to other genomic fragments was observed. These bands, indicated by N, are not of chromosome 7 origin as they did not appear in human-hamster hybrids containing human chromosome 7. The faint band in FIG. 9D indicated by XI in brackets is believed to be caused by the cross-hybridization of sequences due to internal homology with the cDNA.

Since 10-1 detected a strong band on gel blot hybridization of RNA from the T-84 colonic carcinoma cell line, this cDNA was used to screen the library constructed from that source. Fifteen positives were obtained from which clones T6, T6/20, T11, T16-1 and T13-1 were purified and sequenced. Rescreening of the same library with a 0.75 kb Bam HI-Eco RI fragment from the 3' end of T16-1 yielded T16-4.5. A 1.8 kb EcoRI fragment from the 3 end of T16-4.5 yielded T8-B3 and T12a, the latter of which contained a polyadenylation signal and tail. Simultaneously a human lung cDNA library was screened; many clones were isolated including those shown here with the prefix 'CDL'. A pancreas library was also screened, yielding clone CDPJ5.

To obtain copies of this transcript from a CF patient, a cDNA library from RNA of sweat gland epithelial cells from a patient was screened with the 0.75 kb Bam HI-Eco RI fragment from the 3' end of T16-1 and clones C16-1 and C1-1/5, which covered all but exon 1, were isolated. These two clones both exhibit a 3 bp deletion in exon 10 which is not present in any other clone containing that exon. Several clones, including CDLS26-1 from the lung library and T6/20 and T13-1 isolated from T84 were derived from partially processed transcripts. This was confirmed by genomic hybridization and by sequencing across the exon-intron boundaries for each clone. T11 also contained additional sequence at each end. T16-4.5 contained a small insertion near the boundary between exons 10 and 11 that did not correspond to intron sequence. Clones CDLS16A, 11a and 13a from the lung library also contained extraneous sequences of unknown origin. The clone C16-1 also contained a short insertion corresponding to a portion of the γ-transposon of *E. coli;* this element was not detected in the other clones. The 5' clones PA3-5, generated from pancreas RNA and TB2-7 generated from T84 RNA using the anchored PCR technique have identical sequences except for a single nucleotide difference in length at the 5' end as shown in FIGS. 1A–1H. The 3' clone, THZ-4 obtained from T84 RNA contains the 3' sequence of the transcript in concordance with the genomic sequence of this region.

A combined sequence representing the presumptive coding region of the CF gene was generated from overlapping cDNA clones. Since most of the cDNA clones were apparently derived from unprocessed transcripts, further studies were performed to ensure the authenticity of the combined sequence. Each cDNA clone was first tested for localization to chromosome 7 by hybridization analysis with a human-hamster somatic cell hybrid containing a single human chromosome 7 and by pulsed field gel electrophoresis. Fine restriction enzyme mapping was also performed for each clone. While overlapping regions were clearly identifiable for most of the clones, many contained regions of unique restriction patterns.

To further characterize these cDNA clones, they were used as probes in gel hybridization experiments with EcoRI- or HindIII-digested human genomic DNA. As shown in FIGS. 9A–9D, five to six different restriction fragments could be detected with the 10-1 cDNA and a similar number of fragments with other cDNA clones, suggesting the presence of multiple exons for the putative CF gene. The hybridization studies also identified those cDNA clones with unprocessed intron sequences as they showed preferential hybridization to a subset of genomic DNA fragments. For the confirmed cDNA clones, their corresponding genomic DNA segments were isolated and the exons and exon/intron boundaries sequenced. As indicated in FIGS. 7A–7B, a total of 24 exons were identified. Based on this information and the results of physical mapping experiments, the gene locus was estimated to span 250 kb on chromosome 7.

2.6 The Sequence

FIGS. 1A–1H show the nucleotide sequence of the cloned cDNA encoding CFTR together with the deduced amino acid sequence. The first base position corresponds to the first nucleotide in the 5' extension clone PA3-5 which is one nucleotide longer than TB2-7. Arrows indicate position of transcription initiation site by primer extension analysis. Nucleotide 6129 is followed by a poly(dA) tract. Positions of exon junctions are indicated by vertical lines. Potential membrane-spanning segments were ascertained using the algorithm of Eisenberg et al *J. Mol. Biol.* 179:125 (1984). Potential membrane-spanning segments as analyzed and shown in FIG. 11 are enclosed in boxes of FIGS. 1A–1H. In FIG. 11, the mean hydropathy index (Kyte and Doolittle, *J. Molec. Biol.* 157: 105, (1982)) of 9 residue peptides is plotted against the amino acid number. The corresponding positions of features of secondary structure predicted according to Garnier et al, (J. Molec. Biol. 157, 165 (1982)) are indicated in the lower panel. Amino acids comprising putative ATP-binding folds are underlined in FIGS. 1A–1H. Possible sites of phosphorylation by protein kinases A (PKA) or C (PKC) are indicated by open and closed circles, respectively. The open triangle is over the 3 bp (CTT) which are deleted in CF (see discussion below). The cDNA clones in FIGS. 1A–1H were sequenced by the dideoxy chain termination method employing ^{35}S labelled nucleotides by the Dupont Genesis 2000™ automatic DNA sequencer.

The combined cDNA sequence spans 6129 base pairs excluding the poly(A) tail at the end of the 3' untranslated region and it contains an ORF capable of encoding a polypeptide of 1480 amino acids (FIGS. 1A–1H). An ATG (AUG) triplet is present at the beginning of this ORF (base position 133–135). Since the nucleotide sequence surrounding this codon (5'-AGACCAUGCA-3') has the proposed features of the consensus sequence (CC) A/GCCAUGG(G) of an eukaryotic translation initiation site with a highly conserved A at the −3 position, it is highly probable that this AUG corresponds to the first methionine codon for the putative polypeptide.

To obtain the sequence corresponding to the 5' end of the transcript, a primer-extension experiment was performed, as described earlier. As shown in FIG. 10A, a primer extension product of approximately 216 nucleotides could be observed suggesting that the 5' end of the transcript initiated approximately 60 nucleotides upstream of the end of cDNA clone

10-1. A modified polymerase chain reaction (anchored PCR) was then used to facilitate cloning of the 5' end sequences (FIG. **10**B). Two independent 5' extension clones, one from pancreas and the other from T84 RNA, were characterized by DNA sequencing and were found to differ by only 1 base in length, indicating the most probable initiation site for the transcript as shown in FIGS. **1**A–**1**H.

Since most of the initial cDNA clones did not contain a polyA tail indicative of the end of a mRNA, anchored PCR was also applied to the 3' end of the transcript (Frohman et al, 1988, supra). Three 3'-extension oligonucleotides were made to the terminal portion of the cDNA clone T16-4.5. As shown in FIG. **10**C, 3 PCR products of different sizes were obtained. All were consistent with the interpretation that the end of the transcript was approximately 1.2 kb downstream of the HindIII site at nucleotide position 5027 (see FIGS. **1**A–**1**H). The DNA sequence derived from representative clones was in agreement with that of the T84 cDNA clone T12a (See FIGS. **1**A–**1**H and **7**A–**7**B) and the sequence of the corresponding 2.3 kb EcoRI genomic fragment.

3.0 Molecular Genetics of CF

3.1 Sites of Expression

To visualize the transcript for the putative CF gene, RNA gel blot hybridization experiments were performed with the 10-1 cDNA as probe. The RNA hybridization results are shown in FIG. **8**.

RNA samples were prepared from tissue samples obtained from surgical pathology or at autopsy according to methods previously described (A. M. Kimmel, S. L. Berger, eds. *Meth. Enzymol.* 152, 1987). Formaldehyde gels were transferred onto nylon membranes (Zetaprobe™; BioRad Lab). The membranes were then hybridized with DNA probes labeled to high specific activity by the random priming method (A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* 132, 6, 1983) according to previously published procedures (J. Rommens et al, *Am. J. Hum. Genet.* 43, 645–663, 1988). FIG. **8** shows hybridization by the cDNA clone 10-1 to a 6.5 kb transcript in the tissues indicated. Total RNA (10 μg) of each tissue, and Poly A+ RNA (1 μg) of the T84 colonic carcinoma cell line were separated on a 1% formaldehyde gel. The positions of the 28S and 18S rRNA bands are indicated. Arrows indicate the position of transcripts. Sizing was established by comparison to standard RNA markers (BRL Labs). HL60 is a human promyelocytic leukemia cell line, and T84 is a human colon cancer cell line.

Analysis reveals a prominent band of approximately 6.5 kb in size in T84 cells. Similar, strong hybridization signals were also detected in pancreas and primary cultures of cells from nasal polyps, suggesting that the mature mRNA of the putative CF gene is approximately 6.5 kb. Minor hybridization signals, probably representing degradation products, were detected at the lower size ranges but they varied between different experiments. Identical results were obtained with other cDNA clones as probes. Based on the hybridization band intensity and comparison with those detected for other transcripts under identical experimental conditions, it was estimated that the putative CF transcripts constituted approximately 0.01% of total mRNA in T84 cells.

A number of other tissues were also surveyed by RNA gel blot hybridization analysis in an attempt to correlate the expression pattern of the 10-1 gene and the pathology of CF. As shown in FIG. **8**, transcripts, all of identical size, were found in lung, colon, sweat glands (cultured opithelial cells), placenta, liver, and parotid gland but the signal intensities in these tissues varied among different preparations and were generally weaker than that detected in the pancreas and nasal polyps. Intensity varied among different preparations, for example, hybridization in kidney was not detected in the preparation shown in FIG. **8**, but can be discerned in subsequent repeated assays. No hybridization signals could be discerned in the brain or adrenal gland (FIG. **8**), nor in skin fibroblast and lymphoblast cell lines.

In summary, expression of the CF gene appeared to occur in many of the tissues examined, with higher levels in those tissues severely affected in CF. While this epithelial tissue-specific expression pattern is in good agreement with the disease pathology, no significant difference has been detected in the amount or size of transcripts from CF and control tissues, consistent with the assumption that CF mutations are subtle changes at the nucleotide level.

3.2 The Major CF Mutation

FIGS. **17**A–**17**B show the DNA sequence at the F508 deletion. On the left, the reverse complement of the sequence from base position 1649–1664 of the normal sequence (as derived from the cDNA clone T16). The nucleotide sequence is displayed as the output (in arbitrary fluorescence intensity units, y-axis) plotted against time (x-axis) for each of the 2 photomultiplier tubes (PMT#1 and #2) of a DUPONT Genesis 2000™ DNA analysis system. The corresponding nucleotide sequence is shown underneath. On the right is the same region from a mutant sequence (as derived from the cDNA clone C16). Double-stranded plasmid DNA templates were prepared by the alkaline lysis procedure. Five μg of plasmid DNA and 75 ng of oligonucleotide primer were used in each sequencing reaction according to the protocol recommended by Dupont except that the annealing was done at 45° C. for 30 min and that the elongation/termination step was for 10 min at 42° C. The unincorporated fluorescent nucleotides were removed by precipitation of the DNA sequencing reaction product with ethanol in the presence of 2.5 M ammonium acetate at pH 7.0 and rinsed one time with 70% ethanol. The primer used for the T16-1 sequencing was a specific oligonucleotide 5'GTTGGCATGCTTTGATGACGCTTC3' (SEQ ID NO:3) spanning base position 1708-1731 and that for C16-1 was the universal primer SK for the Bluescript vector (Stratagene). FIGS. **18**A–**18**B also show the DNA sequence around the F508 deletion, as determined by manual sequencing. The normal sequence from base position 1726-1651 (from cDNA T16-1) is shown beside the CF sequence (from cDNA C16-1). The left panel shows the sequences from the coding strands obtained with the B primer (5'GTTTTCCTGGAT-TATGCCTGGGCAC3') (SEQ ID NO:4) and the right panel those from the opposite strand with the D primer (5'GTTGGCATGCTTTGATGACG-CTTC3') (SEQ ID NO:5). The brakets indicate the three nucleotides in the normal that are absent in CF (arrowheads). Sequencing was performed as described in F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Nat. Acad. Sci. U.S.A.* 74: 5463 (1977).

To investigate the proportion of CF patients carrying this deletion (F508), genomic DNA samples from patients and their parents were each amplified with oligonucleotide primers flanking the mutation in a polymerase chain reaction and hybridized to ^{32}P-labeled oligonucleotides specific for the normal and the putative mutant sequences (see FIGS. **2**A–**2**G). The results of this analysis are shown in Table 2.

TABLE 2

DISTRIBUTION OF CF AND NON-CF(N) CHROMOSOMES WITH AND WITHOUT THE 3 bp DELETION

	CF chromosomes	N chromosomes
a. without the deletion	69	198
with the deletion	145	0
Total	214	198

	CF chromosomes	
	with the 3 bp deletion	without the deletion
b. CF-PI	62	24
CF-PS	5	9
Unclassified	78	36
Total	145 (68%	69 (32%)

The data for the CF-PI (pancreatic insufficient) and CF-PS (pancreatic sufficient) chromosomes were derived from the CF families used in our linkage analysis. These families were originally selected without knowledge regarding PI or PS; the 15 CF-PS families subsequently identified were not included as part of this calculation. The unclassified CF chromosomes were obtained from the DNA Diagnosis Laboratory at the Hospital for Sick Children in Toronto and for which pancreatic function data were not available.

It can be seen that 68% (145/214) of CF chromosomes in the general patient population had the F508 deletion (Table 2). In contrast none (0/198) of the N chromosomes had the deletion (Table 2; χ^2=207, $p<10^{-57.5}$), suggesting that this sequence alteration is specific to CF and that it is the major mutation causing the disease. No recombination has been detected between the F508 deletion and CF.

Other sequence differences were noted between the normal (T16-4.5) and CF (C1-1/5) cDNA clones. At base position 2629, T16-4.5 showed a C and C1-1/5 had a T, resulting in a Leu to Phe change at the amino acid level. At position 4555, the base was G in T16-4.5 but A in C1-1/5 (Val to Met). These findings are believed to represent sequence polymorphism. Specific oligonucleotide hybridization analysis of patient/family DNA will identify these as other possible mutations. Additional nucleotide differences were observed in the 3' untranslated regions between different cDNA clones and the genomic DNA sequence. Such differences in the sequences and as is appreciated, other sequence modifications are possible; for example, which differences are due to normal sequence polymorphisms and cloning artifacts, all of such differences being essentially equivalent to the sequence as described in FIGS. **1A–1H** in terms of its function and its commercial applications.

The extensive genetic and physical mapping data have directed molecular cloning studies to focus on a small segment of DNA on chromosome 7. Because of the lack of chromosome deletions and rearrangements in CF and the lack of a well-developed functional assay for the CF gene product, the identification of the CF gene required a detailed characterization of the locus itself and comparison between the CF and normal (N) alleles. Random, phenotypically normal, individuals could not be included as controls in the comparison due to the high frequency of symptomless carriers in the population. As a result, only parents of CF patients, each of whom by definition carries an N and a CF chromosome, were suitable for the analysis. Moreover, because of the strong allelic association observed between CF and some of the closely linked DNA markers, it was necessary to exclude the possibility that sequence differences detected between N and CF were polymorphisms associated with the disease locus.

3.3 Identification of RFLPs and Family Studies

To determine the relationship of each of the DNA segments isolated from the chromosome walking and jumping experiments to CF, restriction fragment length polymorphisms (RFLPs) were identified and used to study families where crossover events had previously been detected between CF and other flanking DNA markers. As shown in FIG. **14**, a total of 18 RFLPs were detected in the 500 kb region; 17 of them (from E6 to CE1.0) listed in Table 3; some of them correspond to markers previously reported.

Five of the RFLPs, namely 10-1X.6, T6/20, H1.3 and CE1.0, were identified with cDNA and genomic DNA probes derived from the putative CF gene. The RFLP data are presented in Table 3, with markers in the MET and D7S8 regions included for comparison. The physical distances between these markers as well as their relationship to the MET and D7S8 regions are shown in FIG. **14**.

TABLE 3

RFLPs ASSOCIATED WITH THE CF GENE

Probe name	Enzyme	Frag-length	N[a]	CF-PI[a]	A[b]	*[c]	Reference
metD	BanI	7.6 (kb)	28	48	0.60	0.10	J. E. Spence et al, Am. J. Hum. Genet 39:729 (1986)
		6.8	59	25			
metD	TaqI	6.2	74	75	0.66	0.06	R. White et al, Nature 318:382 (1985
		4.8	19	4			
metH	TaqI	7.5	45	49	0.35	0.05	White et al, supra
		4.0	38	20			
E6	TaqI	4.4	58	62	0.45	0.06	B. Keren et al, Am. J. Hum. Genet. 44:827 (1989)
		3.6	42	17			

TABLE 3-continued

RFLPs ASSOCIATED WITH THE CF GENE

Probe name	Enzyme	Frag-length	N[a]	CF-PI[a]	A[b]	*[c]	Reference
E7	TaqI	3.9	40	16	0.47	0.07	
		3 + 0.9	51	57			
pH131	HinfI	0.4	81	33	0.73	0.15	J. M. Rommens et al, Am. J. Hum. Genet. 43:645 (1988)
		0.3	18	47			
W3D1.4	HindIII	20	82	33	0.68	0.13	B. Kerem et al, supra
		10	22	47			
H2.3A	TaqI	2.1	39	53	0.64	0.09	X. Estivill et al, Nature 326:840 (1987); X. Estivill et al, Genomics 1:257 (1987)
(XV2C)		1.4	37	11			
EG1.4	HincII	3.8	31	69	0.89	0.17	
		2.8	56	7			
EG1.4	BgII	20	27	69	0.89	0.18	
		15	62	9			
JG2E1	PstI	7.8	69	10	0.88	0.18	X. Estivill et al supra and B. Kerem et al supra
(KM19)		6.6	30	70			
E2.6/E.9	MspI	13	34	6	0.85	0.14	
		8.5	26	55			
H2.8A	NcoI	25	22	55	0.87	0.18	
		8	52	9			
E4.1	MspI	12	37	8	0.77	0.11	G. Romeo, personal communication
(Mp6d9)		8.5 + 3.5	38	64			
J44	XbaI	15.3	40	70	0.86	0.13	
		15 + .3	44	6			
10-1X.6	AccI	6.5	67	15	0.90	0.24	
		3.5 + 3	14	60			
10-1X.6	HaeIII	1.2	14	61	0.91	0.25	
		.6	72	15			
T6/20	MspI	8	56	66	0.51	0.54	
		4.3	21	8			
H1.3	NcoI	2.4	53	7	0.87	0.15	
		1 + 1.4	35	69			
CE1.0	NdeI	5.5	81	73	0.41	0.03	
		4.7 + 0.8	8	3			
J32	SacI	15	21	24	0.17	0.02	M. C. Iannuzi et al Am. J. Genet. 44:695 (1989)
		6	47	38			
J3.11	MspI	4.2	36	38	0.29	0.04	B. J. Wainright et al, Nature 318:384 (1985)
		1.8	62	36			
J29	PvuII	9	26	36	0.36	0.06	M. C. Iannuzi et al, supra
		6	55	36			

Notes for Table 3

(a) The number of N and CF-PI (CF with pancreatic insufficiency) chromosomes were derived from the parents in the families used in linkage analysis (Tsui et al, *Cold Spring Harbor Symp. Quant. Biol.* 51:325 (1986)).

(b) Standardized association (A), which is less influenced by the fluctuation of DNA marker allele distribution among the N chromosomes, is used here for the comparison Yule's association coefficient A=(ad–bc)/(ad+bc), where a, b, c, and d are the number of N chromosomes with DNA marker allele 1, CF with 1, N with 2, and CF with 2 respectively. Relative risk can be calculated using the relationship RR=(1+A)/(1–A) or its reverse.

(c) Allelic association (*), calculated according to A. Chakravarti et al, *Am. J. Hum. Genet.* 36:1239, (1984) assuming the frequency of 0.02 for CF chromosomes in the population is included for comparison.

Because of the small number of recombinant families available for the analysis, as was expected from the close distance between the markers studied and CF, and the possibility of misdiagnosis, alternative approaches were necessary in further fine mapping of the CF gene.

3.4 Allelic Association

Allelic association (linkage disequilibrium) has been detected for many closely linked DNA markers. While the utility of using allelic association for measuring genetic distance is uncertain, an overall correlation has been observed between CF and the flanking DNA markers. A strong association with CF was noted for the closer DNA markers, D7S23 and D7S122, whereas little or no association was detected or the more distant markers MET, D7S8 or D7S424 (see FIGS. **1A–1H**).

As shown in Table 3, the degree of association between DNA markers and CF (as measured by the Yule's association coefficient) increased from 0.35 for metH and 0.17 for J32 to 0.91 for 10-1X.6 (only CF-PI patient families were used in the analysis as they appeared to be genetically more homogeneous than CF-PS). The association coefficients appeared to be rather constant over the 300 kb from EG1.4 to H1.3; the fluctuation detected at several locations, most notably at H2.3A, E4.1 and T6/20, were probably due to the variation in the allelic distribution among the N chromosomes (see Table 2). These data are therefore consistent with the result from the study of recombinant families (see FIG. **14**). A similar conclusion could also be made by inspection of the extended DNA marker haplotypes associated with the CF chromosomes (see below). However, the strong allelic association detected over the large physical distance between EB1.4 and H1.3 did not allow further refined mapping of the CF gene. Since J44 was the last genomic DNA clone isolated by chromosome walking and jumping before a cDNA clone was identified, the strong allelic association detected for the JG2E1-J44 interval prompted us to search for candidate gene sequences over this entire interval. It is of interest to note that the highest degree of allelic association was, in fact, detected between CF and the 2 RFLPs detected by 10-1X.6, a region near the major CF mutation.

Table 4 shows pairwise allelic association between DNA markers closely linked to CF. The average number of chromosomes used in these calculations was 75–80 and only chromosomes from CF-PI families were used in scoring CF chromosomes. Similar results were obtained when Yule's standardized association (A) was used).

TABLE 4

	N chromosomes																					
	metD		metH	E6	E7	pH131	W3D1.4	H2.3A	EG1.4		JG2E1	E2.6	H2.8	E4.1	J44	10-1X.6		T6/20	H1.3	CE1.0	J32	J3.11	J29
CF chromosomes	BanI	TaqI	TaqI	TaqI	TaqI	HinfI	HdIII	TaqI	HcII	BgII	PstI	MspI	NcoI	MspI	XbaI	AccI	HaeIII	MspI	NcoI	NdeI	SacI	MspI	PvuII
metD BanI	—	0.35	0.40	0.04	0.04	0.05	0.07	0.27	0.06	0.06	0.07	0.14	0.07	0.09	0.03	0.06	0.10	0.03	0.16	0.05	0.07	0.11	0.02
metD TaqI	0.21	—	0.41	0.13	0.15	0.02	0.01	0.02	0.09	0.15	0.11	0.07	0.24	0.03	0.11	0.08	0.02	0.06	0.13	0.15	0.09	0.09	0.05
metH TaqI	0.81	0.14	—	0.01	0.05	0.06	0.06	0.24	0.05	0.06	0.07	0.13	0.15	0.07	0.04	0.02	0.02	0.07	0.02	0.03	0.21	0.04	0.18
E6 TaqI	0.11	0.30	0.00	—	0.03	0.07	0.06	0.04	0.02	0.03	0.00	0.19	0.02	0.09	0.19	0.09	0.11	0.09	0.15	0.07	0.11	0.20	0.00
E7 TaqI	0.16	0.31	0.02	1.00	—	0.11	0.09	0.03	0.03	0.04	0.01	0.11	0.00	0.07	0.22	0.01	0.02	0.09	0.13	0.06	0.06	0.16	0.04
pH131 HindI	0.45	0.28	0.23	0.36	0.40	—	0.01	0.12	0.04	0.09	0.05	0.06	0.03	0.03	0.08	0.18	0.15	0.20	0.04	0.03	0.06	0.08	0.06
W3D1.4 HindIII	0.45	0.28	0.23	0.45	0.47	0.95	—	0.21	0.02	0.03	0.01	0.08	0.03	0.03	0.10	0.12	0.10	0.23	0.10	0.05	0.05	0.10	0.06
H2.3A TaqI	0.20	0.11	0.15	0.08	0.11	0.36	0.47	—	0.05	0.11	0.07	0.42	0.14	0.29	0.07	0.27	0.22	0.20	0.09	0.23	0.04	0.08	0.12
EG1.4 HincII	0.11	0.06	0.07	0.06	0.07	0.20	0.20	0.24	—	0.95	0.87	0.76	0.86	0.81	0.60	0.07	0.13	0.61	0.56	0.04	0.24	0.14	0.15
EG1.4 BgII	0.03	0.06	0.07	0.08	0.07	0.27	0.27	0.40	1.00	—	0.92	0.77	0.93	0.71	0.55	0.06	0.07	0.58	0.55	0.12	0.28	0.24	0.20
JG2E1 PstI	0.07	0.06	0.03	0.09	0.06	0.30	0.30	0.45	0.93	0.94	—	0.84	1.00	0.76	0.64	0.11	0.11	0.61	0.57	0.13	0.31	0.26	0.22
E2.6/E.9 MspI	0.22	0.06	0.07	0.02	0.03	0.20	0.20	0.34	0.81	0.82	0.92	—	0.83	0.97	0.76	0.56	0.52	0.47	0.70	0.32	0.31	0.25	0.22
H2.8 NcoI	0.05	0.07	0.01	0.08	0.06	0.31	0.31	0.45	0.92	0.93	1.00	0.92	—	0.74	0.65	0.13	0.18	0.60	0.59	0.10	0.28	0.28	0.16
E4.1 MspI	0.12	0.06	0.07	0.05	0.03	0.25	0.25	0.48	0.62	0.86	0.94	1.00	0.93	—	0.71	0.49	0.49	0.49	0.68	0.35	0.27	0.25	0.21
J44 XbaI	0.18	0.05	0.06	0.01	0.01	0.26	0.26	0.43	0.71	0.69	0.80	0.90	0.80	0.85	—	0.33	0.40	0.65	0.64	0.32	0.24	0.22	0.23
10-1X.6 AccI	0.16	0.10	0.24	0.10	0.11	0.42	0.42	0.64	0.54	0.58	0.64	0.70	0.69	0.69	0.59	—	0.91	0.19	0.36	0.56	0.00	0.02	0.03
10-1X.6 HaeIII	0.16	0.10	0.25	0.08	0.11	0.41	0.41	0.65	0.54	0.58	0.64	0.70	0.69	0.69	0.59	1.00	—	0.18	0.43	0.62	0.02	0.02	0.08
T6/20 MspI	0.27	0.07	0.36	0.13	0.13	0.23	0.23	0.29	0.05	0.00	0.01	0.07	0.02	0.01	0.11	0.69	0.59	—	0.56	0.03	0.21	0.18	0.25
H1.3 NcoI	0.08	0.06	0.06	0.03	0.01	0.30	0.39	0.55	0.71	0.78	0.87	0.90	0.87	0.93	0.92	0.54	0.64	0.12	—	0.40	0.19	0.13	0.20
CE1.0 NdeI	0.00	0.04	0.02	0.11	0.11	0.25	0.25	0.08	0.69	0.50	0.55	0.43	0.55	0.37	0.44	0.24	0.24	0.07	0.40	—	0.19	0.20	0.14
J32 SacI	0.03	0.13	0.07	0.17	0.13	0.17	0.24	0.07	0.21	0.21	0.24	0.22	0.24	0.21	0.21	0.27	0.26	0.13	0.21	0.18	—	0.84	0.97
J3.11 MspI	0.14	0.11	0.15	0.07	0.06	0.05	0.05	0.12	0.11	0.10	0.13	0.18	0.19	0.15	0.20	0.28	0.29	0.24	0.14	0.07	0.81	—	0.71
J29 PvuII	0.11	0.12	0.09	0.10	0.10	0.00	0.00	0.09	0.10	0.10	0.14	0.17	0.20	0.16	0.16	0.29	0.29	0.23	0.16	0.06	0.65	0.97	—

Strong allelic association was also detected among subgroups of RFLPs on both the CF and N chromosomes. As shown in Table 4, the DNA markers that are physically close to each other generally appeared to have strong association with each other. For example, strong (in some cases almost complete) allelic association was detected between adjacent markers E6 and E7, between pH131 and W3D1.4 between the AccI and HaeIII polymorphic sites detected by 10-1X.6 and amongst EG1.4, JG2E1, E2.6(E.9), E2.8 and E4.1. The two groups of distal markers in the MET and D7S8 region also showed some degree of linkage disequilibrium among themselves but they showed little association with markers from E6 to CE1.0, consistent with the distant locations for MET and D7S8. On the other hand, the lack of association between DNA markers that are physically close may indicate the presence of recombination hot spots. Examples of these potential hot spots are the region between E7 and pH131, around H2.3A, between J44 and the regions covered by the probes 10-1X.6 and T6/20 (see FIG. **14**). These regions, containing frequent recombination breakpoints, were useful in the subsequent analysis of extended haplotype data for the CF region.

3.5 Haplotype Analysis

Extended haplotypes based on 23 DNA markers were generated for the CF and N chromosomes in the collection of families previously used for linkage analysis. Assuming recombination between chromosomes of different haplotypes, it was possible to construct several lineages of the observed CF chromosomes and, also, to predict the location of the disease locus.

To obtain further information useful for understanding the nature of different CF mutations, the F508 deletion data were correlated with the extended DNA marker haplotypes. As shown in Table 5, five major groups of N and CF haplotypes could be defined by the RFLPs within or immediately adjacent to the putative CF gene (regions 6–8).

TABLE 5

DNA marker haplotypes spanning the CF locus.

		HAPLOTYPES[a]									CF[b] PI (F508)	CF[b] PS (F508)	CF[b] PI others	CF[b] PS others	N
		1	2	3	4	5	6	7	8	9					
I.	(a)	A	A	A	A	A	A	A	A	A	10	1	.	.	.
		A	A	A	A	A	A	.	A	A	3	.	.	.	.
		A	A	A	A	.	A	A	.	A	1	.	.	.	.
		A	A	A	A	.	.	A	.	A	.	.	.	.	1
		A	A	A	A	A	A	A	A	B	10	.	.	.	1
		A	A	.	A	A	A	A	A	B	4	.	.	.	.
		A	A	A	A	.	A	A	A	B	1	.	.	.	.
		A	A	.	A	A	A	A	A	C	1	.	.	.	.
		B	A	A	A	A	A	A	A	A	4	.	.	.	.
		B	A	.	A	A	A	A	A	A	1	.	.	.	.
		B	A	A	A	.	A	A	A	A	.	1	.	.	.
		B	A	A	A	A	A	A	A	.	1	.	.	.	-
		B	A	A	A	.	.	A	.	A	1	.	.	.	-
		A	B	A	A	A	A	A	A	A	1	.	.	.	-
		A	D	A	A	A	A	A	A	A	1	.	.	.	-
		A	G	A	A	A	A	A	A	A	1	.	.	.	-
		B	B	A	A	A	A	A	A	A	1	.	.	.	-
		B	C	A	A	A	A	A	A	A	2	.	.	.	-
		B	B	A	A	.	.	A	.	A	1	.	.	.	-
		D	B	A	A	.	A	.	A	A	1	.	.	.	-
		D	B	B	A	A	A	A	A	A	1	.	.	.	-
		B	A	.	A	A	A	A	A	B	1	.	.	.	.
		C	A	.	A	A	A	A	A	B	1	.	.	.	.
		A	D	A	A	A	A	A	A	B	1	.	.	.	.
		D	C	A	A	A	A	A	A	B	.	1	.	.	.
		A	D	A	A	.	A	A	A	B	1	.	.	.	.
		D	C	A	A	A	A	A	A	B	.	1	.	.	.
		A	D	A	A	.	A	A	A	B	1	.	.	.	.
		D	D	.	A	A	A	A	A	B	.	.	.	.	1
		B	B	.	A	A	A	.	A	B	1	.	.	.	.
		A	B	A	A	A	A	A	A	E	2	.	.	.	.
		A	B	.	A	A	A	A	A	E	1	1	.	.	.
		A	E	B	A	A	A	A	A	E	1	.	.	.	.
		A	C	A	A	A	A	A	A	B	1	.	.	.	.
		A	C	.	C	.	A	A	A	B	.	1	.	.	.
		A	B	A	B	A	A	A	.	A	.	.	.	.	1
		B	C	B	A	.	A	A	A/D	B	1	.	.	.	.
	(b)	A	C	.	A	A	A	A	A	A	.	.	.	1	.
		A	C	A	A	A	A	A	A	.	.	.	1	.	.
		D	C	.	A	A	A	A	A	E	.	.	1	.	.
		D	C	A	A	A	A	A	A	D	.	.	.	.	1
		F	C	.	A	A	A	A	A	E	.	.	1	.	.
		B	C	A	A	A	A	A	A	B	.	.	3	.	.
	(c)	B	C	A	B	C	A	A	D	A	.	.	.	.	1
		B	C	A	B	C	A	A	D	B	.	.	1	.	.
		F	C	A	B	C	A	A	D	B	.	.	.	.	1

TABLE 5-continued

DNA marker haplotypes spanning the CF locus.

		F	A	A	B	C	A	A	D	B	.	.	.	. 1
		A	B	A	B	C	A	A	D	B	.	.	.	. 1
		B	B	A	B	C	A	A	D	B	.	.	.	. 1
		B	D	A	B	C	A	.	D	C	.	.	.	. 1
		A	B	A	B	A	A	.	D	A	.	.	.	. 1
	(d)	D	B	A	A	A	A	A	C	A	.	.	.	. 1
		B	C	B	C	A	A	A	C	B	.	.	.	. 1
											57	5	7	1 14
II.	(a)	B	A	.	B	B	B	A	C	B	.	.	1	. .
		.	B/C	B	B	B	B	A	C	B	.	.	1	. .
		B	A	.	B	.	B	A	A/C	B	.	.	.	1 .
		A	B	B	B	B	B	A	C	B	.	.	1	. .
		B	B	B	B	B	B	A	C	A	.	.	.	. 3
		A	C	B	B	B	B	A	C	A	.	.	.	. 1
		A	C	B	B	B	B	.	C	A	.	.	.	. 1
		F	C	B	B	B	B	A	C	A	.	.	.	. 1
		A	C	B	B	B	B	A	C	B	.	.	.	. 1
		A	C	.	B	B	B	.	C	C	.	.	.	. 1
		B	C	B	B	.	B	A	C	C	.	.	.	1 .
		B	C	B	B	B	B	A	C	B	.	.	.	. 1
		B	C	B	B	B	B	A	C	A	.	.	.	. 1
		B	C	B	B	B	B	A	C	D	.	.	.	. 1
		B	C	.	B	B	B	A	C	B	.	.	.	. 1
		B	C	B	B	B	B	.	C	B	.	.	.	. 1
		D	C	B	B	B	B	A	C	B	.	.	.	. 2
		D	.	B	B	.	B	A	C	B	.	.	.	. 1
		F	C	B	B	B	B	A	C	B	.	.	.	. 1
		C	C	.	B	B	B	A	C	B	.	.	.	. 1
		A	A	A	B	B	B	A	C	B	.	.	.	. 1
		B	G	A	B	B	B	A	C	B	.	.	.	. 1
		F	A	.	B	B	B	A	C	B	.	.	.	. 1
		B	H	.	B	B	B	A	C	B	.	.	.	. 1
		B	B	.	B	B	B	A	C	B	.	.	.	. 1
		A	E	A	B	B	B	A	C	B	.	.	.	. 1
		F	D	A	B	B	B	A	C	B	.	.	.	. 1
		C	D	A	B	B	B	A	C	A	.	.	.	. 1
		B	D	A	B	B	B	A	C	A	.	.	.	. 1
		B	C	A	B	B	B	A	C	A	.	.	.	. 2
		A	C	A	B	B	B	A	C	B	.	.	.	. 1
		A	C	A	B	B	B	.	C	B	.	.	.	. 1
		A	C	A	B	B	B	A	C	C	.	.	.	. 1
		B	C	A	B	B	B	A	C	B	.	.	.	. 1
		D	B/C	.	B	B	B	A	C	A	.	.	.	. 2
		C	C	A	B	B	B	A	C	A	.	.	.	1 .
		D	B	.	B	B	B	A	A/C	B	.	.	.	. 1
		D	B	A	B	.	B	A	C	B	.	.	.	. 1
		A	G	A	B	B	B	A	C	A	.	.	.	. 1
		B	C	.	B	B	B	A	A/C	A	.	.	.	. 1
		A	C	B	D	B	B	A	C	B	.	.	1	. .
		A	C	.	D	.	B	A	C	B	.	.	.	. 1
		B	B	B	B	B	B	A	C	C	.	.	.	. 1
		F	D	A	B	B	B	A	C	C	.	.	.	1 .
		A	A	A	A	A	B	A	C	D	.	.	.	. 1
		.	B/C	A	B	C	B	A	C	B	.	.	.	. 1
		A	B	A	B	B	B/C	A	C	A	.	.	.	. 1
	(b)	A	C	A	B	B	B	A	B	E	.	.	1	. .
		A	C	.	B	B	B	A	B	B	.	.	1	. .
	(c)	E	D	.	B	.	B	A	A	A	.	.	.	. 1
											0	0	6	4 45
III.	(a)	B	C	B	A	A	C	B	A	B	1	.	.	. .
	(b)	B	A	B	A	A	C	B	A	B	.	.	1	. .
		B	C	B	A	A	C	B	A	A	.	.	1	. .
		B	C	B	A	A	C	B	A	B	.	.	.	. 1
		B	C	.	A	A	C	B	A	B	.	.	.	. 2
		A	B	.	A	A	C	B	A	B	.	.	.	. 1
		A	B	.	A	A	C	B	A	C	.	.	.	. 1
		B	B	B	A	A	C	B	A	E	.	.	.	. 2
		D	C	B	A	A	C	B	A	A	.	.	.	1 .
		A	B	B	C	A	C	B	A	B	.	.	.	. 1
		B	B	A	A	A	C	B	A	B	.	.	2	. 1
		B	E	.	A	A	C	B	A	B	.	.	1	. 1
		B	B	A	A	A	C	B	A	A	.	.	.	1 .
		D	A	A	A	A	C	B	A	B	.	.	.	. 1
		D	C	A	A	A	C	B	A	B	.	.	.	. 2
		A	C	.	A	A	C	E	A	B	.	.	1	. 1
		D	B	A	A	A	C	.	A	C	.	.	.	. 1

TABLE 5-continued

DNA marker haplotypes spanning the CF locus.

	(c)	A	A	A	B	B	C	B	A	.	.	.	1	.	.
		F	B	B	B	B	C	B	A	B	.	.	.	.	1
		D	B	B	B	B	C	B	A	A	.	.	.	.	1
											1	0	7	2	17
IV.		F	C	B	A	A	C	B	C	A	.	.	.	1	.
		B	C	A	A	A	C	B	C	.	.	.	.	.	1
		A	B	A	A	A	C	.	C	B	.	.	.	.	1
		A	H	B	A	.	C	.	C	B	.	.	.	.	1
		D	B	B	B	B	C	B	C	B	.	.	.	.	1
											0	0	0	1	4
V.	(a)	B	C	B	B	B	C	A	C	A	.	.	1	.	.
		A	C	B	B	.	.	A	.	A	.	.	1	.	.
		B	B	B	B	B	C	A	C	B	.	.	.	.	1
		B	C	B	B	B	C	A	C	B	.	.	.	.	1
		B	C	.	B	B	C	A	C	B	.	.	.	.	1
		D	.	A	B	B	C	.	C	B	.	.	.	.	1
	(b)	B	C	A	B	C	C	A	C	A	.	.	.	1	.
		B	C	.	B	C	C	.	C	D	.	.	.	.	1
											0	0	2	1	5
Others:		B	C	E	A	A	B	E	A	B	.	.	.	.	1
		B	C	B	A	A	D	B	A	B	.	.	.	.	1
		B	C	B	E	B	A	B	D	A	.	.	.	.	1
		B	C	A	B	B	E	.	C	.	.	.	.	.	1
		B	D	B	B	B	F	A	C	B	.	.	.	.	1
		A	C	.	A	A	C	B	D	A	.	.	.	.	1
		G	B	B	A	A	B/C	A	A/D	B	.	.	.	.	1
											0	0	0	0	7
Unclassified:		.	.	.	.	.	.	.	.	.	4	10	2	18	6
Total:											62	15	24	27	98

(a) The extended haplotype data are derived from the CF families used in previous linkage studies (see footnote (a) of Table 3) with additional CF-PS families collected subsequently (Kerem et al, Am. J. Genet. 44:827 (1989)). The data are shown in groups (regions) to reduce space. The regions are assigned primarily according to pairwise association data shown in Table 4 with regions 6–8 spanning the putative CF locus (the F508 deletion is between regions 6 and 7). A dash (-) is shown at the region where the haplotype has not been determined due to incomplete data or inability to establish phase. Alternative haplotype assignments are also given where data are incomplete. Unclassified includes those chromosomes with more than 3 unknown assignments. The haplotype definitions for each of the 9 regions are:

Region 1 =	metD BanI	metD TaqI	metH TaqI
A =	1	1	1
B =	2	1	2
C =	1	1	2
D =	2	2	1
E =	1	2	.
F =	2	1	1
G =	2	2	2

Region 2 =	E6 TaqI	E7 TaqI	pH131 HinfI	W3D1.4 HindIII
A =	1	2	2	2
B =	2	1	1	1
C =	1	2	1	1
D =	2	1	2	2
E =	2	2	2	1
F =	2	2	1	1
G =	1	2	1	2
H =	1	1	2	2

Region 3 =	H2.3A TaqI
A =	1
B =	2

Region 4 =	EG1.4 HindII	EG1.4 BglII	JG2E1 PstI
A =	1	1	2
B			
C =	2	2	2
D =	1	1	1
E =	1	2	1

Region 5 =	E2.6 MspI	E2.8 NcoI	E4.1 MspI
A =	2	1	2

TABLE 5-continued

DNA marker haplotypes spanning the CF locus.

B =	1	2	1
C =	2	2	2
Region 6 =	J44	10-1X.6	10-1X.6
	XbaI	AccI	HaeIII
A =	1	2	1
B =	2	1	2
C =	1	1	2
D =	1	2	2
E =	2	2	2
F =	2	2	1
Region 7 =	T6/20		
	MspI		
A =	1		
B =	2		
Region 8 =	H1.3	CE1.0	
	NcoI	NdeI	
A =	2	1	
B =	1	2	
C =	1	1	
D =	2	2	
Region 9 =	J32	J3.11	J29
	SacI	MspI	PvuII
A =	1	1	1
B =	2	2	2
C =	2	1	2
D =	2	2	1
E =	2	1	1

(b) Number of chromosomes scored in each class:l
CF-PI(F) = CF chromosomes for CF-PI patients with the F508 deletions;
CF-PS(F) = CF chromosomes for CF-PS patients with the F508 deletions;
CF-PI = Other CF chromosomes from CF-PI patients;
CF-PS = Other CF chromosomes from CF-PS patients;
N = Normal chromosomes derived from carrier parents.

It was apparent that most recombinations between haplotypes occurred between regions 1 and 2 and between regions 8 and 9, again in good agreement with the relatively long physical distance between these regions. Other, less frequent, breakpoints were noted between short distance intervals and they generally corresponded to the hot spots identified by pairwise allelic association studies as shown above. The striking result was that the F508 deletion associated almost exclusively with Group I, the most frequent CF haplotype, supporting the position that this deletion constitutes the major mutation in CF. More important, while the F508 deletion was detected in 89% (62/70) of the CF chromosomes with the AA haplotype (corresponding to the two regions, 6 and 7) flanking the deletion, none was found in the 14 N chromosomes within the same group (χ^2=47.3, $p<10^{-4}$). The F508 deletion was therefore not a common sequence polymorphism associated with the core of the Group I haplotype (see Table 5).

One of the CF chromosomes, detected by the specific oligonucleotide probe for the F508 deletion, was found to belong to a different haplotype group (Group III). None of the 9 other CF chromosomes nor 17 N chromosomes with the same group hybridized to the probe. This specific hybridization result suggests that the mutation harbored on this chromosome is similar to F508. Although recombination or gene conversion are possible mechanisms to explain the presence of this deletion on a non-Group I haplotype, it is more likely that these 2 Group III chromosomes represent a recurrent mutation event, a situation similar to the β^S and β^E mutations at the β globin locus.

Together, the results of the oligonucleotide hybridization study and the haplotype analysis support the fact that the gene locus described here is the CF gene and that the 3 bp (F508) deletion is the most common mutation in CF.

3.6 Other of Mutations

The association of the F508 deletion with 1 common and 1 rare CF haplotype provided further insight into the number of mutational events that could contribute to the present patient population. Based on the extensive haplotype data, the 2 original chromosomes in which the F508 deletion occurred are likely to carry the haplotype -AAAAAAA- (Group Ia) and -CBAACBA- (Group IIIa), as defined in Table 5. The other Group I CF chromosomes carrying the deletion are probably recombination products derived from the original chromosome. If the CF chromosomes in each haplotype group are considered to be derived from the same origin, only 3–4 additional mutational events would be predicted (see Table 5). However, since many of the CF chromosomes in the same group are markedly different from each other, further subdivision within each group is possible. As a result, a higher number of independent mutational events could be considered and the data suggest that at least 7 additional, putative mutations also contribute to the CF-PI phenotype (see Table 4). The mutations leading to the CF-PS subgroup are probably more heterogeneous.

The 7 additional CF-PI mutations are represented by the haplotypes: -CAAAAAA- (Group Ib), -CABCAAD- (Group Ic), ---BBBAC- (Group IIa), -CABBBAB- (Group Va). Although the molecular defect in each of these mutations has yet to be defined, it is clear that none of these mutations severely affect the region corresponding to the oligonucleotide binding sites used in the PCR/hybridization experiment.

3.7 Pancreatic Sufficiency

CF-PS is defined clinically as sufficient pancreatic exocrine function for digestion of food; however, the level of residual pancreatic enzyme activity in the digestive system varies from patient to patient. Previous haplotype data suggested that the CF-PI and CF-PS patients are due to different mutant alleles. Although the basic biochemical defect in CF has yet to be defined, it is possible that the residual pancreatic enzyme activity in CF-PS patients is a direct reflection of the activity of the mutant CF gene product. Thus, the residual exocrine function conferred by a mild (CF-PS) allele, although much lower than that of the normal gene product, would constitute a dominant phenotype over that of more severe (CF-PI) mutations with little or no function. It follows that only patients carrying 2 copies of severe alleles would be CF-PI and that patients carrying 1 or 2 mild alleles would be CF-PS.

To test the above hypothesis, the information on the proportion of CF patients carrying the F508 deletion could be utilized. Assuming that a severe mutation is recessive to a mild mutation and a distribution of CF alleles among the patient population according to the Hardy-Weinberg law, the frequency of severe alleles could be estimated to be 0.92 and that for the mild alleles (M), 0.08 (see Table 6).

TABLE 6

POPULATION ANSLYSIS OF CF-PI AND CF-PS

	Assumed Genotype[a]	Predicted Frequency[b]	Observed[c]	Expected[d]
Pancreatic insufficient(PI)	FF	0.459	21	21.1
	FS	0.331	14	15.2
	SS	0.060	4	2.7
	Total	0.850	39	—
Pancreatic sufficient(PS)	FM	0.106	15[e]	14.8
	SM	0.038	6	6.2
	MM	0.006		
	Total	0.150	21	

[a]Allele designations: F = the 3 bp deletion (deletion of phenylalanine at amino acid position 508); S = uncharacterized severe mutant alleles; M = uncharacterized mild mutant alleles.
[b]Assuming that tht CF-PI mutant phenotype is recessive to the CF-PS mutant phenotype, the frequency of CF-PI mutant alleles, including the 3 bp deletion, could be estimated from the observed proportion of the CF-PI patients in the CF clinic (Corey et al J. Pediatr. 115:274 (1989)) i.e., (0.85) = 0.92. The observed allele frequency for F in the total CF population is 0.68 (Table 3); the frequency for S = 0.92 − 0.68 − 0.24; the frequency forM = 1 − 0.92 − 0.08. The frequency for each genotype was then calculated by using the Hardy-Weinberg Law.

TABLE 6-continued

POPULATION ANSLYSIS OF CF-PI AND CF-PS

	Assumed Genotype[a]	Predicted Frequency[b]	Observed[c]	Expected[d]

[c]The number of CF-PI and CF-PS patients in each category was obtained by oligonucleotide hybridization analysis as illustrated in FIG. 15. The patients were from the CF families used in our linkage analysis with 14 additional CF-PS patients/families from a subsequent study. Since SM and MM could not be distinguished genotypically or phenotypically, they were combined in the analysis.
[d]The expected numbers were calculated for CF-PI and CF-PS after normalization within each group. The x^2 of fit is 0.86, d.f. = 3, $0.74 < p < 0.90$
[e]This number is higher than would be expected (15 observed vs. 9.6 expected) if the F508 deletion is in Hardy-Weinberg equilibrium among all CF chromosomes ($x^2 = 6.48$, d.f. = 1, $p < 0.011$

Since the majority of CF-PI patients were found to be homozygous for the F508 mutation (F), it was reasonable to assume that this mutation corresponded to one of the severe alleles. Given the observed frequency of F (0.68) in the studied CF population, the frequency of the remaining severe alleles (S) could be derived. The proportion of FF, SS, MM, FS, FM and SM patients was then calculated. Since individuals with SM and MM could not be distinguished phenotypically or genotypically, they were combined in the analysis. As shown in Table 6, the observed frequencies for all 5 groups of patients were as expected from this hypothesis.

The above analysis thus provides strong support for our position that CF-PI is due to the presence of 2 severe alleles and that a CF-PS patient carries either a single severe allele or 2 mild alleles. This model also explains the lower frequency of the F508 deletion in the CF-PS than in the CF-PI population and the excess number of CF-PS patients with one copy of the deletion (see note in Table 6).

Given the predicted dominant phenotype conferred by the M alleles, it was necessary to examine the CF chromosomes in CF-PS patients individually in order to identify those carrying the M alleles. As shown in Table 7, five of the 7 representative CF-PS patients carry one copy of the P508 deletion; at least 5 different haplotypes could be assigned to the other CF chromosomes.

TABLE 7

	Haplotypes of CF chromosomes in CF-PS individuals and families with MI									
Family #	1	2	3	4	5	6	7	8	9	CF Alleles
	(a) CF-PS individuals									
3	A	A	A	A	A	A	—	—	A	F (Group Ia)
	D	C	B	A	A	C	B	A	A	M (predicted, Group IIIb)
14	B	A	A	A	—	A	A	A	A	F (Group Ia)
	B	C	B	B	—	B	A	C	C	M (predicted, Group IIa)

TABLE 7-continued

Family #	Haplotypes of CF chromosomes in CF-PS individuals and families with MI 1	2	3	4	5	6	7	8	9	CF Alleles
27	A	B	—	A	A	A	A	A	E	F (Group Ia)
	A	C	—	A	A	A	A	A	A	M (predicted, Group Ib)
29	A	C	—	C	—	A	A	A	B	F (Group Ia)
	B	A	—	B	—	B	A	A/C	B	M (predicted, Group IIa)
40	D	A	A	A	A	A	A	A	B	F (Group Ia)
	F	C	B	A	A	C	B	C	A	M (predicted, Group IV)
51	C	C	A	B	B	B/C	A	C	A	M (predicted, Group IIa)
	F	D	A	B	B	B/C	A	C	C	M (predicted, Group IIa)
54	B	C	A	B	C	C	A	C	A	M or S (predicted, Group Vb)
	B	B	A	A	A	C	B	A	A	M (predicted, Group IIIb)
					(b) Families with MI					
4	B	A	A	A	A	A	A	A	A	F (Group Ia)
	B	A	A	A	A	A	A	A	A	F (Group Ia)
10	D	B	A	A	—	A	—	A	A	F (Group Ia)
	A	D	A	A	—	A	A	A	B	F (Group Ia)
23	A	E	B	A	A	A	A	A	E	F (Group Ia)
	B	C	A	A	A	A	A	A	B	S (predicted, Group Ib)
28	A	A	—	A	A	A	A	A	C	F (Group Ia)
	A	A	—	A	A	A	A	A	B	F (Group Ia)
33	B	B	—	A	A	A	—	A	B	F (Group Ia)
	B	A	—	A	A	A	A	A	B	F (Group Ia)
49	A	A	A	A	A	A	A	A	B	F (Group Ia)
	A	A	A	A	A	A	A	A	B	F (Group Ia)

(a) The haplotype definitions are the same as in Table 5.
(b) Allele designations are the same as in Table 6: F = the F508 deletion; S = uncharacterized severe mutant allele; M = uncharacterized mild mutant allele.

These latter observations provide further support that the majority of CF-PS patients are compound heterozygotes.

4.0 CFTR Protein

As discussed with respect to the DNA sequence of FIGS. **1A–1H**, analysis of the sequence of the overlapping cDNA clones predicted an unprocessed polypeptide of 1480 amino acids with a molecular mass of 168,138 daltons. As later described, due to polymorphisms in the protein, the molecular weight of the protein can vary due to possible substitutions or deletion of certain amino acids. The molecular weight will also change due to the addition of carbohydrate units to form a glycoprotein. It is also understood that the functional protein in the cell will be similar to the unprocessed polypeptide, but may be modified due to cell metabolism.

Accordingly, the invention provides purified normal CFTR polypeptide characterized by a molecular weight of about 170,000 daltons and having epithelial cell transmembrane ion conductance activity. The normal CFTR polypeptide, which is substantially free of other human proteins, is encoded by the aforementioned DNA sequences and according to one embodiment, that of FIGS. **1A–1H**. Such polypeptide displays the immunological or biological activity of normal CFTR polypeptide. As will be later discussed, the CFTR polypeptide and fragments thereof may be made by chemical or enzymatic peptide synthesis or expressed in an appropriate cultured cell system. The invention also provides purified mutant CFTR polypeptide which is characterized by cystic fibrosis-associated activity in human epithelial cells. Such mutant CFTR polypeptide, as substantially free of other human proteins, can be encoded by the mutant DNA sequence.

4.1 Structure of CFTR

The most characteristic feature of the predicted protein is the presence of two repeated motifs, each of which consists of a set of amino acid residues capable of spanning the membrane several times followed by sequence resembling consensus nucleotide (ATP)-binding folds (NBFs) (FIGS. **11, 12** and **16A–16B**). These characteristics are remarkably similar to those of the mammalian multidrug resistant P-glycoprotein and a number of other membrane-associated proteins, thus implying that the predicted CF gene product is likely to be involved in the transport of substances (ions) across the membrane and is probably a member of a membrane protein super family.

FIG. **13** is a schematic model of the predicted CFTR protein. In FIG. **13**, cylinders indicate membrane spanning helices, hatched spheres indicate NBFS. The stippled sphere is the polar R-domain. The 6 membrane spanning helices in each half of the molecule are depicted as cylinders. The inner cytoplasmically oriented NBFs are shown as hatched spheres with slots to indicate the means of entry by the nucleotide. The large polar R-domain which links the two halves is represented by an stippled sphere. Charged individual amino acids within the transmembrane segments and on the R-domain surface are depicted as small circles

containing the charge sign. Net charges on the internal and external loops joining the membrane cylinders and on regions of the NBFs are contained in open squares. Sites for phosphorylation by protein kinases A or C are shown by closed and open triangles respectively. K,R,H,D, and E are standard nomenclature for the amino acids, lysine, arginine, histidine, aspartic acid and glutaxic acid respectively.

Each of the predicted membrane-associated regions of the CFTR protein consists of 6 highly hydrophobic segments capable of spanning a lipid bilayer according to the algorithms of Kyte and Doolittle and of Garnier et al (*J. Mol. Biol.* 120, 97 (1978) (FIG. **13**). The membrane-associated regions are each followed by a large hydrophilic region containing the NBFs. Based on sequence alignment with other known nucleotide binding proteins, each of the putative NBFs in CFTR comprises at least 150 residues (FIG. **13**). The 3 bp deletion detected in the majority of CF patients is located between the 2 most highly conserved segments of the first NBF in CFTR. The amino acid sequence identity between the region surrounding the phenylalanine deletion and the corresponding regions of a number of other proteins suggests that this region is of functional importance (FIGS. **16A–16B**). A hydrophobic amino acid, usually one with an aromatic side chain, is present in most of these proteins at the position corresponding to F508 of the CFTR protein. It is understood that amino acid polymorphisms may exist as a result of DNA polymorphisms.

FIGS. **16A–16B** show alignment of the 3 most conserved segments of the extended NBF's of CFTR with comparable regions of other proteins. These 3 segments consist of residues 433–473, 488–513, and 542–584 of the N-terminal half and 1219–1259, 1277–1302, and 1340–1382 of the C-terminal half of CFTR. The heavy overlining points out the regions of greatest similarity. Additional general homology can be seen even without the introduction of gaps.

Despite the overall symmetry in the structure of the protein and the sequence conservation of the NBFs, sequence homology between the two halves of the predicted CFTR protein is modest. This is demonstrated in FIG. **12**, where amino acids 1–1480 are represented on each axis. Lines on either side of the identity diagonal indicate the positions of internal similarities. Therefore, while four sets of internal sequence identity can be detected as shown in FIG. **12**, using the Dayhoff scoring matrix as applied by Lawrence et al. (C. B. Lawrence, D. A. Goldman, and R. T. Hood, *Bull Math Biol.* 48, 569 (1966)), three of these are only apparent at low threshold settings for standard deviation. The strongest identity is between sequences at the carboxyl ends of the NBFs. Of the 66 residues aligned 27% are identical and another 11% are functionally similar. The overall weak internal homology is in contrast to the much higher degree (>70%) in P-glycoprotein for which a gene duplication hypothesis has been proposed (Gros et al, *Cell* 47, 371, 1986, C. Chen et al, *Cell* 47, 381, 1986, Gerlach et al, *Nature,* 324, 485, 1986, Gros et al, *Mol. Cell. Biol.* 8, 2770, 1988). The lack of conservation in the relative positions of the exon-intron boundaries may argue against such a model for CFTR (FIGS. **2A–2G**).

Since there is apparently no signal-peptide, sequence at the amino-terminus of CFTR, the highly charged hydrophilic segment preceding the first transmembrane sequence is probably oriented in the cytoplasm. Each of the 2 sets of hydrophobic helices are expected to form 3 transversing loops across the membrane and little sequence of the entire protein is expected to be exposed to the exterior surface, except the region between transmembrane segment 7 and 8. It is of interest to note that the latter region contains two potential sites for N-linked glycosylation.

Each of the membrane-associated regions is followed by a NBF as indicated above. In addition, a highly charged cytoplasmic domain can be identified in the middle of the predicted CFTR polypeptide, linking the 2 halves of the protein. This domain, named the R-domain, is operationally defined by a single large exon in which 69 of the 241 amino acids are polar residues arranged in alternating clusters of positive and negative charges. Moreover, 9 of the 10 consensus sequences required for phosphosphorylation by protein kinase A (PKA), and, 7 of the potential substrate sites for protein kinase C (PKc) found in CFTR are located in this exon.

4.2 Function of CFTR

Properties of CFTR can be derived from comparison to other membrane-associated proteins (FIGS. **16A–16B**). In addition to the overall structural similarity with the mammalian P-glycoprotein, each of the two predicted domains in CFTR also shows remarkable resemblance to the single domain structure of hemolysin B of *E. coli* and the product of the White gene of Drosophila. These latter proteins are involved in the transport of the lytic peptide of the hemolysin system and of eye pigment molecules, respectively. The vitamin B12 transport system of *E. coli,* BtuD and MbpX which is a liverwort chloroplast gene whose function is unknown also have a similar structural motif. Furthermore, the CFTR protein shares structural similarity with several of the periplasmic solute transport systems of gram negative bacteria where the transmembrane region and the ATP-binding folds are contained in separate proteins which function in concert with a third substrate-binding polypeptide.

The overall structural arrangement of the transmembrane domains in CFTR is similar to several cation channel proteins and some cation-translocating ATPases as well as the recently described adenylate cyclase of bovine brain. The functional significance of this topological classification, consisting of 6 transmembrane domains, remains speculative.

Short regions of sequence identity have also been detected between the putative transmembrane regions of CFTR and other membrane-spanning proteins. Interestingly, there are also sequences, 18 amino acids in length situated approximately 50 residues from the carboxyl terminus or CFTR and the raf serine/threonine kinase protooncogene of *Xenopus laevis* which are identical at 12 of these positions.

Finally, an amino acid sequence identity (10/13 conserved residues) has been noted between a hydrophilic segment (position 701–713) within the highly charged R-domain of CFTR and a region immediately preceding the first transmembrane loop of the sodium channels in both rat brain and eel. The charged R-domain of CFTR is not shared with the topologically closely related P-glycoprotein; the 241 amino acid linking-peptide is apparently the major difference between the two proteins.

In summary, features of the primary structure of the CFTR protein indicate its possession of properties suitable to participation in the regulation and control of ion transport in the epithelial cells of tissues affected in CF. Secure attachment to the membrane in two regions serve to position its three major intracellular domains (nucleotide-binding folds 1 and 2 and the R-domain) near the cytoplasmic surface of the cell membrane where they can modulate ion movement through channels formed either by CFTR transmembrane segments themselves or by other membrane proteins.

In view of the genetic data, the tissue-specificity, and the predicted properties of the CFTR protein, it is reasonable to

conclude that CFTR is directly responsible for CF. It, however, remains unclear how CFTR is involved in the regulation of ion conductance across the apical membrane of epithelial cells.

It is possible that CFTR serves as an ion channel itself. As depicted in FIG. **13**, 10 of the 12 transmembrane regions contain one or more amino acids with charged side chains, a property similar to the brain sodium channel and the GABA receptor chloride channel subunits, where charged residues are present in 4 of the 6, and 3 of the 4, respective membrane-associated domains per subunit or repeat unit. The amphipathic nature of these transmembrane segments is believed to contribute to the channel-forming capacity of these molecules. Alternatively, CFTR may not be an ion channel but instead serve to regulate ion channel activities. In support of the latter assumption, none of the purified polypeptides from trachea and kidney that are capable of reconstituting chloride channels in lipid membranes (Landry et al, *Science* 224:1469 (1989)) appear to be CFTR if judged on the basis of the molecular mass.

In either case, the presence of ATP-binding domains in CFTR suggests that ATP hydrolysis is directly involved and required for the transport function. The high density of phosphorylation sites for PKA and PKC and the clusters of charged residues in the R-domain may both serve to regulate this activity. The deletion of a phenylalanine residue in the NBF may prevent proper binding of ATP or the conformational change which this normally elicits and consequently result in the observed insensitivity to activation by PKA- or PKC-mediated phosphorylation of the CF apical chloride conductance pathway. Since the predicted protein contains several domains and belongs to a family of proteins which frequently function as parts of multi-component molecular systems, CFTR may also participate in epithelial tissue functions of activity or regulation not related to ion transport.

With the isolated CF gene (cDNA) now in hand it is possible to define the basic biochemical defect in CF and to further elucidate the control of ion transport pathways in epithelial cells in general. Most important, knowledge gained thus far from the predicted structure of CFTR together with the additional information from studies of the protein itself provide a basis for the development of improved means of treatment of the disease. In such studies, antibodies have been raised to the CFTR protein as later described.

4.3 Protein Purification

The CFTR protein can be purified by methods selected on the basis of properties as revealed by its sequence. For example, since it possesses distinctive properties of an integral membrane protein, a membrane fraction of the epithelial cells in which it is highly expressed (e.g., the cultured colonic carcinoma cell line, T84) is first isolated using established methods (J. E. Langridge, et al, *Biochim. Biophys. Acts.* 751: 318 (1983)). The peripheral proteins of these membranes are those removed by extraction with high salt concentrations, high pH or chaotropic agents such as lithium diiodosalicylate. All of the integral proteins remaining including the CFTR protein are then solubilized using a detergent such as octyl glucoside (Landry, et al, supra), CHAPS (D. J. Beros et al, *J. Biol. Chem.* 262: 10613 (1987)), or other compounds of similar action. Making use of the nucleotide binding domains of CFTR, cibacron-blue (S. T. Thompson et al. *Proc. Nat. Acad. Sci. U.S.A.* 72: 669 (1975)) affinity chromatography is then used to bind the CFTR protein and remove it from other integral proteins of the detergent stabilized mixture. Since CFTR is a glycoprotein, differential lectin chromatography can bring about further purification (Riordan et al. *J. Biol. Chem.* 254: 1270 (1979)). Final purification to homogeneity is then achieved using other standard protein purification procedures; i.e., ion exchange chromatography, gel permeation chromatography, adsorption chromatography or isoelectric focussing as necessary. Alternatively, use is made of single step purification procedures, such as immuno-affinity chromatography using immobilized antibodies to the CFTR protein (or fragments thereof) or preparative polyacrylamide gel electrophoresis using advanced instrumentation such as the Applied Biosystems "230A HPEC System". Based on experience in the purification of P-glycoprotein (Riordan et al, supra), another member of the general category of nucleotide binding transport-associated membrane proteins, the purification of the CFTR protein is facilitated.

In addition to purification from tissues and cells in which the CFTR protein is highly expressed, similar procedures are used to purify CFTR from cells transfected with vectors containing the CF gene (cDNA) as described above. Protein products resulting from expression of modified version of the cDNA sequence are purified in a similar manner. Criteria of the homogeneity of protein so provided include those standard to the field of protein chemistry including one and two dimensional gel electrophoresis and N-terminal amino acid determination. The purified protein is used in further physical biochemical analysis to determine features of its secondary and tertiary structure, to aid in the design of drugs to promote the proper functioning of the mutant CF forms. In preparation for use in protein therapy, the absence of potentially toxic contaminating substances is considered. It is recognized that the hydrophobic nature of the protein necessitates the inclusion of amphiphilic compounds such as detergents and others (J. V. Ambud Kar and P. C. Maloney *J. Biol. Chem,* 261: 10079 (1986)) at all stages of its handling.

5.0 CF Screening

5.1 DNA Based Diagnosis

Given the knowledge of the major mutation as disclosed herein, carrier screening and prenatal diagnosis can be carried out as follows.

The high risk population for cystic fibrosis is Caucasians. For example, each Caucasian woman and/or man of childbearing age would be screened to determine if she or he was a carrier (approximately a 5% probability for each individual). If both are carriers, they are a couple at risk for a cystic fibrosis child. Each child of the at risk couple has a 25% chance of being affected with cystic fibrosis. The procedure for determining carrier status using the probes disclosed herein is as follows.

One major application of the DNA sequence information of the normal and mutant CF genes is in the area of genetic testing, carrier detection and prenatal diagnosis. Individuals carrying mutations in the CF gene (disease carrier or patients) may be detected at the DNA level with the use of a variety of techniques. The genomic DNA used for the diagnosis may be obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen and autopsy material. The DNA may be used directly for detection of specific sequence or may be amplified enzymatically in vitro by using PCR (Saiki et al. *Science* 230: 1350–1353, (1985), Saiki et al. *Nature* 324:

163–166 (1986)) prior to analysis. RNA or its cDNA form may also be used for the same purpose. Recent reviews of this subject have been presented by Caskey, (*Science* 236: 1223–8 (1989) and by Landegren et al (*Science* 242: 229–237 (1989).

The detection of specific DNA sequences may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al. *Cold Spring Harbour Symp. Quant. Biol.* 51: 257–261 (1986)), direct DNA sequencing (Church and Gilbert, *Proc. Nat. Acad. Sci. U. S. A.* 81: 1991–1995 (1988)), the use of restriction enzymes (Flavell et al. *Cell* 15: 25 (1978), Geever et al *Proc. Nat. Acad. Sci. U.S.A.* 78: 5081 (1981)), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis, *Cold Spring Harbour Sym. Quant. Biol.* 51: 275–284 (1986)), RNase protection (Myers, R. M., Larin, Y., and T. Maniatis *Science* 230: 1242 (1985)), chemical cleavage (Cotton et al *Proc. Nat. Acad. Sci. U.S.A.* 85: 4397–4401, (1985)) and the ligase-mediated detection procedure; (Landegren et al *Science* 241:1077 (1988)).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labelled radioactively with isotopes (such as ^{32}P) or non-radioactively (with tags such as biotin (Ward and Langer et al. *Proc. Nat. Acad. Sci. U. S. A.* 78: 6633–6657 (1981)), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis The presence or absence of these specific sequences are visualized by methods such as autoradiography or fluorometric (Landegren et al, 1989, supra) or colorimetric reactions (Gebeyehu et al. *Nucleic Acids Research* 15: 4513–4534 (1987)). An embodiment of this oligonucleotide screening method has been applied in the detection of the F508 deletion as described herein.

Sequence differences between normal and mutants may be revealed by the direct DNA sequencing method of Church and Gilbert (supra). Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR (Wrichnik et al, *Nucleic Acid Res.* 15:529–542 (1987); Wong et al, *Nature* 330:384–386 (1987); Stoflet et al, *Science* 239:491–494 (1988)). In the latter procedure, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent-tags.

Sequence alterations may occasionally generate fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization (Southern, *J. Mol. Biol* 98: 503 (1975)). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme; fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. For example, the PCR product with the 3 bp deletion is clearly distinguishable from the normal sequence on an 8% non-denaturing polyacrylamide gel. DNA fragments of different sequence compositions may be distinguished on denaturing formamide gradient gel in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperatures (Myers, supra). In addition, sequence alterations, in particular small deletions, may be detected as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis, as have been detected for the 3 bp (F508) mutation and in other experimental systems (Nagamine et al, *Am. J. Hum. Genet,* 45:337–339 (1989)). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length in a PCR. For example, one invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase (Myers, supra) and S1 protection (Berk, A. J., and P. A. Sharpe *Proc. Nat. Acad. Sci. U.S.A.* 75: 1274 (1978)), the chemical cleavage method (Cotton, supra) or the ligase-mediated detection procedure (Landegren supra).

In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments may also be visualized by methods where the individual DNA samples are not immobilized on membranes. The probe and target sequences may be both in solution or the probe sequence may be immobilized (Saiki et al, *Proc. Natl. Acad. Sci USA,* 86:6230–6234 (1989)). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving colorigenic reactions and fluorometry involving fluorogenic reactions, may be used to identify specific individual genotypes.

Since more than one mutation is anticipated in the CF gene, a multiples system is an ideal protocol for screening CF carriers and detection of specific mutations. For example, a PCR with multiple, specific oligonucleotide primers and hybridization probes, may be used to identify all possible mutations at the same time (Chamberlain et al. *Nucleic Acids Research* 16: 1141–1155 (1988)). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki et al, supra).

5.2 Detecting the Major Mutation

These detection methods may be applied to prenatal diagnosis using amniotic fluid cells, chorionic villi biopsy or sorting fetal cells from maternal circulation. The test for CF carriers in the population may be incorporated as an essential component in a broad-scale genetic testing program for common diseases.

According to an embodiment of the invention, the portion of the DNA segment that is informative for a mutation, such as the mutation according to this embodiment, that is, the portion that immediately surrounds the F508 deletion, can then be amplified by using standard PCR techniques (as reviewed in Landegren, Ulf, Robert Kaiser, C. Thomas Caskey, and Leroy Hood, DNA Diagnostics—Molecular Techniques and Automation, in *Science* 242: 229–237 (1988)). It is contemplated that the portion of the DNA segment which is used may be a single DNA segment or a mixture of different DNA segments. A detailed description of this technique now follows.

A specific region of genomic DNA from the person or fetus is to be screened. Such specific region is defined by the oligonucleotide primers is (5'GTTTTCCTGG-ATTATGCCTGGGCAC3') (SEQ ID NO:6) and C16D (5'GTTGGCATGCTTTGATGACGCTTC3') (SEQ ID NO:7). The specific regions were amplified by the polymerase chain reaction (PCR). 200–400 ng of genomic DNA, from either cultured lymphoblasts or peripheral blood samples of CF individuals and their parents, were used in each PCR with the oligonucleotides primers indicated above. The oligonucleotides were purified with Oligonucleotide Purification Cartridges™ (Applied Biosystems) or NENSORB™ PREP columns (Dupont) with procedures recommended by the suppliers. The primers were annealed at 62° C. for 45 sec, extended at 72° C. for 120 sec (with 2 units of Taq DNA polymerase) and denatured at 94° C. for 60 sec, for 28 cycles with a final cycle of 7% min for extension in a Perkin-Elmer/Cetus automatic thermocycler with a Step-Cycle program (transition setting at 1.5 min). Portions of the PCR products were separated by electrophoresis on 1.4% agarose gels, transferred to Zetabind™; (Biorad) membrane according to standard procedures. The two oligonucleotide probes of FIG. **15** (10 ng each) were labeled separately with 10 units of T4 polynucleotide kinase (Pharmacia) in a 10 μl reaction containing 50 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM spermidine, 1 mM EDTA and 30–40 μCi of $\gamma[^{32}P]$—ATP for 20–30 min at 37° C. The unincorporated radionucleotides were removed with a Sephadex G-25 column before use. The hybridization conditions were as described previously (J. M. Rommens et al *Am. J. Hum. Genet.* 43,645 (1988)) except that the temperature was 37° C. The membranes were washed twice at room temperature with 5×SSC and twice at 39° C. with 2×SSC (1×SSC=150 mM NaCl and 15 mM Na citrate). Autoradiography was performed at room temperature overnight. Autoradiographs show the hybridization results of genomic DNA with the 2 specific oligonucleotide probes as indicated in FIG. **15**. Probe C detects the normal DNA sequence and Probe$^-$F detects the mutant sequence. Genomic DNA sample from each family member was amplified by the polymerase chain reaction and the products separated by electrophoresis on a 1.4% agarose gel and then transferred to Zetabind (Biorad) membrane according to standard procedures. Water blank and plasmid DNA, T16 and C16, corresponding to the normal sequence (N) and the F508 deletion (CF), respectively, were included as controls.

The 3 bp deletion was also revealed by polyacrylamide gel electrophoresis. When the PCR generated by the above-mentioned C16B and C16D primers were applied to an 8% polyacrylamide gel, electrophoresed for 2 hrs at 20V/cm in a 90 mM Tris-borate buffer (pH 8.3), DNA fragments of a different mobility were clearly detectable for individuals without the 3 bp deletion, heterozygous or homozygous for the deletion. In addition, an extra DNA band, presumably the heteroduplex between normal and mutant DNA strands, was noted in heterozygotes. Similar alteration in gel mobility for heteroduplexes formed during PCR has also been reported for experimental systems where small deletions are involved (Nagamine at al supra). These mobility shifts may be used as the basis for the non-radioactive genetic screening tests.

5.3 CF Screening Programs

It is appreciated that only 70% of the carriers can be detected using the specific F508 probes of this particular embodiment of the invention. Thus, if an individual tested is not a carrier using the F508 probes, their carrier status can not be excluded, they may carry some other mutation as previously noted. However, if both the individual and the spouse of the individual tested are a carrier for the F508 mutation, it can be stated with certainty that they are an at risk couple. The sequence of the gene as disclosed herein is an essential prerequisite for the determination of the other mutations.

Prenatal diagnosis is a logical extension of carrier screening. A couple can be identified as at risk for having a cystic fibrosis child in one of two ways: if they already have a cystic fibrosis child, they are both, by definition, obligate carriers of the disease, and each subsequent child has a 25% chance of being affected with cystic fibrosis. A major advantage, of the present invention eliminates the need for family pedigree analysis, whereas, according to this invention, a gene mutation screening program as outlined above or other similar method can be used to identify a genetic mutation that leads to a protein with altered function. This is not dependent on prior ascertainment of the family through an affected child. Fetal DNA samples, for example, can be obtained, as previously mentioned, from amniotic fluid cells and chorionic villi specimens. Amplification by standard PCR techniques can then be performed on this template DNA.

If both parents are shown to be carriers with the F508 deletion, the interpretation of the results would be the following. If there is hybridization of the fetal DNA to the normal (no deletion, as shown in FIG. **15**) probe, the fetus will not be affected with cystic fibrosis, although it may be a CF carrier (50% probability for each fetus of an at risk couple). If the fetal DNA hybridizes only to the F508 deletion probe and not to the normal probe (as shown in FIG. **15**), the fetus will be affected with cystic fibrosis.

It is appreciated that for this and other mutations in the CF gene, a range of different specific procedures can be used to provide a complete diagnosis for all potential CF carriers or patients. A complete description of these procedures is later described.

The invention therefore provides a method and kit for determining if a subject is a CF carrier or CF patient. In summary, the screening method comprises the steps of:

providing a biological sample of the subject to be screened; and providing an assay for detecting in the biological sample, the presence of at least a member from the group consisting of the normal CF gene, normal CF gene products, a mutant CF gene, mutant CF gene products and mixtures thereof.

The method may be further characterized by including at least one more nucleotide probe which is a different DNA sequence fragment of, for example, the DNA of FIGS. **1A–1H**, or a different DNA sequence fragment of human chromosome 7 and located to either side of the DNA sequence of FIGS. **1A–1H**.

A kit, according to an embodiment of the invention, suitable for use in the screening technique and for assaying for the presence of the CF gene by an immunoassay comprises:

(a) an antibody which specifically binds to a gene product of the CF gene;

(b) reagent means for detecting the binding of the antibody to the gene product; and

(c) the antibody and reagent means each being present in amounts effective to perform the immunoassay.

The kit for assaying for the presence for the CF gene may also be provided by hybridization techniques. The kit comprises:

(a) an oligonucleotide probe which specifically binds to the CF gene;

(b) reagent means for detecting the hybridization of the oligonucleotide probe to the CF gene; and

(c) the probe and reagent means each being present in amounts effective to perform the hybridization assay.

5.4 Antibodies to Detect CFTR

As mentioned, antibodies to epitopes within the CFTR protein are raised to provide extensive information on the characteristics of the protein and other valuable information which includes:

1. To enable visualization at the protein in cells and tissues in which it is expressed by immunoblotting ("Western blots") following polyacrylamide gel electrophoresis. This allows an estimation of the molecular size of the mature protein including the contribution from the cells of post-translationally added moieties including oligosaccharide chains and phosphate groups, for example. Immunocytochemical techniques including immunofluorescence and immuno-electronmicroscopy can be used to establish the subcellular localization of the protein in cell membranes. The antibodies can also be used to provide another technique in detecting any of the other CF mutations which result in the synthesis of a protein with an altered size.
2. Antibodies to distinct domains of the protein can be used to determine the topological arrangement of the protein in the cell membrane. This provides information on segments of the protein which are accessible to externally added modulating agents for purposes of drug therapy.
3. The structure-function relationships of portions of the protein can be examined using specific antibodies. For example, it is possible to introduce into cells antibodies recognizing each of the charged cytoplasmic loops which join the transmembrane sequences as well as portions of the nucleotide binding folds and the R-domain. The influence of these antibodies on functional parameters of the protein provide insight into cell regulatory mechanisms and potentially suggest means or modulating the activity of the defective protein in a CF patient.
4. Antibodies with the appropriate avidity also enable immunoprecipitation and immuno-affinity purification of the protein. Immunoprecipitation will facilitate characterization of synthesis and post translational modification including ATP binding and phosphorylation. Purification will be required for studies of protein structure and for reconstitution of its function, as well as protein based therapy.

In order to prepare the antibodies, fusion proteins containing defined portions of CFTR polypeptides have been synthesized in bacteria by expression of corresponding DNA sequences in a suitable cloning vehicle whereas smaller peptides were synthesized chemically as described in Table 8. The fusion proteins were purified, for example, by affinity chromatography on glutathione-agarose and the peptides were coupled to a carrier protein (hemocyanin), mixed with Freund's adjuvant and injected into rabbits. Following booster injections at bi-weekly intervals, the rabbits were bled and sera isolated. The stained fusion proteins are shown in FIG. **19**A. Lane 1, uninduced control plasmid; lane 2, IPTG-induced control plasmid expressing just glutathione-S-transferase (GST); lane 3, affinity purified GST band at 27 kilodaltons (kD); lane 4 is uninduced, lane 5 is induced and lane 6 is the purified fusion protein #1 of Table 8. In FIG. **19**B, the gel electrophoresis is of lysates from bacteria transformed with pGEX plasmids containing fusion proteins #5 of Table 8 for lanes 1 and 2 and fusion proteins #2 of Table 8 for lanes 3 and 4. Lane 1 of FIG. **19**B is for the uninduced plasmid whereas lane 2 is for the induced plasmid to express the fusion protein #5. Lane 3 of FIG. **19**B is for the uninduced plasmid whereas lane 4 is for the induced plasmid to express the fusion protein #2. Immunoblots of fusion protein #1 probed with antisera obtained from the second bleeds of two different rabbits are shown in FIG. **20**. The staining is with alkaline-phosphatase conjugated second antibody (Blake et al. *Anal. Biochem.* 136:175, (1984)). Both of these immune sera stain the 32 kD fusion protein whereas the preimmune sera do not. FIG. **21** shows the reactivity of one of these immune sera with a band of approximately 200 kD in size in membranes isolated from T-84 colonic carcinoma cells which express the CFTR transcript at a high level. This band is in the size range which might be expected for the CFTR protein which has a predicted molecular weight of 169 kD prior to post-translational modifications.

Sera from rabbits immunized with the LKH conjugate of peptide #2 were screened again both pure peptide and KLH as shown in FIG. **22**. In this Figure, H denotes hemocyanin; P1, peptide #1; P2, peptide #2. Amounts of protein or peptide dotted in ng are indicated. This antiserumn detects as little as 1 ng of the peptide and does not react at all with control paptide #1.

Thus, it is possible to raise polyclonal antibodies specific for both fusion proteins containing portions of the CFTR protein and peptides corresponding to short segments of its sequence. Similarly, mice can be injected with KLH conjugates of peptides 1, 2 and 7 of Table 8 to initiate the production of monoclonal antibodies to these segments of CFTR protein. Monoclonal antibodies can be similarly raised to other domains of the CFTR protein.

As for the generation of polyclonal antibodies, immunogens for the raising of monoclonal antibodies (mAbs) to the CFTR protein are bacterial fusion proteins (Smith et al, *Gene* 67:31 (1988)) containing portions of the CFTR polypeptide or synthetic peptides corresponding to short (12 to 25 amino acids in length) segments of the sequence. The essential methodology is that of Kohler and Milstein (*Nature* 256: 495 (1975)).

Balb/c mice are immunized by intraperitoneal injection with 500 μg of pure fusion protein or synthetic peptize in incomplete Freund's adjuvant. A second injection is given after 14 days, a third after 21 days and a fourth after 28 days. Individual animals, so immunized are sacrificed one, two and four weeks following the final injection. Spleens are removed, their cells dissociated, collected and fused with Sp2/O-Ag14 myeloma cells according to Gefter et al, *Somatic Cell Genetics* 3:231 (1977). The fusion mixture is distributed in culture medium selective for the propagation of fused cells which are grown until they are about 25% confluent. At this time, culture supernatants are tested for the presence of antibodies reacting with a particular CFTR antigen. An alkaline phosphatase labelled anti-mouse second antibody is then used for detection of positives. Cells from positive culture wells are then expanded in culture, their supernatants collected for further testing and the cells stored deep frozen in cryoprotectant-containing medium. To obtain large quantities of a mAb, producer cells are injected into the

peritoneum at 5×10^6 cells per animal, and ascites fluid is obtained. Purification is by chromatography on Protein G- or Protein A-agarose according to Ey et al, *Immunochemistry* 15:429 (1977).

Reactivity of these mAbs with the CFTR protein is confirmed by polyacrylamide gel electrophoresis of membranes isolated from epithelial cells in which it is expressed and immunoblotting (Towbin et al. *Proc. Natl. Acad. Sci. USA* 76:4350 (1979)).

In addition to the use of monoclonal antibodies specific for each of the different domains of the CFTR protein to probe their individual functions, other mAbs, which can distinguish between the normal and mutant forms of CFTR protein, are used to detect the mutant protein in epithelial cell samples obtained from patients, such as nasal mucosa biopsy "brushings" (R. De-Lough and J. Rutland, *J. Clin. Pathol.* 42, 613 (1989)) or skin biopsy specimens containing sweat glands.

Antibodies capable of this distinction are obtained by differentially screening hybridomas from paired sets of mice immunized with a peptide containing the phenylalanine at amino acid position 508 (e.g. GTIKENIIFGVSY) or a peptide which is identical except for the absence of F508 (GTIKENIIGVSY). mAbs capable of recognizing the other mutant forms of CFTR protein present in patients in addition or instead of F508 deletion are obtained using similar monoclonal antibody production strategies.

Antibodies to normal and CF versions of CFTR protein and of segments thereof are used in diagnostically immunocytochemical and immunofluorescence light microscopy and immunoelectron microscopy to demonstrate the tissue, cellular and subcellular distribution of CFTR within the organs of CF patients, carriers and non-CF individuals.

Antibodies are used to therapeutically modulate by promoting the activity of the CFTR protein in CF patients and in cells of CF patients. Possible modes of such modulation might involve stimulation due to cross-linking of CFTR protein molecules with multivalent antibodies in analogy with stimulation or some cell surface membrane receptors, such as the insulin receptor (O'Brien et al, *Euro. Mol. Biol. Organ. J.* 6:4003 (1987)), epidermal growth factor receptor (Schreiber et al, *J. Biol. Chem.* 258:846 (1983)) and T-cell receptor-associated molecules such as CD4 (Veillette et al *Nature,* 338:257 (1989)).

Antibodies are used to direct the delivery of therapeutic agents to the cells which express detective CFTR protein in CF. For this purpose, the antibodies are incorporated into a vehicle such as a liposome (Matthay et al, *Cancer Res.* 46:4904 (1986)) which carries the therapeutic agent such as a drug or the normal gene.

TABLE 8

CFTR FRAGMENTS USED TO RAISE ANTIBODIES

GST[a] fusion proteins containing CFTR residues	CFTR Domain of FIG. 13
1. 204–249	TM3, Ext. 2, TMA
2. 347–698	NBF-1, N-term ½ R-domain
3. 710–757	Neg. charged middle of R-domain
4. 758–796	Pos. charged segment of R-domain
5. 1188–1480	C-term. cyto.domain with NBF-2

TABLE 8-continued

CFTR FRAGMENTS USED TO RAISE ANTIBODIES

KLH[b] conjugates containing CFTR peptides:	CFTR Domain of FIG. 13
1. 28–45	N-term. cytoplasmic
2. 58–75	N-term. cytoplasmic
3. 104–117	1st extracellular
4. 139–153	2nd cytoplasmic
5. 279–294	N-term. of 3rd cytoplasmic
6. 500–512	NBF-1: around the F508 deletion
7. 725–739	Charged middle of R-domain
8. 933–946	5th cytoplasmic
9. 1066–1084	6th cytoplasmic

[a]restriction fragments coding for these fragments ligated to 3' end of glutathione S-transferase (GST) of *Schistosoma japonicum* in pGEX plasmid expression vector as identified in Smith et al, Gene 67:31, (1988).
[b]Peptides coupled through an N-terminal cysteine to the carrier protein keyhole limpet hemocyanin (KLH) according to Green et al Cell 28:477 (1982). TM denotes transmembrane sequences.

5.5 RFLP Analysis

This invention provides a number of benefits stemming directly from the discovery and characterization of the CF gene which are of immediate practical application. The amino acid sequence of CFTR provides insight into the structure and function of the protein as well as the molecular mechanisms in which CFTR participates and which are defective in cystic fibrosis. This information enables the generation of further tools and concepts, in research on and therapy for this disease.

Carrier detection, DNA diagnosis and family counselling are some of the applications of the invention. Previously DNA-based genetic testing for CF has primarily been available to families with affected children and to their close relatives. Knowledge of the CF mutations at the DNA sequence level permits testing of any random individual; our estimate shows that 46% of CF patients without a previous family history can be accurately diagnosed by DNA analysis, and 68% of the CF carriers in the population can be identified via the F508 deletion.

Given that the carrier frequency in the North American population is approximately 1 in 20, it is feasible to screen all women and/or men of child-bearing age, for example, for their carrier status. Carrier detection using probes specific for the F508 deletion will pick up 70% of the carriers. The remaining carriers will be detected by a battery of probes specific for the various haplotype groups identified above.

Since the F508 deletion constitutes about 70% of all CF mutations, RFLP analysis may be used in supplement to the direct deletion testing for family members or close relative of CF patients. About 55% of the CF parents not carrying the F508 mutation are expected to be informative for the DNA marker JG2E1 (KM19) (Kerem et al *Am. J. Hum. Genet* 44:827–834 (1989); Estivill et al, *Genomics* 1:257 (1987)) based on retrospective analysis of our CF linkage families; an additional 39% would be informative if E6 (Taq I) (Kerem et al supra) and J3.11 (Msp I) (Wainright et al *Nature* (1985)) were also tested; virtually all parents would be informative if H2.3 (XV2C-Taq I) (Kerem et al, supra; Estivill et al, *Nature* (1987)), E2.6 (E.9) (Msp I) (probe available on request), E4.1 (Mp6d.9) (Msp I) (probe available upon request; Estivill et al, *Am. J. Hum. Genet.* (1989)), J44 (E3.1) (Xba I) (probe available on request) and metD

(Ban I) (Spence et al, *Am. J. Hum. Genet* (1986), (ATCC #40219) were included.

The utility of theme probes lies in the fact that they recognize polymorphic restriction sites. Thus, the probes are typically not defined by their sequence across the particular polymorphic site, but rather, can be utilized based on knowledge of flanking sequences, allowing for polymerase chain reaction (PCR) generation of the region in question, as would be known by one skilled in the art.

For example, the probe E2.6 (Msp I) is completely defined by two flanking oligomers:

5'GTGATCCAGTTTGCTCTCCA3' (SEQ ID NO:10), and 5'GGAATCACTCTTCCTGATAT3' (SEQ ID NO:11). Use of this E2.6 PCR generated probe to detect an Msp I polymorphism will detect two different alleles; either one 850 bp fragment, or a 490 bp and a 360 bp fragment, depending on the presence or absence of the Msp I site. Similarly, the probe J44 (E3.1) (Xba I) is completely defined by two flanking oligomers:

5'CAATGTGATTGGTGAAACTA3' (SEQ ID NO:12), and 5'CTTCTCCTCCTAGACACCTGCAT3' (SEQ ID NO:13). Use of this J44 (E3.1) PCR generated probe to detect an Xba I polymorphism will detect two different alleles: either an 860 bp fragment or a 610 bp and a 250 bp fragment, depending on the presence or absence of the Xba I site.

The linked RFLPs may also be used in risk calculation for individuals who do not carry the F508 deletion. A general risk estimate procedure has been discussed in Beaudet et al *Am. J. Hum. Genet* 44:319–326).

For prenatal diagnosis, microvillar intestinal enzyme analysis (Brock, *Lancet* 2: 941 (1983)) may be performed to increase the confidence of diagnosis in cases where DNA diagnosis is inconclusive.

DNA diagnosis is currently being used to assess whether a fetus will be born with cystic fibrosis, but historically this has only been done after a particular set of parents has already had one cystic fibrosis child which identifies them as obligate carriers. However, in combination with carrier detection as outlined above, DNA diagnosis for all pregnancies of carrier couples will be possible. If the parents have already had a cystic fibrosis child, an extended haplotype analysis can be done on the fetus and thus the percentage of false positive or false negative will be greatly reduced. If the parents have not already had an affected child and the DNA diagnosis on the fetus is being performed on the basis of carrier detection, haplotype analysis can still be performed.

Although it has been thought for many years that there is a great deal of clinical heterogeneity in the cystic fibrosis disease, it is now emerging that there are two general categories, called pancreatic sufficiency (CF-PS) and pancreatic insufficiency (CF-PI). If the mutations related to these disease categories are well characterized, one can associate a particular mutation with a clinical phenotype of the disease. This allows changes in the treatment of each patient. Thus the nature of the mutation will to a certain extent predict the prognosis of the patient and indicate a specific treatment.

6.0 Molecular Biology of Cystic Fibrosis

The postulate that CFTR may regulate the activity of ion channels, particularly the outwardly rectifying C1 channel implicated as the functional defect in CF, can be tested by the injection and translation of full length in vitro transcribed CFTR mRNA in Xenopus oocytes. The ensuing changes in ion currents across the oocyte membrane can be measured as the potential is clamped at a fixed value. CFTR may regulate endogenous oocyte channels or it may be necessary to also introduce epithelial cell RNA to direct the translation of channel proteins. Use of mRNA coding for normal and for mutant CFTR, as provided by this invention, makes these experiments possible.

Other modes of expression in heterologous cell system also facilitate dissection of structure-function relationships. The complete CFTR DNA sequence ligated into a plasmid expression vector is used to transfect cells so that its influence on ion transport can be assessed. Plasmid expression vectors containing part of the normal CFTR sequence along with portions of modified sequence at selected sites can be used in vitro mutagenesis experiments performed in order to identify those portions of the CFTR protein which are crucial for regulatory function.

6.1 Expression of DNA Sequence

The DNA sequence can be manipulated in studies to understand the expression of the gene and its product, and, to achieve production of large quantities of the protein for functional analysis, antibody production, and patient therapy. The changes in the sequence may or may not alter the expression pattern in terms of relative quantities, tissue-specificity and functional properties. The partial or full-length cDNA sequences, which encode for the subject protein, unmodified or modified, may be ligated to bacterial expression vectors such as the pRIT (Nilsson et al. *EMBO J.* 4: 1075–1080 (1985)), pGEX (Smith and Johnson, *Gene* 67: 31–40 (1988)) or pATH (Spindler et al. *J. Virol.* 49: 132–141 (1984)) plasmids which can be introduced into *E. coli* cells for production of the corresponding proteins which may be isolated in accordance with the previously discussed protein purification procedures. The DNA sequence can also be transferred from its existing context to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal virus, yeast artificial chromosomes (YAC) (Burke et al. *Science* 236: 806–812, (1987)), somatic cells, and other simple or complex organisms, such as bacteria, fungi (Timberlake and Marshall, *Science* 244: 1313–1317 (1989), invertebrates, plants (Gasser and Fraley, *Science* 244: 1293 (1989), and pigs (Pursel et al. *Science* 244: 1281–1288 (1989)).

For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus (SV) 40, promoter in the pSV2 vector (Mulligan and Berg, *Proc. Natl. Acad. Sci USA,* 78:2072–2076 (1981)) and introduced into cells, such as monkey COS-1 cells (Gluzman, *Cell,* 23:175–182 (1981)), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg, *J. Mol. Appln. Genet.* 1:327–341 (1982)) and mycophoenolic acid (Mulligan and Berg, supra).

DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR.

The cDNA sequence (or portion& derived from it), or a mini gene (a cDNA with an intron and its own promoter) is introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the

transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the simian virus (SV)40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV 40 are readily available (Mulligan et al *Proc. Natl. Acad. Sci. USA* 78:1078–2076, (1981); Gorman et al *Proc Natl. Acad. Sci USA* 79: 6777–6781 (1982)). Alternatively, the CFTR endogenous promoter may be used. The level of expression of the cDNA can be manipulated with this type of vector, either by using promoters that have different activities (for example, the baculovirus pAC373 can express cDNAs at high levels in *S. frungiperda* cells (M. D. Summers and G. E. Smith in, Genetically Altered Viruses and the Environment (B. Fields, et al, eds.) vol. 22 no 319–328, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y., 1985) or by using vectors that contain promoters amenable to modulation, for example the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee et al, *Nature* 294:228 (1982)). The expression of the cDNA can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

In addition, some vectors contain selectable, markers [such as the gpt (Mulligan et Berg supra) or neo (Southern and Berg *J. Mol. Appln. Genet* 1:327–341 (1982)) bacterial genes that permit isolation of cells, by chemical selection, that have stable, long term expression of the vectors (and therefore the cDNA) in the recipient cell. The vectors can be maintained in the cells as episomal, freely replicating entities by fusing regulatory elements of viruses such at papilloma (Sarver et al *Mol. Cell Biol.* 1:486 (1981)) or Epstein-Barr (Sugden et al *Mol. Cell Biol.* 5:410 (1985)). Alternatively, one can also produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the cDNA as well) to create cell lines that can produce high levels or the gene product (Alt et al. *J. Biol. Chem.* 253: 1357 (1978)).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, *Virology* 52:466 (1973)) or strontium phosphate (Brash et al *Mol. Cell Biol.* 7:2013 (1987)), electroporation (Neumann et al *EMBO J* 1:841 (1982)), lipofection (Felgner et al *Proc Natl. Acad. Sci USA* 84:7413 (1987)) DEAE dextran (McCuthan et al *J. Natl Cancer Inst.* 41:351 1968)), microinjection (Mueller et al *Cell* 15:579 1978)), protoplast fusion (Schafner, *Proc Natl. Aca. Sci USA* 72:2163) or pellet guns (Klein et al, *Nature* 327: 70 (1987)). Alternatively, the cDNA can be introduced by infection with virus vectors. Systems are developed that uses for example, retroviruses (Bernstein et al. *Genetic Engineering* 7: 235, (1985)), adenovirumes (Ahmad et al *J. Virol* 57:267 (1986)) or Herpes virus (Spaete et al *Cell* 30:295 (1982)).

These eukaryotic expression systems can be used for many studies of the CF gene and the CFTR product. These include, for example: (1) determination that the gene is properly expressed and that all post-translational modifications necessary for full biological activity have been properly completed (2) identify regulatory elements located in the 5' region of the CF gene and their role in the tissue- or temporal-regulation of the expression of the CF gene (3) production of large amounts of the normal protein for isolation and purification (4) to use cells expressing the CFTR protein as an assay system for antibodies generated against the CFTR protein or an assay system to test the effectiveness of drugs, (5) study the function of the normal complete protein, specific portions of the protein, or of naturally occurring or artificially produced mutant proteins. Naturally occurring mutant proteins exist in patients with CF while artificially produced mutant protein can be designed by site directed sequence-alterations. These latter studies can probe the function of any desired amino acid residue in the protein by mutating the nucleotides coding for that amino acid.

Using the above techniques, the expression vectors containing the CF gene sequence or fragments thereof can be introduced into human cells, mammalian cells from other species or non-mammalian cells as desired. The choice of cell is determined by the purpose of the treatment. For example, one can use monkey COS cells (Gluzman, *Cell* 23:175 (1981)), that produce high levels of the SV40 T antigen and permit the replication of vectors containing the SV40 origin of replication, can be used to show that the vector can express the protein, product, since function is not required. Similar treatment could be performed with Chinese hamster ovary (CHO) or mouse NIH 3T3 fibroblasts or with human fibroblasts or lymphoblasts.

The recombinant cloning vector, according to this invention, then comprises the selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that normal CFTR polypeptide can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be specifically selected from the group consisting of the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

The host cell, which may be transfected with the vector of this invention, may be selected from the group consisting of *E. coli,* Pseudomonas, *Bacillus subtilis, Bacillus stearothermophilus* or other bacili; other bacteria; yeast; fungi; insect; mouse or other animal; or plant hosts; or human tissue cells.

It is appreciated that for the mutant DNA sequence similar systems are employed to express and produce the mutant product.

6.2 Protein Function Considerations

To study the function of the CFTR protein, it is preferable to use epithelial cells as recipients, since proper functional expression may require the presence of other pathways or gene products that are only expressed in such cells. Calls that can be used include, for example, human epithelial cell lines such as T84 (ATCC #CRL 248) or PANC-1 (ATCC # CLL 1469), or the T43 immortalized CF nasal epithelium cell line (Jettan et al, *Science* (1989)) and primary (Yanhoskes et al. *Ann. Rey. Resp. Dis,* 132: 1281 (1985)) or transformed (Scholte et al. *Exp. Cell. Res.* 182: 559(1989)) human nasal polyp or airways cells, pancreatic cells (Harris

and Coleman *J. Cell. Sci.* 87: 695 (1987)), or sweat gland cells (Collie et al. *In Vitro* 21: 597 (1985)) derived from normal or CF subjects. The CF cells can be used to test for the functional activity of mutant CF genes. Current functional assays available include the study of the movement of anions (Cl or I) across cell membranes as a function of stimulation of cells by agents that raise intracellular AMP levels and activate chloride channels (Stutto et al. *Proc. Nat. Acad. Sci. U.S.A.* 82: 6677 (1985)). Other assays include the measurement or changes in cellular potentials by patch clamping of whole cells or of isolated membranes (Frizzell et al. *Science* 233: 558 (1986), Welsch and Liedtke *Nature* 322: 467 (1986)) or the study of ion fluxes in epithelial sheets of confluent cells (Widdicombe et al. *Proc. Nat. Acad. Sci.* 82: 6167 (1985)). Alternatively, RNA made from the CF gene could be injected into Xenopuns oocytes. The oocyte will translate RNA into protein and allow its study. As other more specific assays are developed these can also be used in the study of transfected CFTR protein function.

"Domain-switching" experiments between CFTR and the human multidrug resistance P-glycoprotein can also be performed to further the study of the CFTR protein. In these experiments, plasmid expression vectors are constructed by routine techniques from fragments or the CFTR sequence and fragments of the sequence of P-glycoprotein ligated together by DNA ligase so that a protein containing the respective portions of these two proteins will be synthesized by a host cell transfected with the plasmid. The latter approach has the advantage that many experimental parameters associated with multidrug resistance can be measured. Hence, it is now possible to assess the ability of segments of CFTR to influence these parameters.

These studies of the influence of CFTR on ion transport will serve to bring the field of epithelial transport into the molecular arena. This is the first transport related molecule from epithelial cells for which the complete primary structure is shown. Knowledge of CFTR can be used to better understand at a molecular level the characteristics of the epithelial cell membrane in this area. For example, the molecules in closest proximity to CFTR can be determined by cross-linking experiments. The hypothesis that the role of CFTR is to regulate ion channels would predict that these channels would necessarily fall into that category. The large, high quality cDNA libraries constructed for the cloning of CFTR cDNAs will also be useful for the molecular cloning of cDNAs for polypeptides constituting other epithelial ion transport systems, including other channels as well as co-, counter-, and active-transport systems.

6.3 Therapies

It is understood that the major aim of the various biochemical studies using the compositions of this invention is the development of therapies to circumvent or overcome the CF defect, using both the pharmacological and the "gene-therapy" approaches.

In the pharmacological approach, drugs which circumvent or overcome the CF defect are sought. Initially, compounds may be tested essentially at random, and screening systems are required to discriminate among many candidate compounds. This invention provides host cell systems, expressing various of the mutant CF genes, which are particularly well suited for use as first level screening systems. Preferably, a cell culture system using mammalian cells (most preferably human cells) transfected with an expression vector comprising a DNA sequence coding for CFTR protein containing a CF-generating mutation, for example the F508 deletion, is used in the screening process. Candidate drugs are tested by incubating the cells in the presence of the candidate drug and measuring those cellular functions dependent on CFTR, especially by measuring ion currents where the transmembrane potential is clamped at a fixed value. To accommodate the large number of assays, however, more convenient assays are based, for example, on the use of ion-sensitive fluorescent dyes. To detect changes in Cl^-ion concentration SPQ or its analogues are useful.

Alternatively, a cell-free system could be used. Purified CFTR could be reconstituted into articifial membranes and drugs could be screened in a cell-free assay (Al-Aqwatt, *Science,* (1989)).

At the second level, animal testing is required. It is possible to develop a model of CF by interfering with the normal expression of the counterpart of the CF gene in an animal such as the mouse. The "knock-out" of this gene by introducing a mutant form of it into the germ line of animals will provide a strain of animals with CF-like syndromes. This enables testing of drugs which showed a promise in the first level cell-based screen.

As further knowledge is gained about the nature of the protein and its function, it will be possible to predict structures of proteins or other compounds that interact with the CFTR protein. That in turn will allow for certain predictions to be made about potential drugs that will interact with this protein and have some effect on the treatment of the patients. Ultimately such drugs may be designed and synthesized chemically on the basis of structures predicted to be required to interact with domains of CFTR. This approach is reviewed in Capsey and Delvatte, *Genetically Engineered Human Therapeutic Drugs* Stockton Press, New York, 1988. These potential drugs must also be tested in the screening system.

6.3.1 Protein Replacement Therapy

Treatment of CF can be performed by replacing the defective protein with normal protein, by modulating the function of the defective protein or by modifying another step in the pathway in which CFTR participates in order to correct the physiological abnormality.

To be able to replace the defective protein with the normal version, one must have reasonably large amounts of pure CFTR protein. Pure protein can be obtained as described earlier from cultured cell systems. Delivery of the protein to the affected airways tissue will require its packaging in lipid-containing vesicles that facilitate the incorporation of the protein into the cell membrane. It may also be feasible to use vehicles that incorporate proteins such as surfactant protein, such as SAP(Val) or SAP(Phe) that performs this function naturally, at least for lung alveolar cells. (PCT Patent Application WO/8803170, Whitsett et al, May 7, 1988 and PCT Patent Application WO89/04327, Benson et al, May 18, 1989). The CFTR-containing vesicles are introduced into the airways by inhalation or irrigation, techniques that are currently used in CF treatment (Boat et al, supra).

6.3.2 Drug Therapy

Modulation of CFTR function can be accomplished by the use of therapeutic agents (drugs). These can be identified by random approaches using a screening program in which their effectiveness in modulating the defective CFTR protein is monitored in vitro. Screening programs can use cultured cell systems in which the defective CFTR protein is expressed. Alternatively, drugs can be designed to modulate CFTR activity from knowledge of the structure and function

correlations of CFTR protein and from knowledge of the specific defect in the various CFTR mutant proteins (Capsey and Delvatte, supra). It is possible that each mutant CFTR protein will require a different drug for specific modulation. It will then be necessary to identify the specific mutation(s) in each CF patient before initiating drug therapy.

Drugs can be designed to interact with different aspects of CFTR protein structure or function. For example, a drug (or antibody) can bind to a structural fold of the protein to correct a defective structure. Alternatively, a drug might bind to a specific functional residue and increase its affinity for a substrate or cofactor. Since it is known that members of the class of proteins to which CFTR has structural homology can interact, bind and transport a variety of drugs, it is reasonable to expect that drug-related therapies may be effective in treatment of CF.

A third mechanism for enhancing the activity of an effective drug would be to modulate the production or the stability of CFTR inside the cell. This increase in the amount of CFTR could compensate for its defective function.

Drug therapy can also be used to compensate for the defective CFTR function by interactions with other components of the physiological or biochemical pathway necessary for the expression of the CFTR function. These interactions can lead to increases or decreases in the activity of these ancillary proteins. The methods for the identification of these drugs would be similar to those described above for CFTR-related drugs.

In other genetic disorders, it has been possible to correct for the consequences of altered or missing normal functions by use of dietary modifications. This has taken the form of removal of metabolites, as in the case of phenylketonuria, where phenylalanine is removed from the diet in the first five years of life to prevent mental retardation, or by the addition of large amounts of metabolites to the diet, as in the case of adenosime deaminase deficiency where the functional correction of the activity of the enzyme can be produced by the addition of the enzyme to the diet. Thus, once the details of the CFTR function have been elucidated and the basic defect in CF has been defined, therapy may be achieved by dietary manipulations.

The second potential therapeutic approach is so-called "gene-therapy" in which normal copies of the CF gene are introduced in to patients so as to successfully code for normal protein in the key epithelial cells of affected tissues. It is most crucial to attempt to achieve this with the airway epithelial cells of the respiratory tract. The CF gene is delivered to these cells in a form in which it can be taken up and code for sufficient protein to provide regulatory function. As a result, the patient's quality and length of life will be greatly extended. Ultimately, of course, the aim is to deliver the gene to all affected tissues.

6.3.3 Gene Therapy

One approach to therapy of CF is to insert a normal version of the CF gene into the airway epithelium of affected patients. It is important to note that the respiratory system is the primary cause of mordibity and mortality in CF; while pancreatic disease is a major feature, it is relatively well treated today with enzyme supplementation. Thus, somatic cell gene therapy (for a review, see T. Friedmann, *Science* 244:1275 (1989)) targeting the airway would alleviate the most severe problems associated with CF.

A. Retroviral Vectors

Retroviruses have been considered the preferred vector for experiments in somatic gene therapy, with a high efficiency of infection and stable integration and expression (Orkin et al *Prog. Med. Genet* 7:130, (1988)). A possible drawback is that cell division is necessary for retroviral integration, so that the targeted cells in the airway may have to be nudged into the cell cycle prior to retroviral infection, perhaps by chemical means. The full length CF gene cDNA can be cloned into a retroviral vector and driven from either its endogenous promoter or from the retroviral LRT (long terminal repeat). Expression of levels of the normal protein as low as 10% or the endogenous mutant protein in CF patients would be expected to be beneficial, since this is a recessive disease. Delivery of the virus could be accomplished by aerosol or instillation into the trachea.

B. Other Viral Vectors

Other delivery systems which can be utilized include adeno-associated virus (AAV, McLaughlin et al, *J. Virol* 62:1963 (1986)), vaccinia virus (Moss et al *Annu. Rev.Immuno,* 5:305, 1987)), bovine papilloma virus [Rasmussen et al, *Methods Enzymol* 139:642 (1987)) or member of the herpeavirus group such as Epstein-Barr virus (Margolskee et al *Mol. Cell. Biol* 8:2937 (1988)). Though much would need to be learned about their basic biology, the idea of using a viral vector with natural tropism for the respiratory tree (e.g. respiratory syncytial virus, echovirus, Coxsackie virus, etc.) is possible.

C. Non-viral Gene Transfer

Other methods of inserting the CF gene into respiratory epithelium may also be productive; many of these are lower efficiency and would potentially require infection in vitro, selection of transfectants, and reinplantation. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. A particularly attractive idea is the use of liposome, which might be possible to carry out in vivo (Ostro, *Liposomes,* Marcel-Dekker, 1987). Synthetic cationic lipids such as DOTMA (Felger et al *Proc. Natl. Acad.Sci USA* 84:7413 (1987)) may increase the efficiency and ease of carrying out this approach.

6.4 CF Animal Models

The creation of a mouse or other animal model for CF will be crucial to understanding the disease and for testing of possible therapies (for general review of creating animal models, see Erickson, *Am. J. Hum. Genet* 43:582 (1988)). Currently no animal model of the CF exists. The evolutionary conservation or the CF gene (as demonstrated by the cross-species hybridization blots for E4.3 and H1.6), as is shown in FIGS. **4A–4D**, indicate that an orthologous gene exists in the mouse (hereafter to be denoted mCF, and its corresponding protein as mCFTR), and this will be possible to clone in mouse genomic and cDNA libraries using the human CF gene probes. It is expected that the generation of a specific mutation in the mouse gene analogous to the F508 mutation will be most optimum to reproduce the phenotype, though complete inactivation of the mCFTR gene will also be a useful mutant to generate.

A. Mutagenesis

Inactivation of the mCF gene can be achieved by chemical (e.g. Johnson et al *Proc. Natl. Acad. Sci. USA* 78:3138 (1981)) or X-ray mutagenesis (Popp et al *J. Mol. Biol.* 127:141 (1979)) of mouse gametes, followed by fertilization. Offspring heterozygous for inactivation of mCFTR can then be identified by Southern blotting to demonstrate loss of one allele by dosage, or failure to inherit one parental allele if an RFLP marker is being assessed. This approach has previously been successfully used to identify mouse mutants for α-globin (Whitney et al *Proc. Natl. Acad. Sci. USA* 77:1087 (1980)), phenylalanine hydroxylase

(McDonald et al *Pediatr. Res* 23:63 (1988)), and carbonic anhydrase II (Lewis et al *Proc. Natl. Acad. Sci. USA* 85:1962, (1988)).

B. Transgenics

A normal or mutant version of CFTR or mCFTR can be inserted into the mouse germ line using now standard techniques of oocyte injection (Camper, *Trends in Genetics* (1988)); alternatively, if it is desirable to inactivate or replace the endogenous mCF gene, the homologous recombination system using embryonic stem (ES) cells (Capecchi, *Science* 244:1288 (1989)) may be applied.

1. Oocyte Injection

Placing one or more copies of the normal or mutant mCF gene at a random location in the mouse germline can be accomplished by microinjection of the pronucleus of a just-fertilized mouse oocyte, followed by reimplantation into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of human CF gene sequences. The same protocol can be used to insert a mutant mCF gene. To generate a mouse model, one would want to place this transgene in a mouse background where the endogenous mCF gene has been inactivated, either by mutagenesis (see above) or by homologous recombination (see below). The transgene can be either: a) a complete genomic sequence, though the size of this (about 250 kb) would require that it be injected as a yeast artificial chromosome or a chromosome fragment; b) a cDNA with either the natural promoter or a heterologous promoter; c) a "minigene" containing all of the coding region and various other elements such as introns, promoter, and 3' flanking elements found to be necessary for optimum expression.

2. Retroviral Infection of Early Embryos

This alternative involves inserting the CFTR or mCF gene into a retroviral vector and directly infecting mouse embroyos at early stages of development generating a chimera (Soriano et al *Cell* 46:19 (1986)). At least some of these will lead to germline transmission.

3. ES Cells and Homologous Recombination

The embryonic stem cell approach (Capecchi, supra and Capecchi, *Trends Genet* 5:70 (1989)) allows the possibility of performing gene transfer and then screening the resulting totipotent cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of mouse blastocysts, and a proportion of the resulting is mice will show germline transmission from the recombinant line. There are several ways this could be useful in the generation of a mouse model for CF:

a) Inactivation of the mCF gene can be conveniently accomplished by designing a DNA fragment which contains sequences from a mCFTR axon flanking a selectable marker such as neo. Homologous recombination will lead to insertion of the neo sequences in the middle of an exon, inactivating mCFTR. The homologous recombination events (usually about 1 in 1000) can be recognized from the heterologous ones by DNA analysis of individual clones (usually using PCR, Kim et al *Nucleic Acids Res.* 16:8887 (1988), Joyner et al *Nature* 338:153 (1989); Zimmer et al supra, p. 150) or by using a negative selection against the heterologous events (such as the use of an HSV TK gene at the end of the construct, followed by the gancyclovir selection, Mansour et al, *Nature* 336:348 (1988)). This inactivated mCFTR mouse can then be used to introduce a mutant CF gene or mCF gene containing the F508 abnormality or any other desired mutation.

b) It is possible that specific mutants of mCFTR cDNA be created in one step. For example, one can make a construct containing mCF intron 9 sequences at the 5' end, a selectable neo gene in the middle, and intro 9+exon 10 (containing the mouse version of the F508 mutation) at the 3' end. A homologous recombination event would lead to the insertion of the neo gene in intron 9 and the replacement of exon 10 with the mutant version.

c) If the presence of the selectable neo marker in the intron altered expresson of the mCF gene, it would be possible to excise it in a second homologous recombination step.

d) It is also possible to create mutations in the mouse germline by injecting oligonucleotides containing the mutation of interest and screening the resulting cells by PCR.

This embodiment of the invention has considered primarily a mouse model for cystic fibrosis. FIGS. **4**A–**4**D show cross-species hybridization not only to mouse DNA, but also to bovine, hamster and chicken DNA. Thus, it is contemplated that an orthologous gene will exist in many other species also. It is thus contemplated that it will be possible to generate other animal models using similar technology.

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 43

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGAATTCTC GAGATCTTTT TTTTTTTT 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAAGTCCA AGGATTTAG 19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTGGCATGC TTTGATGACG CTTC 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTTTCCTGG ATTATGCCTG GGCAC 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGGCATGC TTTGATGACG CTTC 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTTTCCTGG ATTATGCCTG GGCAC 25

-continued

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTGGCATGC TTTGATGACG CTTC 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Thr Ile Lys Glu Asn Ile Ile Phe Gly Val Ser Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Thr Ile Lys Glu Asn Ile Ile Gly Val Ser Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGATCCAGT TTGCTCTCCA 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAATCACTC TTCCTGATAT 20

(2) INFORMATION FOR SEQ ID NO:12:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAATGTGATT GGTGAAACTA 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTTCTCCTCC TAGACACCTG CAT 23

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAAGAAAATA TCATCTTTGG TGTT 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAGAAAATA TCATTGGTGT T 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6130 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 133..4572

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTGGAAGC AAATGACATC ACAGCAGGTC AGAGAAAAAG GGTTGAGCGG CAGGCACCCA 60

GAGTAGTAGG TCTTTGGCAT TAGGAGCTTG AGCCCAGACG GCCCTAGCAG GGACCCCAGC 120

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GCCCGAGAGA CC ATG CAG AGG TCG CCT CTG GAA AAG GCC AGC GTT GTC        168
              Met Gln Arg Ser Pro Leu Glu Lys Ala Ser Val Val
                1               5                  10

TCC AAA CTT TTT TTC AGC TGG ACC AGA CCA ATT TTG AGG AAA GGA TAC      216
Ser Lys Leu Phe Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr
         15                  20                  25

AGA CAG CGC CTG GAA TTG TCA GAC ATA TAC CAA ATC CCT TCT GTT GAT      264
Arg Gln Arg Leu Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp
     30                  35                  40

TCT GCT GAC AAT CTA TCT GAA AAA TTG GAA AGA GAA TGG GAT AGA GAG      312
Ser Ala Asp Asn Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu
 45                  50                  55                  60

CTG GCT TCA AAG AAA AAT CCT AAA CTC ATT AAT GCC CTT CGG CGA TGT      360
Leu Ala Ser Lys Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys
                 65                  70                  75

TTT TTC TGG AGA TTT ATG TTC TAT GGA ATC TTT TTA TAT TTA GGG GAA      408
Phe Phe Trp Arg Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu
             80                  85                  90

GTC ACC AAA GCA GTA CAG CCT CTC TTA CTG GGA AGA ATC ATA GCT TCC      456
Val Thr Lys Ala Val Gln Pro Leu Leu Leu Gly Arg Ile Ile Ala Ser
         95                  100                 105

TAT GAC CCG GAT AAC AAG GAG GAA CGC TCT ATC GCG ATT TAT CTA GGC      504
Tyr Asp Pro Asp Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly
    110                 115                 120

ATA GGC TTA TGC CTT CTC TTT ATT GTG AGG ACA CTG CTC CTA CAC CCA      552
Ile Gly Leu Cys Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro
125                 130                 135                 140

GCC ATT TTT GGC CTT CAT CAC ATT GGA ATG CAG ATG AGA ATA GCT ATG      600
Ala Ile Phe Gly Leu His His Ile Gly Met Gln Met Arg Ile Ala Met
                145                 150                 155

TTT AGT TTG ATT TAT AAG AAG ACT TTA AAG CTG TCA AGC CGT GTT CTA      648
Phe Ser Leu Ile Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu
            160                 165                 170

GAT AAA ATA AGT ATT GGA CAA CTT GTT AGT CTC CTT TCC AAC AAC CTG      696
Asp Lys Ile Ser Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu
        175                 180                 185

AAC AAA TTT GAT GAA GGA CTT GCA TTG GCA CAT TTC GTG TGG ATC GCT      744
Asn Lys Phe Asp Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala
    190                 195                 200

CCT TTG CAA GTG GCA CTC CTC ATG GGG CTA ATC TGG GAG TTG TTA CAG      792
Pro Leu Gln Val Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln
205                 210                 215                 220

GCG TCT GCC TTC TGT GGA CTT GGT TTC CTG ATA GTC CTT GCC CTT TTT      840
Ala Ser Ala Phe Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe
                225                 230                 235

CAG GCT GGG CTA GGG AGA ATG ATG ATG AAG TAC AGA GAT CAG AGA GCT      888
Gln Ala Gly Leu Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala
            240                 245                 250

GGG AAG ATC AGT GAA AGA CTT GTG ATT ACC TCA GAA ATG ATT GAA AAT      936
Gly Lys Ile Ser Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn
        255                 260                 265

ATC CAA TCT GTT AAG GCA TAC TGC TGG GAA GAA GCA ATG GAA AAA ATG      984
Ile Gln Ser Val Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met
    270                 275                 280

ATT GAA AAC TTA AGA CAA ACA GAA CTG AAA CTG ACT CGG AAG GCA GCC     1032
Ile Glu Asn Leu Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala
285                 290                 295                 300

TAT GTG AGA TAC TTC AAT AGC TCA GCC TTC TTC TTC TCA GGG TTC TTT     1080
Tyr Val Arg Tyr Phe Asn Ser Ser Ala Phe Phe Phe Ser Gly Phe Phe
                305                 310                 315
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GTG GTG TTT TTA TCT GTG CTT CCC TAT GCA CTA ATC AAA GGA ATC ATC     1128
Val Val Phe Leu Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile
            320                 325                 330

CTC CGG AAA ATA TTC ACC ACC ATC TCA TTC TGC ATT GTT CTG CGC ATG     1176
Leu Arg Lys Ile Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met
        335                 340                 345

GCG GTC ACT CGG CAA TTT CCC TGG GCT GTA CAA ACA TGG TAT GAC TCT     1224
Ala Val Thr Arg Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser
    350                 355                 360

CTT GGA GCA ATA AAC AAA ATA CAG GAT TTC TTA CAA AAG CAA GAA TAT     1272
Leu Gly Ala Ile Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr
365                 370                 375                 380

AAG ACA TTG GAA TAT AAC TTA ACG ACT ACA GAA GTA GTG ATG GAG AAT     1320
Lys Thr Leu Glu Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn
                385                 390                 395

GTA ACA GCC TTC TGG GAG GAG GGA TTT GGG GAA TTA TTT GAG AAA GCA     1368
Val Thr Ala Phe Trp Glu Glu Gly Phe Gly Glu Leu Phe Glu Lys Ala
            400                 405                 410

AAA CAA AAC AAT AAC AAT AGA AAA ACT TCT AAT GGT GAT GAC AGC CTC     1416
Lys Gln Asn Asn Asn Asn Arg Lys Thr Ser Asn Gly Asp Asp Ser Leu
        415                 420                 425

TTC TTC AGT AAT TTC TCA CTT CTT GGT ACT CCT GTC CTG AAA GAT ATT     1464
Phe Phe Ser Asn Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile
    430                 435                 440

AAT TTC AAG ATA GAA AGA GGA CAG TTG TTG GCG GTT GCT GGA TCC ACT     1512
Asn Phe Lys Ile Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr
445                 450                 455                 460

GGA GCA GGC AAG ACT TCA CTT CTA ATG ATG ATT ATG GGA GAA CTG GAG     1560
Gly Ala Gly Lys Thr Ser Leu Leu Met Met Ile Met Gly Glu Leu Glu
                465                 470                 475

CCT TCA GAG GGT AAA ATT AAG CAC AGT GGA AGA ATT TCA TTC TGT TCT     1608
Pro Ser Glu Gly Lys Ile Lys His Ser Gly Arg Ile Ser Phe Cys Ser
            480                 485                 490

CAG TTT TCC TGG ATT ATG CCT GGC ACC ATT AAA GAA AAT ATC ATC TTT     1656
Gln Phe Ser Trp Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe
        495                 500                 505

GGT GTT TCC TAT GAT GAA TAT AGA TAC AGA AGC GTC ATC AAA GCA TGC     1704
Gly Val Ser Tyr Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys
    510                 515                 520

CAA CTA GAA GAG GAC ATC TCC AAG TTT GCA GAG AAA GAC AAT ATA GTT     1752
Gln Leu Glu Glu Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile Val
525                 530                 535                 540

CTT GGA GAA GGT GGA ATC ACA CTG AGT GGA GGT CAA CGA GCA AGA ATT     1800
Leu Gly Glu Gly Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala Arg Ile
                545                 550                 555

TCT TTA GCA AGA GCA GTA TAC AAA GAT GCT GAT TTG TAT TTA TTA GAC     1848
Ser Leu Ala Arg Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp
            560                 565                 570

TCT CCT TTT GGA TAC CTA GAT GTT TTA ACA GAA AAA GAA ATA TTT GAA     1896
Ser Pro Phe Gly Tyr Leu Asp Val Leu Thr Glu Lys Glu Ile Phe Glu
        575                 580                 585

AGC TGT GTC TGT AAA CTG ATG GCT AAC AAA ACT AGG ATT TTG GTC ACT     1944
Ser Cys Val Cys Lys Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr
    590                 595                 600

TCT AAA ATG GAA CAT TTA AAG AAA GCT GAC AAA ATA TTA ATT TTG CAT     1992
Ser Lys Met Glu His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu His
605                 610                 615                 620

GAA GGT AGC AGC TAT TTT TAT GGG ACA TTT TCA GAA CTC CAA AAT CTA     2040
Glu Gly Ser Ser Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu
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                625                 630                 635

CAG CCA GAC TTT AGC TCA AAA CTC ATG GGA TGT GAT TCT TTC GAC CAA     2088
Gln Pro Asp Phe Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln
            640                 645                 650

TTT AGT GCA GAA AGA AGA AAT TCA ATC CTA ACT GAG ACC TTA CAC CGT     2136
Phe Ser Ala Glu Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg
        655                 660                 665

TTC TCA TTA GAA GGA GAT GCT CCT GTC TCC TGG ACA GAA ACA AAA AAA     2184
Phe Ser Leu Glu Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys
    670                 675                 680

CAA TCT TTT AAA CAG ACT GGA GAG TTT GGG GAA AAA AGG AAG AAT TCT     2232
Gln Ser Phe Lys Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser
685                 690                 695                 700

ATT CTC AAT CCA ATC AAC TCT ATA CGA AAA TTT TCC ATT GTG CAA AAG     2280
Ile Leu Asn Pro Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys
                705                 710                 715

ACT CCC TTA CAA ATG AAT GGC ATC GAA GAG GAT TCT GAT GAG CCT TTA     2328
Thr Pro Leu Gln Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu
            720                 725                 730

GAG AGA AGG CTG TCC TTA GTA CCA GAT TCT GAG CAG GGA GAG GCG ATA     2376
Glu Arg Arg Leu Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile
        735                 740                 745

CTG CCT CGC ATC AGC GTG ATC AGC ACT GGC CCC ACG CTT CAG GCA CGA     2424
Leu Pro Arg Ile Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg
    750                 755                 760

AGG AGG CAG TCT GTC CTG AAC CTG ATG ACA CAC TCA GTT AAC CAA GGT     2472
Arg Arg Gln Ser Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly
765                 770                 775                 780

CAG AAC ATT CAC CGA AAG ACA ACA GCA TCC ACA CGA AAA GTG TCA CTG     2520
Gln Asn Ile His Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu
                785                 790                 795

GCC CCT CAG GCA AAC TTG ACT GAA CTG GAT ATA TAT TCA AGA AGG TTA     2568
Ala Pro Gln Ala Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu
            800                 805                 810

TCT CAA GAA ACT GGC TTG GAA ATA AGT GAA GAA ATT AAC GAA GAA GAC     2616
Ser Gln Glu Thr Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp
        815                 820                 825

TTA AAG GAG TGC TTT TTT GAT GAT ATG GAG AGC ATA CCA GCA GTG ACT     2664
Leu Lys Glu Cys Phe Phe Asp Asp Met Glu Ser Ile Pro Ala Val Thr
    830                 835                 840

ACA TGG AAC ACA TAC CTT CGA TAT ATT ACT GTC CAC AAG AGC TTA ATT     2712
Thr Trp Asn Thr Tyr Leu Arg Tyr Ile Thr Val His Lys Ser Leu Ile
845                 850                 855                 860

TTT GTG CTA ATT TGG TGC TTA GTA ATT TTT CTG GCA GAG GTG GCT GCT     2760
Phe Val Leu Ile Trp Cys Leu Val Ile Phe Leu Ala Glu Val Ala Ala
                865                 870                 875

TCT TTG GTT GTG CTG TGG CTC CTT GGA AAC ACT CCT CTT CAA GAC AAA     2808
Ser Leu Val Val Leu Trp Leu Leu Gly Asn Thr Pro Leu Gln Asp Lys
            880                 885                 890

GGG AAT AGT ACT CAT AGT AGA AAT AAC AGC TAT GCA GTG ATT ATC ACC     2856
Gly Asn Ser Thr His Ser Arg Asn Asn Ser Tyr Ala Val Ile Ile Thr
        895                 900                 905

AGC ACC AGT TCG TAT TAT GTG TTT TAC ATT TAC GTG GGA GTA GCC GAC     2904
Ser Thr Ser Ser Tyr Tyr Val Phe Tyr Ile Tyr Val Gly Val Ala Asp
    910                 915                 920

ACT TTG CTT GCT ATG GGA TTC TTC AGA GGT CTA CCA CTG GTG CAT ACT     2952
Thr Leu Leu Ala Met Gly Phe Phe Arg Gly Leu Pro Leu Val His Thr
925                 930                 935                 940

CTA ATC ACA GTG TCG AAA ATT TTA CAC CAC AAA ATG TTA CAT TCT GTT     3000
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Leu Ile Thr Val Ser Lys Ile Leu His His Lys Met Leu His Ser Val
                945                 950                 955

CTT CAA GCA CCT ATG TCA ACC CTC AAC ACG TTG AAA GCA GGT GGG ATT     3048
Leu Gln Ala Pro Met Ser Thr Leu Asn Thr Leu Lys Ala Gly Gly Ile
            960                 965                 970

CTT AAT AGA TTC TCC AAA GAT ATA GCA ATT TTG GAT GAC CTT CTG CCT     3096
Leu Asn Arg Phe Ser Lys Asp Ile Ala Ile Leu Asp Asp Leu Leu Pro
        975                 980                 985

CTT ACC ATA TTT GAC TTC ATC CAG TTG TTA TTA ATT GTG ATT GGA GCT     3144
Leu Thr Ile Phe Asp Phe Ile Gln Leu Leu Leu Ile Val Ile Gly Ala
    990                 995                 1000

ATA GCA GTT GTC GCA GTT TTA CAA CCC TAC ATC TTT GTT GCA ACA GTG     3192
Ile Ala Val Val Ala Val Leu Gln Pro Tyr Ile Phe Val Ala Thr Val
1005                1010                1015                1020

CCA GTG ATA GTG GCT TTT ATT ATG TTG AGA GCA TAT TTC CTC CAA ACC     3240
Pro Val Ile Val Ala Phe Ile Met Leu Arg Ala Tyr Phe Leu Gln Thr
                1025                1030                1035

TCA CAG CAA CTC AAA CAA CTG GAA TCT GAA GGC AGG AGT CCA ATT TTC     3288
Ser Gln Gln Leu Lys Gln Leu Glu Ser Glu Gly Arg Ser Pro Ile Phe
            1040                1045                1050

ACT CAT CTT GTT ACA AGC TTA AAA GGA CTA TGG ACA CTT CGT GCC TTC     3336
Thr His Leu Val Thr Ser Leu Lys Gly Leu Trp Thr Leu Arg Ala Phe
        1055                1060                1065

GGA CGG CAG CCT TAC TTT GAA ACT CTG TTC CAC AAA GCT CTG AAT TTA     3384
Gly Arg Gln Pro Tyr Phe Glu Thr Leu Phe His Lys Ala Leu Asn Leu
    1070                1075                1080

CAT ACT GCC AAC TGG TTC TTG TAC CTG TCA ACA CTG CGC TGG TTC CAA     3432
His Thr Ala Asn Trp Phe Leu Tyr Leu Ser Thr Leu Arg Trp Phe Gln
1085                1090                1095                1100

ATG AGA ATA GAA ATG ATT TTT GTC ATC TTC TTC ATT GCT GTT ACC TTC     3480
Met Arg Ile Glu Met Ile Phe Val Ile Phe Phe Ile Ala Val Thr Phe
                1105                1110                1115

ATT TCC ATT TTA ACA ACA GGA GAA GGA GAA GGA AGA GTT GGT ATT ATC     3528
Ile Ser Ile Leu Thr Thr Gly Glu Gly Glu Gly Arg Val Gly Ile Ile
            1120                1125                1130

CTG ACT TTA GCC ATG AAT ATC ATG AGT ACA TTG CAG TGG GCT GTA AAC     3576
Leu Thr Leu Ala Met Asn Ile Met Ser Thr Leu Gln Trp Ala Val Asn
        1135                1140                1145

TCC AGC ATA GAT GTG GAT AGC TTG ATG CGA TCT GTG AGC CGA GTC TTT     3624
Ser Ser Ile Asp Val Asp Ser Leu Met Arg Ser Val Ser Arg Val Phe
    1150                1155                1160

AAG TTC ATT GAC ATG CCA ACA GAA GGT AAA CCT ACC AAG TCA ACC AAA     3672
Lys Phe Ile Asp Met Pro Thr Glu Gly Lys Pro Thr Lys Ser Thr Lys
1165                1170                1175                1180

CCA TAC AAG AAT GGC CAA CTC TCG AAA GTT ATG ATT ATT GAG AAT TCA     3720
Pro Tyr Lys Asn Gly Gln Leu Ser Lys Val Met Ile Ile Glu Asn Ser
                1185                1190                1195

CAC GTG AAG AAA GAT GAC ATC TGG CCC TCA GGG GGC CAA ATG ACT GTC     3768
His Val Lys Lys Asp Asp Ile Trp Pro Ser Gly Gly Gln Met Thr Val
            1200                1205                1210

AAA GAT CTC ACA GCA AAA TAC ACA GAA GGT GGA AAT GCC ATA TTA GAG     3816
Lys Asp Leu Thr Ala Lys Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu
        1215                1220                1225

AAC ATT TCC TTC TCA ATA AGT CCT GGC CAG AGG GTG GGC CTC TTG GGA     3864
Asn Ile Ser Phe Ser Ile Ser Pro Gly Gln Arg Val Gly Leu Leu Gly
    1230                1235                1240

AGA ACT GGA TCA GGG AAG AGT ACT TTG TTA TCA GCT TTT TTG AGA CTA     3912
Arg Thr Gly Ser Gly Lys Ser Thr Leu Leu Ser Ala Phe Leu Arg Leu
1245                1250                1255                1260
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CTG AAC ACT GAA GGA GAA ATC CAG ATC GAT GGT GTG TCT TGG GAT TCA     3960
Leu Asn Thr Glu Gly Glu Ile Gln Ile Asp Gly Val Ser Trp Asp Ser
                1265                1270                1275

ATA ACT TTG CAA CAG TGG AGG AAA GCC TTT GGA GTG ATA CCA CAG AAA     4008
Ile Thr Leu Gln Gln Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys
            1280                1285                1290

GTA TTT ATT TTT TCT GGA ACA TTT AGA AAA AAC TTG GAT CCC TAT GAA     4056
Val Phe Ile Phe Ser Gly Thr Phe Arg Lys Asn Leu Asp Pro Tyr Glu
        1295                1300                1305

CAG TGG AGT GAT CAA GAA ATA TGG AAA GTT GCA GAT GAG GTT GGG CTC     4104
Gln Trp Ser Asp Gln Glu Ile Trp Lys Val Ala Asp Glu Val Gly Leu
    1310                1315                1320

AGA TCT GTG ATA GAA CAG TTT CCT GGG AAG CTT GAC TTT GTC CTT GTG     4152
Arg Ser Val Ile Glu Gln Phe Pro Gly Lys Leu Asp Phe Val Leu Val
1325                1330                1335                1340

GAT GGG GGC TGT GTC CTA AGC CAT GGC CAC AAG CAG TTG ATG TGC TTG     4200
Asp Gly Gly Cys Val Leu Ser His Gly His Lys Gln Leu Met Cys Leu
                1345                1350                1355

GCT AGA TCT GTT CTC AGT AAG GCG AAG ATC TTG CTG CTT GAT GAA CCC     4248
Ala Arg Ser Val Leu Ser Lys Ala Lys Ile Leu Leu Leu Asp Glu Pro
            1360                1365                1370

AGT GCT CAT TTG GAT CCA GTA ACA TAC CAA ATA ATT AGA AGA ACT CTA     4296
Ser Ala His Leu Asp Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu
        1375                1380                1385

AAA CAA GCA TTT GCT GAT TGC ACA GTA ATT CTC TGT GAA CAC AGG ATA     4344
Lys Gln Ala Phe Ala Asp Cys Thr Val Ile Leu Cys Glu His Arg Ile
    1390                1395                1400

GAA GCA ATG CTG GAA TGC CAA CAA TTT TTG GTC ATA GAA GAG AAC AAA     4392
Glu Ala Met Leu Glu Cys Gln Gln Phe Leu Val Ile Glu Glu Asn Lys
1405                1410                1415                1420

GTG CGG CAG TAC GAT TCC ATC CAG AAA CTG CTG AAC GAG AGG AGC CTC     4440
Val Arg Gln Tyr Asp Ser Ile Gln Lys Leu Leu Asn Glu Arg Ser Leu
                1425                1430                1435

TTC CGG CAA GCC ATC AGC CCC TCC GAC AGG GTG AAG CTC TTT CCC CAC     4488
Phe Arg Gln Ala Ile Ser Pro Ser Asp Arg Val Lys Leu Phe Pro His
            1440                1445                1450

CGG AAC TCA AGC AAG TGC AAG TCT AAG CCC CAG ATT GCT GCT CTG AAA     4536
Arg Asn Ser Ser Lys Cys Lys Ser Lys Pro Gln Ile Ala Ala Leu Lys
        1455                1460                1465

GAG GAG ACA GAA GAA GAG GTG CAA GAT ACA AGG CTT TAGAGAGCAG         4582
Glu Glu Thr Glu Glu Glu Val Gln Asp Thr Arg Leu
    1470                1475                1480

CATAAATGTT GACATGGGAC ATTTGCTCAT GGAATTGGAG CTCGTGGGAC AGTCACCTCA   4642

TGGAATTGGA GCTCGTGGAA CAGTTACCTC TGCCTCAGAA AACAAGGATG AATTAAGTTT   4702

TTTTTTAAAA AAGAAACATT TGGTAAGGGG AATTGAGGAC ACTGATATGG GTCTTGATAA   4762

ATGGCTTCCT GGCAATAGTC AAATTGTGTG AAAGGTACTT CAAATCCTTG AAGATTTACC   4822

ACTTGTGTTT TGCAAGCCAG ATTTTCCTGA AAACCCTTGC CATGTGCTAG TAATTGGAAA   4882

GGCAGCTCTA AATGTCAATC AGCCTAGTTG ATCAGCTTAT TGTCTAGTGA AACTCGTTAA   4942

TTTGTAGTGT TGGAGAAGAA CTGAAATCAT ACTTCTTAGG GTTATGATTA AGTAATGATA   5002

ACTGGAAACT TCAGCGGTTT ATATAAGCTT GTATTCCTTT TTCTCTCCTC TCCCCATGAT   5062

GTTTAGAAAC ACAACTATAT TGTTTGCTAA GCATTCCAAC TATCTCATTT CCAAGCAAGT   5122

ATTAGAATAC CACAGGAACC ACAAGACTGC ACATCAAAAT ATGCCCCATT CAACATCTAG   5182

TGAGCAGTCA GGAAAGAGAA CTTCCAGATC CTGGAAATCA GGGTTAGTAT TGTCCAGGTC   5242

TACCAAAAAT CTCAATATTT CAGATAATCA CAATACATCC CTTACCTGGG AAAGGGCTGT   5302
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TATAATCTTT CACAGGGGAC AGGATGGTTC CCTTGATGAA GAAGTTGATA TGCCTTTTCC 5362

CAACTCCAGA AAGTGACAAG CTCACAGACC TTTGAACTAG AGTTTAGCTG GAAAAGTATG 5422

TTAGTGCAAA TTGTCACAGG ACAGCCCTTC TTTCCACAGA AGCTCCAGGT AGAGGGTGTG 5482

TAAGTAGATA GGCCATGGGC ACTGTGGGTA GACACACATG AAGTCCAAGC ATTTAGATGT 5542

ATAGGTTGAT GGTGGTATGT TTTCAGGCTA GATGTATGTA CTTCATGCTG TCTACACTAA 5602

GAGAGAATGA GAGACACACT GAAGAAGCAC CAATCATGAA TTAGTTTTAT ATGCTTCTGT 5662

TTTATAATTT TGTGAAGCAA AATTTTTTCT CTAGGAAATA TTTATTTTAA TAATGTTTCA 5722

AACATATATT ACAATGCTGT ATTTTAAAAG AATGATTATG AATTACATTT GTATAAAATA 5782

ATTTTTATAT TTGAAATATT GACTTTTTAT GGCACTAGTA TTTTTATGAA ATATTATGTT 5842

AAAACTGGGA CAGGGGAGAA CCTAGGGTGA TATTAACCAG GGGCCATGAA TCACCTTTTG 5902

GTCTGGAGGG AAGCCTTGGG GCTGATCGAG TTGTTGCCCA CAGCTGTATG ATTCCCAGCC 5962

AGACACAGCC TCTTAGATGC AGTTCTGAAG AAGATGGTAC CACCAGTCTG ACTGTTTCCA 6022

TCAAGGGTAC ACTGCCTTCT CAACTCCAAA CTGACTCTTA AGAAGACTGC ATTATATTTA 6082

TTACTGTAAG AAAATATCAC TTGTCAATAA AATCCATACA TTTGTGTA 6130

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1480 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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

Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr Arg Gln Arg Leu
             20                  25                  30

Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp Ser Ala Asp Asn
         35                  40                  45

Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu Leu Ala Ser Lys
     50                  55                  60

Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys Phe Phe Trp Arg
 65                  70                  75                  80

Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu Val Thr Lys Ala
                 85                  90                  95

Val Gln Pro Leu Leu Leu Gly Arg Ile Ile Ala Ser Tyr Asp Pro Asp
            100                 105                 110

Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly Ile Gly Leu Cys
        115                 120                 125

Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro Ala Ile Phe Gly
    130                 135                 140

Leu His His Ile Gly Met Gln Met Arg Ile Ala Met Phe Ser Leu Ile
145                 150                 155                 160

Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu Asp Lys Ile Ser
                165                 170                 175

Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu Asn Lys Phe Asp
            180                 185                 190

Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala Pro Leu Gln Val
        195                 200                 205
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Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln Ala Ser Ala Phe
    210                 215                 220

Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe Gln Ala Gly Leu
225                 230                 235                 240

Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala Gly Lys Ile Ser
                245                 250                 255

Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn Ile Gln Ser Val
            260                 265                 270

Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met Ile Glu Asn Leu
        275                 280                 285

Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala Tyr Val Arg Tyr
    290                 295                 300

Phe Asn Ser Ser Ala Phe Phe Phe Ser Gly Phe Phe Val Val Phe Leu
305                 310                 315                 320

Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile Leu Arg Lys Ile
                325                 330                 335

Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met Ala Val Thr Arg
            340                 345                 350

Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser Leu Gly Ala Ile
        355                 360                 365

Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr Lys Thr Leu Glu
    370                 375                 380

Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn Val Thr Ala Phe
385                 390                 395                 400

Trp Glu Glu Gly Phe Gly Glu Leu Phe Glu Lys Ala Lys Gln Asn Asn
                405                 410                 415

Asn Asn Arg Lys Thr Ser Asn Gly Asp Asp Ser Leu Phe Phe Ser Asn
            420                 425                 430

Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile Asn Phe Lys Ile
        435                 440                 445

Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr Gly Ala Gly Lys
    450                 455                 460

Thr Ser Leu Leu Met Met Ile Met Gly Glu Leu Glu Pro Ser Glu Gly
465                 470                 475                 480

Lys Ile Lys His Ser Gly Arg Ile Ser Phe Cys Ser Gln Phe Ser Trp
                485                 490                 495

Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe Gly Val Ser Tyr
            500                 505                 510

Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys Gln Leu Glu Glu
        515                 520                 525

Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile Val Leu Gly Glu Gly
    530                 535                 540

Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala Arg Ile Ser Leu Ala Arg
545                 550                 555                 560

Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp Ser Pro Phe Gly
                565                 570                 575

Tyr Leu Asp Val Leu Thr Glu Lys Glu Ile Phe Glu Ser Cys Val Cys
            580                 585                 590

Lys Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr Ser Lys Met Glu
        595                 600                 605

His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu His Glu Gly Ser Ser
    610                 615                 620
```

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```
Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu Gln Pro Asp Phe
625                 630                 635                 640

Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln Phe Ser Ala Glu
                645                 650                 655

Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg Phe Ser Leu Glu
            660                 665                 670

Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys Gln Ser Phe Lys
        675                 680                 685

Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser Ile Leu Asn Pro
    690                 695                 700

Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys Thr Pro Leu Gln
705                 710                 715                 720

Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu Glu Arg Arg Leu
                725                 730                 735

Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile Leu Pro Arg Ile
            740                 745                 750

Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg Arg Arg Gln Ser
        755                 760                 765

Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly Gln Asn Ile His
    770                 775                 780

Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu Ala Pro Gln Ala
785                 790                 795                 800

Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu Ser Gln Glu Thr
                805                 810                 815

Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp Leu Lys Glu Cys
            820                 825                 830

Phe Phe Asp Asp Met Glu Ser Ile Pro Ala Val Thr Thr Trp Asn Thr
        835                 840                 845

Tyr Leu Arg Tyr Ile Thr Val His Lys Ser Leu Ile Phe Val Leu Ile
    850                 855                 860

Trp Cys Leu Val Ile Phe Leu Ala Glu Val Ala Ala Ser Leu Val Val
865                 870                 875                 880

Leu Trp Leu Leu Gly Asn Thr Pro Leu Gln Asp Lys Gly Asn Ser Thr
                885                 890                 895

His Ser Arg Asn Asn Ser Tyr Ala Val Ile Ile Thr Ser Thr Ser Ser
            900                 905                 910

Tyr Tyr Val Phe Tyr Ile Tyr Val Gly Val Ala Asp Thr Leu Leu Ala
        915                 920                 925

Met Gly Phe Phe Arg Gly Leu Pro Leu Val His Thr Leu Ile Thr Val
    930                 935                 940

Ser Lys Ile Leu His His Lys Met Leu His Ser Val Leu Gln Ala Pro
945                 950                 955                 960

Met Ser Thr Leu Asn Thr Leu Lys Ala Gly Gly Ile Leu Asn Arg Phe
                965                 970                 975

Ser Lys Asp Ile Ala Ile Leu Asp Asp Leu Leu Pro Leu Thr Ile Phe
            980                 985                 990

Asp Phe Ile Gln Leu Leu Leu Ile Val Ile Gly Ala Ile Ala Val Val
        995                 1000                1005

Ala Val Leu Gln Pro Tyr Ile Phe Val Ala Thr Val Pro Val Ile Val
    1010                1015                1020

Ala Phe Ile Met Leu Arg Ala Tyr Phe Leu Gln Thr Ser Gln Gln Leu
1025                1030                1035                1040

Lys Gln Leu Glu Ser Glu Gly Arg Ser Pro Ile Phe Thr His Leu Val
```

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```
               1045                1050                1055

Thr Ser Leu Lys Gly Leu Trp Thr Leu Arg Ala Phe Gly Arg Gln Pro
            1060                1065                1070

Tyr Phe Glu Thr Leu Phe His Lys Ala Leu Asn Leu His Thr Ala Asn
        1075                1080                1085

Trp Phe Leu Tyr Leu Ser Thr Leu Arg Trp Phe Gln Met Arg Ile Glu
    1090                1095                1100

Met Ile Phe Val Ile Phe Phe Ile Ala Val Thr Phe Ile Ser Ile Leu
1105                1110                1115                1120

Thr Thr Gly Glu Gly Glu Gly Arg Val Gly Ile Ile Leu Thr Leu Ala
               1125                1130                1135

Met Asn Ile Met Ser Thr Leu Gln Trp Ala Val Asn Ser Ser Ile Asp
            1140                1145                1150

Val Asp Ser Leu Met Arg Ser Val Ser Arg Val Phe Lys Phe Ile Asp
        1155                1160                1165

Met Pro Thr Glu Gly Lys Pro Thr Lys Ser Thr Lys Pro Tyr Lys Asn
    1170                1175                1180

Gly Gln Leu Ser Lys Val Met Ile Ile Glu Asn Ser His Val Lys Lys
1185                1190                1195                1200

Asp Asp Ile Trp Pro Ser Gly Gly Gln Met Thr Val Lys Asp Leu Thr
               1205                1210                1215

Ala Lys Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu Asn Ile Ser Phe
            1220                1225                1230

Ser Ile Ser Pro Gly Gln Arg Val Gly Leu Leu Gly Arg Thr Gly Ser
        1235                1240                1245

Gly Lys Ser Thr Leu Leu Ser Ala Phe Leu Arg Leu Leu Asn Thr Glu
    1250                1255                1260

Gly Glu Ile Gln Ile Asp Gly Val Ser Trp Asp Ser Ile Thr Leu Gln
1265                1270                1275                1280

Gln Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys Val Phe Ile Phe
               1285                1290                1295

Ser Gly Thr Phe Arg Lys Asn Leu Asp Pro Tyr Glu Gln Trp Ser Asp
            1300                1305                1310

Gln Glu Ile Trp Lys Val Ala Asp Glu Val Gly Leu Arg Ser Val Ile
        1315                1320                1325

Glu Gln Phe Pro Gly Lys Leu Asp Phe Val Leu Val Asp Gly Gly Cys
    1330                1335                1340

Val Leu Ser His Gly His Lys Gln Leu Met Cys Leu Ala Arg Ser Val
1345                1350                1355                1360

Leu Ser Lys Ala Lys Ile Leu Leu Leu Asp Glu Pro Ser Ala His Leu
               1365                1370                1375

Asp Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu Lys Gln Ala Phe
            1380                1385                1390

Ala Asp Cys Thr Val Ile Leu Cys Glu His Arg Ile Glu Ala Met Leu
        1395                1400                1405

Glu Cys Gln Gln Phe Leu Val Ile Glu Glu Asn Lys Val Arg Gln Tyr
    1410                1415                1420

Asp Ser Ile Gln Lys Leu Leu Asn Glu Arg Ser Leu Phe Arg Gln Ala
1425                1430                1435                1440

Ile Ser Pro Ser Asp Arg Val Lys Leu Phe Pro His Arg Asn Ser Ser
               1445                1450                1455

Lys Cys Lys Ser Lys Pro Gln Ile Ala Ala Leu Lys Glu Glu Thr Glu
            1460                1465                1470
```

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```
Glu Glu Val Gln Asp Thr Arg Leu
        1475                1480


(2) INFORMATION FOR SEQ ID NO:18:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 110 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile Asn Phe Lys Ile
1               5                   10                  15

Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr Gly Ala Gly Lys
            20                  25                  30

Thr Ser Leu Leu Met Met Ile Met Gly Ile Ser Phe Cys Ser Gln Phe
        35                  40                  45

Ser Trp Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe Gly Val
    50                  55                  60

Ser Tyr Asp Gly Glu Gly Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala
65                  70                  75                  80

Arg Ile Ser Leu Ala Arg Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu
                85                  90                  95

Leu Asp Ser Pro Phe Gly Tyr Leu Asp Val Leu Thr Glu Lys
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:19:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 111 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu Asn Ile Ser Phe Ser Ile
1               5                   10                  15

Ser Pro Gly Gln Arg Val Gly Leu Leu Gly Arg Thr Gly Ser Gly Lys
            20                  25                  30

Ser Thr Leu Leu Ser Ala Phe Leu Arg Asp Ser Ile Thr Leu Gln Gln
        35                  40                  45

Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys Val Phe Ile Phe Ser
    50                  55                  60

Gly Thr Phe Arg Val Asp Gly Gly Cys Val Leu Ser His Gly His Lys
65                  70                  75                  80

Gln Leu Met Cys Leu Ala Arg Ser Val Leu Ser Lys Ala Lys Ile Leu
                85                  90                  95

Leu Leu Asp Glu Pro Ser Ala His Leu Asp Pro Val Thr Tyr Gln
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:20:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 110 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
```

-continued

(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Pro Ser Arg Lys Glu Val Lys Ile Leu Lys Gly Leu Asn Leu Lys Val
1               5                   10                  15

Gln Ser Gly Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Thr Val Gln Leu Met Gln Arg Ile Gly Val Val Ser Gln Glu
        35                  40                  45

Pro Val Leu Phe Ala Thr Thr Ile Ala Glu Asn Ile Arg Tyr Gly Arg
    50                  55                  60

Glu Asn Val Gly Glu Arg Gly Ala Gln Leu Ser Gly Gly Gln Lys Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Asn Pro Lys Ile Leu Leu
                85                  90                  95

Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Ala
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Pro Thr Arg Pro Asp Ile Pro Val Leu Gln Gly Leu Ser Leu Glu Val
1               5                   10                  15

Lys Lys Gly Gln Thr Leu Ala Leu Val Gly Ser Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Val Val Gln Leu Leu Glu Arg Leu Gly Ile Val Ser Gln Glu
        35                  40                  45

Pro Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly Asp
    50                  55                  60

Asn Ser Arg Gly Asp Lys Gly Thr Leu Leu Ser Gly Gly Gln Lys Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Gln Pro His Ile Leu Leu
                85                  90                  95

Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Pro Ser Arg Ser Glu Val Gln Ile Leu Lys Gly Leu Asn Leu Lys Val
1               5                   10                  15

Lys Ser Gly Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys
            20                  25                  30
```

-continued

```
Ser Thr Thr Val Gln Leu Met Gln Arg Ile Gly Val Val Ser Gln Glu
        35                  40                  45

Pro Val Leu Phe Ala Thr Thr Ile Ala Glu Asn Ile Arg Tyr Gly Arg
    50                  55                  60

Glu Asp Val Gly Glu Arg Gly Ala Gln Leu Ser Gly Gly Gln Lys Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Asn Pro Lys Ile Leu Leu
                85                  90                  95

Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Ala
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Pro Thr Arg Pro Asn Ile Pro Val Leu Gln Gly Leu Ser Leu Glu Val
1               5                   10                  15

Lys Lys Gly Gln Thr Leu Ala Leu Val Gly Ser Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Val Val Gln Leu Leu Glu Arg Leu Gly Glu Val Ser Gln Glu
        35                  40                  45

Pro Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly Asp
    50                  55                  60

Asn Ser Arg Gly Asp Lys Gly Thr Gln Leu Ser Gly Gly Gln Lys Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Gln Pro His Ile Leu Leu
                85                  90                  95

Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Pro Ser Arg Ala Asn Ile Lys Ile Leu Lys Gly Leu Asn Leu Lys Val
1               5                   10                  15

Lys Ser Gly Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Thr Val Gln Leu Leu Gln Arg Ile Gly Val Val Ser Gln Glu
        35                  40                  45

Pro Val Leu Ser Phe Thr Thr Ile Ala Glu Asn Ile Arg Tyr Gly Arg
    50                  55                  60

Gly Val Gly Asp Arg Gly Ala Gln Leu Ser Gly Gly Gln Lys Gln Arg
65                  70                  75                  80

Ile Ala Ile Ala Arg Ala Leu Val Arg Asn Pro Lys Ile Leu Leu Leu
```

-continued

```
               85                  90                  95

Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Ala
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Pro Thr Arg Ala Asn Val Pro Asn Leu Gln Gly Leu Ser Leu Glu Val
1               5                   10                  15

Lys Lys Gly Gln Thr Leu Ala Leu Val Gly Ser Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Val Val Gln Leu Leu Glu Arg Leu Gly Ile Val Ser Gln Glu
        35                  40                  45

Pro Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly Asp
    50                  55                  60

Asn Ser Arg Gly Asp Lys Gly Thr Gln Leu Ser Gly Gly Gln Lys Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Ile Arg Gln Pro Arg Val Leu Leu
                85                  90                  95

Leu Asp Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Asp Thr Arg Lys Asp Val Glu Ile Tyr Lys Asp Leu Ser Phe Thr Leu
1               5                   10                  15

Leu Lys Glu Gly Lys Thr Tyr Ala Phe Val Gly Glu Ser Gly Cys Gly
            20                  25                  30

Lys Ser Thr Ile Leu Lys Leu Ile Glu Ile Gly Val Val Ser Gln Asp
        35                  40                  45

Pro Leu Leu Phe Ser Asn Ser Ile Lys Asn Asn Ile Lys Tyr Ser Leu
    50                  55                  60

Tyr Ser Leu Ser Asn Ala Ser Lys Leu Ser Gly Gly Gln Lys Gln Arg
65                  70                  75                  80

Ile Ser Ile Ala Arg Ala Ile Met Arg Asn Pro Lys Ile Leu Ile Leu
                85                  90                  95

Asp Glu Ala Thr Ser Ser Leu Asp Asn Lys Ser Glu Tyr
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid

-continued

(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Ile Ser Arg Pro Asn Val Pro Ile Tyr Lys Asn Leu Ser Phe Thr Cys
  1               5                  10                  15

Asp Ser Lys Lys Thr Thr Ala Ile Val Gly Glu Thr Gly Ser Gly Lys
            20                  25                  30

Ser Thr Phe Met Asn Leu Leu Leu Arg Phe Ser Ile Val Ser Gln Glu
        35                  40                  45

Pro Met Leu Phe Asn Met Ser Ile Tyr Glu Asn Ile Lys Phe Gly Arg
    50                  55                  60

Glu Asp Ala Pro Tyr Gly Lys Ser Leu Ser Gly Gly Gln Lys Gln Arg
65                  70                  75                  80

Ile Ala Ile Ala Arg Ala Leu Leu Arg Glu Pro Lys Ile Leu Leu Leu
                85                  90                  95

Asp Glu Ala Thr Ser Ser Leu Asp Ser Asn Ser Glu Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
Pro Ser Arg Pro Ser Glu Ala Val Leu Lys Asn Val Ser Leu Asn Phe
1               5                  10                  15

Ser Ala Gly Gln Phe Thr Phe Ile Val Gly Lys Ser Gly Ser Gly Lys
            20                  25                  30

Ser Thr Leu Ser Asn Leu Leu Leu Arg Ile Thr Val Val Glu Gln Arg
        35                  40                  45

Cys Thr Leu Phe Asn Asp Thr Leu Arg Lys Asn Ile Leu Leu Gly Ser
    50                  55                  60

Thr Asp Ser Gly Thr Gly Gly Val Thr Leu Ser Gly Gly Gln Gln Gln
65                  70                  75                  80

Arg Val Ala Ile Ala Arg Ala Phe Ile Arg Asp Thr Pro Ile Leu Phe
                85                  90                  95

Leu Asp Glu Ala Val Ser Ala Leu Asp Ile Val His Arg Asn
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Pro Ser Ala Pro Thr Ala Phe Val Tyr Lys Asn Met Asn Phe Asp Met
1               5                  10                  15

Phe Cys Gly Gln Thr Leu Gly Ile Ile Gly Glu Ser Gly Thr Gly Lys
```

-continued

```
            20                  25                  30

Ser Thr Leu Val Leu Leu Leu Thr Lys Ile Ser Val Val Glu Gln Lys
        35                  40                  45

Pro Leu Leu Phe Asn Gly Thr Ile Arg Asp Asn Leu Thr Tyr Gly Leu
    50                  55                  60

Gln Asp Glu Arg Ile Asp Thr Thr Leu Leu Ser Gly Gly Gln Ala Gln
65                  70                  75                  80

Arg Leu Cys Ile Ala Arg Ala Leu Leu Arg Lys Ser Lys Ile Leu Ile
                85                  90                  95

Leu Asp Glu Cys Thr Ser Ala Leu Asp Ser Val Ser Ser Ser
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:30:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 110 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Tyr Lys Pro Asp Ser Pro Val Ile Leu Asp Asn Ile Asn Ile Ser Ile
 1               5                  10                  15

Lys Gln Gly Glu Val Ile Gly Ile Val Gly Arg Ser Gly Ser Gly Lys
            20                  25                  30

Ser Thr Leu Ile Lys Leu Ile Gln Arg Val Gly Val Val Leu Gln Asp
        35                  40                  45

Asn Val Leu Leu Asn Arg Ser Ile Ile Asp Asn Ile Ser Leu Ala Pro
    50                  55                  60

Gly Met Ser Gly Glu Gln Gly Ala Gly Leu Ser Gly Gly Gln Arg Gln
65                  70                  75                  80

Arg Ile Ala Ile Ala Arg Ala Leu Val Asn Asn Pro Lys Ile Leu Ile
                85                  90                  95

Phe Asp Glu Ala Thr Ser Ala Leu Asp Tyr Ala Ser Glu His
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:31:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 111 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ile Pro Ala Pro Arg Lys His Leu Leu Lys Asn Val Cys Gly Val Ala
1               5                   10                  15

Tyr Pro Gly Glu Leu Leu Ala Val Met Gly Ser Ser Gly Ala Gly Lys
            20                  25                  30

Thr Thr Leu Leu Asn Ala Leu Ala Phe Arg Cys Ala Tyr Val Gln Gln
        35                  40                  45

Asp Asp Leu Phe Ile Gly Leu Ile Ala Arg Glu His Leu Ile Phe Gln
    50                  55                  60

Ala Met Val Arg Pro Gly Arg Val Lys Gly Leu Ser Gly Gly Glu Arg
65                  70                  75                  80
```

-continued

```
Lys Arg Leu Ala Phe Ala Ser Glu Ala Leu Thr Asp Pro Pro Leu Leu
                85                  90                  95

Ile Cys Asp Glu Pro Thr Ser Gly Leu Asp Ser Phe Thr Ala His
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
Lys Ser Leu Gly Asn Leu Lys Ile Leu Asp Arg Val Ser Leu Tyr Val
1               5                   10                  15

Pro Lys Phe Ser Leu Ile Ala Leu Leu Gly Pro Ser Gly Ser Gly Lys
            20                  25                  30

Ser Ser Leu Leu Arg Ile Leu Ala Gly Met Ser Phe Val Phe Gln His
        35                  40                  45

Tyr Ala Leu Phe Lys His Met Thr Val Tyr Glu Asn Ile Ser Phe Gly
    50                  55                  60

Leu Arg Leu Arg Phe Glu Tyr Pro Ala Gln Leu Ser Gly Gly Gln Lys
65                  70                  75                  80

Gln Arg Val Ala Leu Ala Arg Ser Leu Ala Ile Gln Pro Asp Leu Leu
                85                  90                  95

Leu Asp Glu Pro Phe Gly Ala Leu Asp Gly Glu Leu Arg Arg
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Gln Asp Val Ala Glu Ser Thr Arg Leu Gly Pro Leu Ser Gly Glu Val
   1               5                  10                  15

Arg Ala Gly Arg Ile Leu His Leu Val Gly Pro Asn Gly Ala Gly Lys
              20                  25                  30

Ser Thr Leu Leu Ala Arg Ile Ala Gly Tyr Leu Ser Gln Gln Gln Thr
        35                  40                  45

Pro Pro Phe Ala Thr Pro Val Trp His Tyr Leu Thr Leu His Gln His
       50                  55                  60

Asp Lys Thr Arg Gly Arg Ser Thr Asn Gln Leu Ser Gly Gly Glu Trp
65                  70                  75                  80

Gln Arg Val Arg Leu Ala Ala Val Val Leu Gln Ile Thr Leu Leu Leu
                85                  90                  95

Leu Asp Glu Pro Met Asn Ser Leu Asp Val Ala Gln Gln Ser Ala
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids

-continued

(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Phe Tyr Tyr Gly Lys Phe His Ala Leu Lys Asn Ile Asn Leu Asp Thr
1               5                   10                  15

Ala Lys Asn Gln Val Thr Ala Phe Ile Gly Pro Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Leu Leu Arg Thr Phe Asn Lys Val Gly Met Val Phe Gln Lys
        35                  40                  45

Pro Thr Pro Phe Pro Met Ser Ile Tyr Asp Asn Ile Ala Phe Gly Val
    50                  55                  60

Arg Leu Phe His Gln Ser Gly Tyr Ser Leu Ser Gly Gly Gln Gln Gln
65                  70                  75                  80

Arg Leu Cys Ile Ala Arg Gly Ile Ala Ile Arg Pro Glu Val Leu Leu
                85                  90                  95

Leu Asp Glu Pro Cys Ser Ala Leu Asp Pro Ile Ser Thr Gly
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Arg Arg Tyr Gly Gly His Glu Val Leu Lys Gly Val Ser Leu Gln Ala
1               5                   10                  15

Arg Ala Gly Asp Val Ile Ser Ile Ile Gly Ser Ser Gly Ser Gly Lys
            20                  25                  30

Ser Thr Phe Leu Arg Cys Ile Asn Phe Gly Ile Met Val Phe Gln His
        35                  40                  45

Phe Asn Leu Trp Ser His Met Thr Val Leu Glu Asn Val Met Glu Ala
    50                  55                  60

Pro Ile Gln Val Gly Lys Tyr Pro Val His Leu Ser Gly Gly Gln Gln
65                  70                  75                  80

Gln Arg Val Ser Ile Ala Arg Ala Leu Ala Met Glu Pro Asp Val Leu
                85                  90                  95

Leu Phe Asp Glu Pro Thr Ser Ala Leu Asp Pro Glu Leu Val Gly
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Lys Ala Trp Gly Glu Val Val Val Ser Lys Asp Ile Asn Ile Asp Ile
  1               5                  10                  15
```

-continued

```
His Glu Gly Glu Phe Val Val Phe Val Gly Pro Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Leu Leu Arg Met Ile Ala Gly Val Gly Met Val Phe Gln Ser
        35                  40                  45

Tyr Ala Leu Tyr Pro His Leu Ser Val Ala Glu Asn Met Ser Phe Gly
    50                  55                  60

Leu Lys Pro Ala Asp Arg Lys Pro Lys Ala Leu Ser Gly Gly Arg Gln
65                  70                  75                  80

Gln Arg Val Ala Ile Gly Arg Thr Leu Val Ala Glu Pro Ser Val Phe
                85                  90                  95

Leu Leu Asp Glu Pro Leu Ser Asn Leu Asp Ala Ala Leu Arg Val
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:37:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 111 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Thr Pro Asp Gly Asp Val Thr Ala Val Asn Asp Leu Asn Phe Thr Leu
1               5                   10                  15

Arg Ala Gly Glu Thr Leu Gly Ile Val Gly Glu Ser Gly Ser Gly Lys
            20                  25                  30

Ser Gln Thr Ala Phe Ala Leu Met Gly Ile Ser Met Ile Phe Gln Asp
        35                  40                  45

Pro Met Thr Ser Leu Asn Pro Tyr Met Arg Val Gly Glu Gln Leu Met
    50                  55                  60

Glu Val Leu Met Lys Met Tyr Pro His Glu Phe Ser Gly Gly Met Arg
65                  70                  75                  80

Gln Arg Val Met Ile Ala Met Ala Leu Leu Cys Arg Pro Lys Leu Leu
                85                  90                  95

Ile Ala Asp Glu Pro Thr Thr Ala Leu Asp Val Thr Val Gln Ala
            100                 105                 110


(2) INFORMATION FOR SEQ ID NO:38:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 111 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: Not Relevant
          (D) TOPOLOGY: Not Relevant

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gln Pro Pro Lys Thr Leu Lys Ala Val Asp Gly Val Thr Leu Arg Leu
1               5                   10                  15

Tyr Glu Gly Glu Thr Leu Gly Val Val Gly Glu Ser Gly Cys Gly Lys
            20                  25                  30

Ser Thr Phe Ala Arg Ala Ile Ile Gly Ile Gln Met Ile Phe Gln Asp
        35                  40                  45

Pro Leu Ala Ser Leu Asn Pro Arg Met Thr Ile Gly Glu Ile Ile Ala
    50                  55                  60

Glu Pro Leu Arg Asn Arg Tyr Pro His Glu Phe Ser Gly Gly Gln Cys
65                  70                  75                  80
```

-continued

```
Gln Arg Ile Gly Ile Ala Arg Ala Leu Ile Leu Glu Pro Lys Leu Ile
                85                  90                  95

Ile Cys Asp Asp Ala Val Ser Ala Leu Asp Val Ser Ile Gln Ala
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Lys Ala Val Pro Gly Val Lys Ala Leu Ser Gly Ala Ala Leu Asn Val
1               5                   10                  15

Tyr Pro Gly Arg Val Met Ala Leu Val Gly Glu Asn Gly Ala Gly Lys
            20                  25                  30

Ser Thr Met Met Lys Val Leu Thr Gly Ala Gly Ile Ile His Gln Glu
        35                  40                  45

Leu Asn Leu Ile Pro Gln Leu Thr Ile Ala Glu Asn Ile Phe Leu Gly
    50                  55                  60

Arg Glu Phe Val Asp Lys Leu Val Gly Asp Leu Ser Ile Gly Asp Gln
65                  70                  75                  80

Gln Met Val Glu Ile Ala Lys Val Leu Ser Phe Glu Ser Lys Val Ile
                85                  90                  95

Ile Met Asp Glu Pro Thr Cys Ala Leu Ile Asp Thr Glu Thr Glu
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
Val Asp Asn Leu Cys Gly Pro Gly Val Asn Asp Val Ser Phe Thr Leu
1               5                   10                  15

Arg Lys Gly Glu Ile Leu Gly Val Ser Gly Leu Met Gly Ala Gly Arg
            20                  25                  30

Thr Glu Leu Met Lys Val Leu Tyr Gly Ile Ser Glu Asp Arg Lys Arg
        35                  40                  45

Asp Gly Leu Val Leu Gly Met Ser Val Lys Glu Asn Met Ser Leu Thr
    50                  55                  60

Ala Leu Arg Tyr Glu Gln Ala Ile Gly Leu Leu Ser Gly Gly Asn Gln
65                  70                  75                  80

Gln Lys Val Ala Ile Ala Arg Gly Leu Met Thr Arg Pro Lys Val Leu
                85                  90                  95

Ile Leu Asp Glu Pro Thr Pro Gly Val Asp Val Gly Ala Lys Lys
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Leu Thr Gly Ala Arg Gly Asn Asn Leu Lys Asp Val Thr Leu Thr Leu
1               5                   10                  15

Pro Val Gly Leu Phe Thr Cys Ile Thr Gly Val Ser Gly Ser Gly Lys
            20                  25                  30

Ser Thr Leu Ile Asn Asp Thr Leu Phe Thr Tyr Thr Gly Val Phe Thr
        35                  40                  45

Pro Val Arg Glu Leu Phe Ala Gly Val Pro Glu Ser Arg Ala Arg Gly
    50                  55                  60

Tyr Thr Pro Gly Gly Gln Ser Ala Thr Thr Leu Ser Gly Gly Glu Ala
65                  70                  75                  80

Gln Arg Val Lys Leu Ala Arg Glu Leu Ser Lys Arg Gly Leu Tyr Ile
                85                  90                  95

Leu Asp Glu Pro Thr Thr Gly Leu His Phe Ala Asp Ile Gln Gln
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Lys Ser Tyr Gly Gly Lys Ile Val Val Asn Asp Leu Ser Phe Thr Ile
 1               5                   10                  15

Ala Ala Gly Glu Cys Phe Gly Leu Leu Gly Pro Asn Gly Ala Gly Lys
            20                  25                  30

Ser Thr Ile Ile Arg Met Ile Leu Gly Ile Gly Ile Val Ser Gln Glu
        35                  40                  45

Asp Asn Leu Asp Leu Glu Phe Thr Val Arg Glu Asn Leu Leu Val Tyr
    50                  55                  60

Gly Arg Tyr Phe Asn Thr Arg Val Ala Asp Leu Ser Gly Gly Met Lys
65                  70                  75                  80

Arg Arg Leu Thr Leu Ala Gly Ala Leu Ile Asn Asp Pro Gln Leu Leu
                85                  90                  95

Ile Leu Asp Glu Pro Thr Thr Gly Leu Asp Pro His Ala Arg His
            100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: Not Relevant
(D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Ala Tyr Leu Gly Gly Arg Gln Ala Leu Gln Gly Val Thr Phe His Met
1               5                   10                  15
```

-continued

```
Gln Pro Gly Glu Met Ala Phe Leu Thr Gly His Ser Gly Ala Gly Lys
            20                  25                  30

Ser Thr Leu Leu Lys Leu Ile Cys Gly Ile Gly Met Ile Phe Gln Asp
        35                  40                  45

His His Leu Leu Met Asp Arg Thr Val Tyr Asp Asn Val Ala Ile Pro
    50                  55                  60

Leu Ile Ile Ala Lys Asn Phe Pro Ile Gln Leu Ser Gly Gly Glu Gln
65                  70                  75                  80

Gln Arg Val Gly Ile Ala Arg Ala Val Val Asn Lys Pro Ala Val Leu
                85                  90                  95

Leu Ala Asp Glu Pro Thr Gly Asn Leu Asp Asp Ala Leu Ser Glu
            100                 105                 110
```

We claim:

1. An isolated polypeptide comprising an amino acid sequence

(a) according to FIGS. **1**A–**1**H for amino acid residue positions from 1 to 1480 comprising a normal CFTR polypeptide.

2. A polypeptide according to claim **1** made by chemical or enzymatic peptide synthesis.

3. An isolated mutant CFTR polypeptide, comprising at least one CF mutation of a normal CFTR polypeptide having the sequence according to FIGS. **1**A–**1**H for amino acid residue positions 1 to 1480, wherein said CF mutation includes the deletion of phenylalanine from amino acid residue position 508 of FIGS. **1**A–**1**H.

* * * * *