EXHIBIT B
Part 1 of 2

United States Patent [19]

Goeddel et al.

[11] Patent Number: 4,766,075

[45] Date of Patent: Aug. 23, 1988

[54] HUMAN TISSUE PLASMINOGEN ACTIVATOR

[75] Inventors: **David V. Goeddel**, Hillsborough; **William J. Kohr**, San Mateo; **Diane Pennica**, Foster City; **Gordon A. Vehar**, San Carlos, all of Calif.

[73] Assignee: **Genentech, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **483,052**

[22] Filed: **Apr. 7, 1983**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 398,003, Jul. 14, 1982, and a continuation-in-part of Ser. No. 374,860, May 5, 1982.

[51] **Int. Cl.**[4] **C12N 5/00**; C12N 15/00; C12N 9/48; C12N 9/72; C12N 1/20; C12N 1/00; C07H 17/00

[52] **U.S. Cl.** **435/240.2**; 435/172.3; 435/212; 435/215; 435/253; 435/320; 536/27; 935/14; 935/29; 935/32; 935/70; 935/73

[58] **Field of Search** 435/172.3, 253, 317, 435/240, 212, 215, 320, 240.2; 536/27; 935/14, 27, 29, 32, 72, 73, 70

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Primary Examiner—Thomas G. Wiseman
Assistant Examiner—Jayme A. Huleatt

[57] **ABSTRACT**

Human tissue phasminogen activator (t-PA) is produced in useful quantities using recombinant DNA techniques. The invention disclosed thus enables the production of t-PA free of contaminants with which it is ordinarily associated in its native cellular environment. Methods, expression vehicles and various host cells useful in its production are also disclosed.

11 Claims, 14 Drawing Sheets

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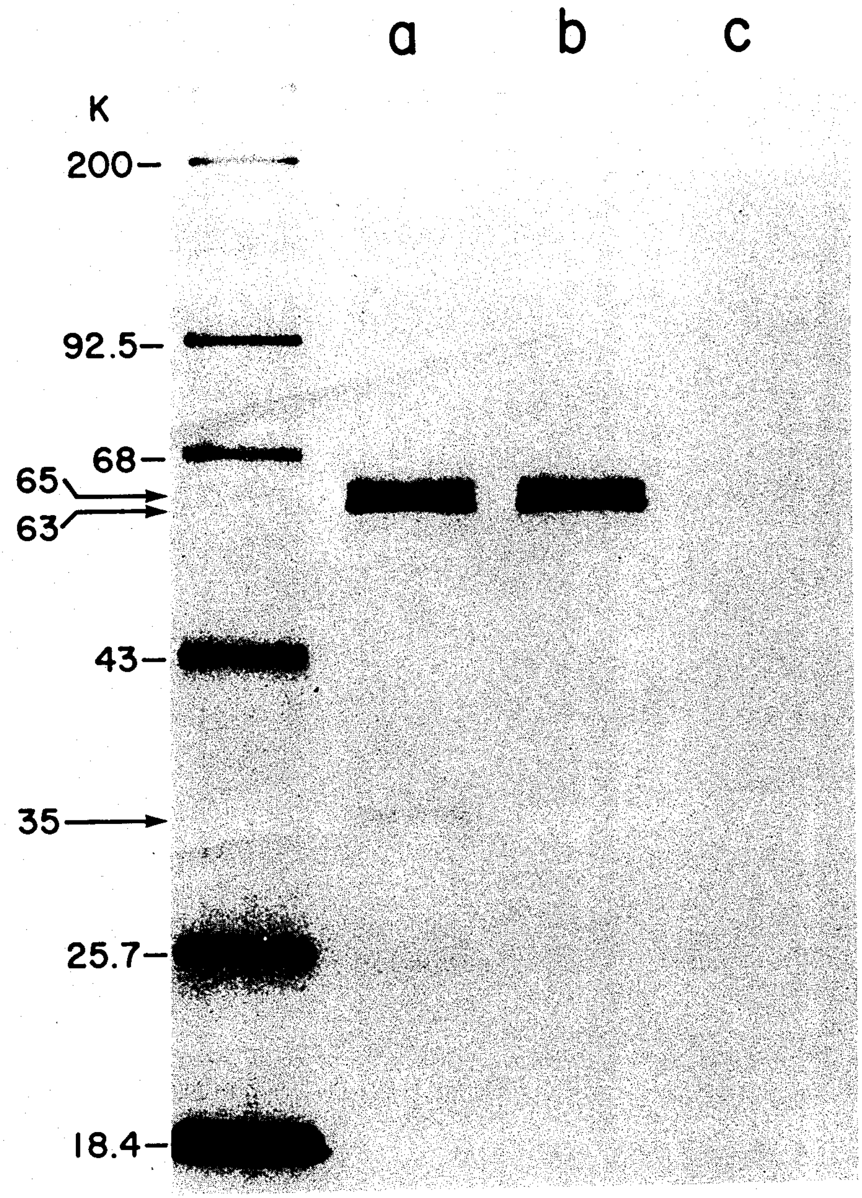


Fig.1.

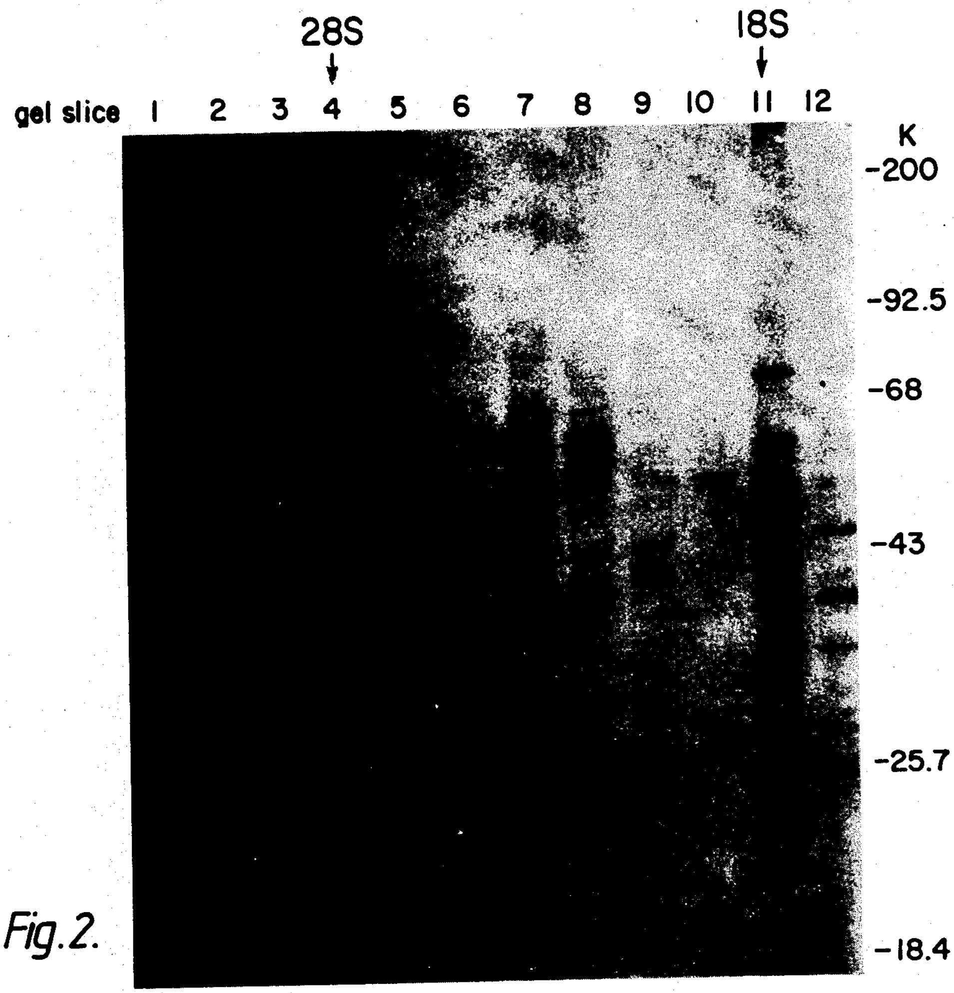


Fig. 2.

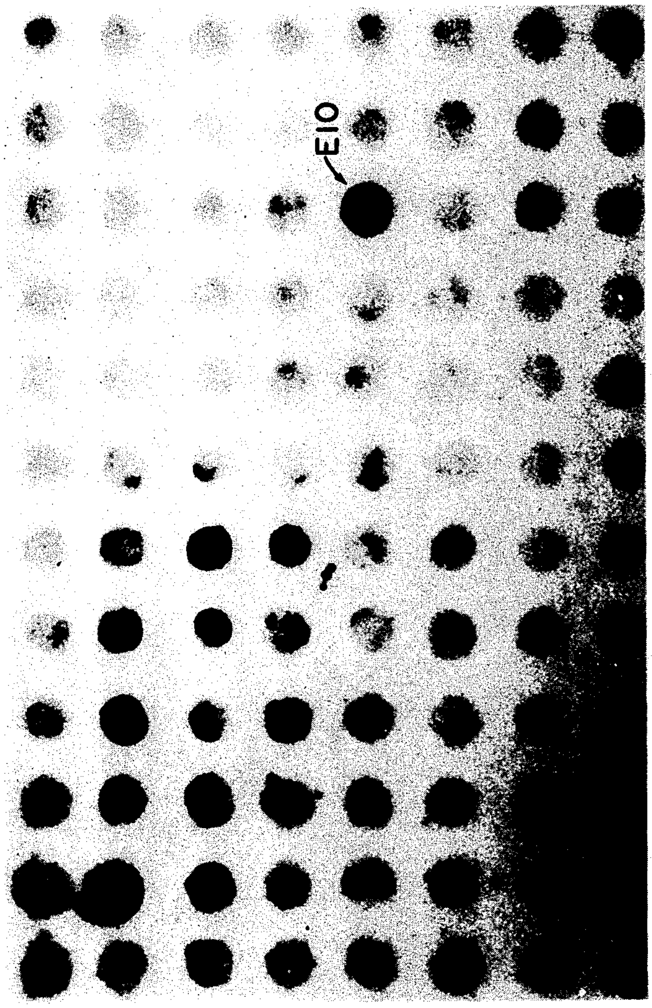
COLONY HYBRIDIZATION

RNA Gel Slice 7 cDNA Clones

vs.

^{32}P-TC$\binom{A}{G}$CA$\binom{A}{G}$TA$\binom{C}{T}$TCCCA Probe

Fig.3.



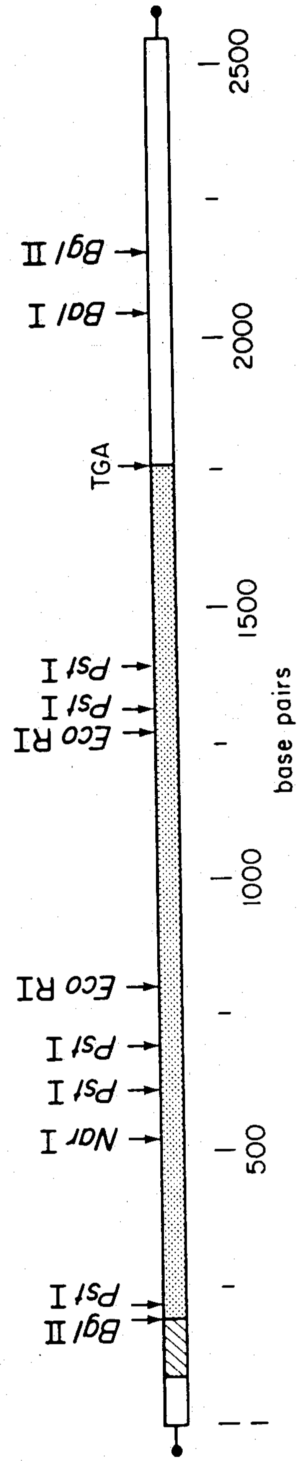

Bgl II
Bal I
TGA
Pst I
Pst I
Eco RI
Eco RI
Pst I
Pst I
Nar I
Pst I
Bgl II
2500
2000
1500
1000
500
base pairs

Fig. 4.

```
GTTCTGAGCACAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGGAAGGAGCAAGCCGTGA

                            -35                     -30
                            met asp ala met lys arg gly leu
ATTTAAGGGACGCTGTGAAGCAATC   ATG GAT GCA ATG AAG AGA GGG CTC

                            -20
cys cys val leu leu leu cys gly ala val phe val ser pro ser
TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC

        -10                                     1
gln glu ile his ala arg phe arg arg gly ala arg SER TYR GLN
CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA TCT TAC CAA

                        10
VAL ILE CYS ARG ASP GLU LYS THR GLN MET ILE TYR GLN GLN HIS
GTG ATC TGC AGA GAT GAA AAA ACG CAG ATG ATA TAC CAG CAA CAT

    20                                      30
GLN SER TRP LEU ARG PRO VAL LEU ARG SER ASN ARG VAL GLU TYR
CAG TCA TGG CTG CGC CCT GTG CTC AGA AGC AAC CGG GTG GAA TAT

                        40
CYS TRP CYS ASN SER GLY ARG ALA GLN CYS HIS SER VAL PRO VAL
TGC TGG TGC AAC AGT GGC AGG GCA CAG TGC CAC TCA GTG CCT GTC

    50                                      60
LYS SER CYS SER GLU PRO ARG CYS PHE ASN GLY GLY THR CYS GLN
AAA AGT TGC AGC GAG CCA AGG TGT TTC AAC GGG GGC ACC TGC CAG

                        70
GLN ALA LEU TYR PHE SER ASP PHE VAL CYS GLN CYS PRO GLU GLY
CAG GCC CTG TAC TTC TCA GAT TTC GTG TGC CAG TGC CCC GAA GGA

    80                                      90
PHE ALA GLY LYS CYS CYS GLU ILE ASP THR ARG ALA THR CYS TYR
TTT GCT GGG AAG TGC TGT GAA ATA GAT ACC AGG GCC ACG TGC TAC

                        100
GLU ASP GLN GLY ILE SER TYR ARG GLY THR TRP SER THR ALA GLU
GAG GAC CAG GGC ATC AGC TAC AGG GGC ACG TGG AGC ACA GCG GAG

    110                                     120
SER GLY ALA GLU CYS THR ASN TRP ASN SER SER ALA LEU ALA GLN
AGT GGC GCC GAG TGC ACC AAC TGG AAC AGC AGC GCG TTG GCC CAG

                        130
LYS PRO TYR SER GLY ARG ARG PRO ASP ALA ILE ARG LEU GLY LEU
AAG CCC TAC AGC GGG CGG AGG CCA GAC GCC ATC AGG CTG GGC CTG

    140                                     150
GLY ASN HIS ASN TYR CYS ARG ASN PRO ASP ARG ASP SER LYS PRO
GGG AAC CAC AAC TAC TGC AGA AAC CCA GAT CGA GAC TCA AAG CCC

                        160
TRP CYS TYR VAL PHE LYS ALA GLY LYS TYR SER SER GLU PHE CYS
TGG TGC TAC GTC TTT AAG GCG GGG AAG TAC AGC TCA GAG TTC TGC

    170                                     180
SER THR PRO ALA CYS SER GLU GLY ASN SER ASP CYS TYR PHE GLY
AGC ACC CCT GCC TGC TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG
```

Fig. 5A.

```
                                190
ASN GLY SER ALA TYR ARG GLY THR HIS SER LEU THR GLU SER GLY
AAT GGG TCA GCC TAC CGT GGC ACG CAC AGC CTC ACC GAG TCG GGT

    200                                         210
ALA SER CYS LEU PRO TRP ASN SER MET ILE LEU ILE GLY LYS VAL
GCC TCC TGC CTC CCG TGG AAT TCC ATG ATC CTG ATA GGC AAG GTT

                                220
TYR THR ALA GLN ASN PRO SER ALA GLN ALA LEU GLY LEU GLY LYS
TAC ACA GCA CAG AAC CCC AGT GCC CAG GCA CTG GGC CTG GGC AAA

    230                                         240
HIS ASN TYR CYS ARG ASN PRO ASP GLY ASP ALA LYS PRO TRP CYS
CAT AAT TAC TGC CGG AAT CCT GAT GGG GAT GCC AAG CCC TGG TGC

                                250
HIS VAL LEU LYS ASN ARG ARG LEU THR TRP GLU TYR CYS ASP VAL
CAC GTG CTG AAG AAC CGC AGG CTG ACG TGG GAG TAC TGT GAT GTG

    260                                         270
PRO SER CYS SER THR CYS GLY LEU ARG GLN TYR SER GLN PRO GLN
CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG TAC AGC CAG CCT CAG

                                280
PHE ARG ILE LYS GLY GLY LEU PHE ALA ASP ILE ALA SER HIS PRO
TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC ATC GCC TCC CAC CCC

    290                                         300
TRP GLN ALA ALA ILE PHE ALA LYS HIS ARG ARG SER PRO GLY GLU
TGG CAG GCT GCC ATC TTT GCC AAG CAC AGG AGG TCG CCC GGA GAG

                                310
ARG PHE LEU CYS GLY GLY ILE LEU ILE SER SER CYS TRP ILE LEU
CGG TTC CTG TGC GGG GGC ATA CTC ATC AGC TCC TGC TGG ATT CTC

    320                                         330
SER ALA ALA HIS CYS PHE GLN GLU ARG PHE PRO PRO HIS HIS LEU
TCT GCC GCC CAC TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC CTG

                                340
THR VAL ILE LEU GLY ARG THR TYR ARG VAL VAL PRO GLY GLU GLU
ACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG GAG

    350                                         360
GLU GLN LYS PHE GLU VAL GLU LYS TYR ILE VAL HIS LYS GLU PHE
GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC

                                370
ASP ASP ASP THR TYR ASP ASN ASP ILE ALA LEU LEU GLN LEU LYS
GAT GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG CAG CTG AAA

    380                                         390
SER ASP SER SER ARG CYS ALA GLN GLU SER SER VAL VAL ARG THR
TCG GAT TCG TCC CGC TGT GCC CAG GAG AGC AGC GTG GTC CGC ACT

                                400
VAL CYS LEU PRO PRO ALA ASP LEU GLN LEU PRO ASP TRP THR GLU
GTG TGC CTT CCC CCG GCG GAC CTG CAG CTG CCG GAC TGG ACG GAG

    410                                         420
CYS GLU LEU SER GLY TYR GLY LYS HIS GLU ALA LEU SER PRO PHE
TGT GAG CTC TCC GGC TAC GGC AAG CAT GAG GCC TTG TCT CCT TTC
```

Fig. 5B.

430
TYR SER GLU ARG LEU LYS GLU ALA HIS VAL ARG LEU TYR PRO SER
TAT TCG GAG CGG CTG AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC

440 450
SER ARG CYS THR SER GLN HIS LEU LEU ASN ARG THR VAL THR ASP
AGC CGC TGC ACA TCA CAA CAT TTA CTT AAC AGA ACA GTC ACC GAC

460
ASN MET LEU CYS ALA GLY ASP THR ARG SER GLY GLY PRO GLN ALA
AAC ATG CTG TGT GCT GGA GAC ACT CGG AGC GGC GGG CCC CAG GCA

470 480
ASN LEU HIS ASP ALA CYS GLN GLY ASP SER GLY GLY PRO LEU VAL
AAC TTG CAC GAC GCC TGC CAG GGC GAT TCG GGA GGC CCC CTG GTG

490
CYS LEU ASN ASP GLY ARG MET THR LEU VAL GLY ILE ILE SER TRP
TGT CTG AAC GAT GGC CGC ATG ACT TTG GTG GGC ATC ATC AGC TGG

500 510
GLY LEU GLY CYS GLY GLN LYS ASP VAL PRO GLY VAL TYR THR LYS
GGC CTG GGC TGT GGA CAG AAG GAT GTC CCG GGT GTG TAC ACC AAG

520 527
VAL THR ASN TYR LEU ASP TRP ILE ARG ASP ASN MET ARG PRO OP
GTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC ATG CGA CCG TGA

CCAGGAACACCCGACTCCTCAAAAGCAAATGAGATCCCGCCTCTTCTTCTTCAGAAGACA

CTGCAAAGGCGCAGTGCTTCTCTACAGACTTCTCCAGACCCACCACACCGCAGAAGCGGG

ACGAGACCCTACAGGAGAGGGAAGAGTGCATTTTCCCAGATACTTCCCATTTTGGAAGT

TTTCAGGACTTGGTCTGATTTCAGGATACTCTGTCAGATGGGAAGACATGAATGCACACT

AGCCTCTCCAGGAATGCCTCCTCCCTGGGCAGAAAGTGGCCATGCCACCCTGTTTTCAGCTA

AAGCCCAACCTCCTGACCTGTCACCGTGAGCAGCTTTGGAAACAGGACCACAAAAATGAA

AGCATGTCTCAATAGTAAAAGATAACAAGATCTTTCAGGAAAGACGGATTGCATTAGAA

ATAGACAGTATATTTATAGTCACAAGAGCCCAGCAGGGCCTCAAAGTTGGGGCAGGCTGGC

TGGCCCGTCATGTTCCTCAAAAGCACCCTTGACGTCAAGTCTCCTTCCCCTTTCCCCACT

CCCTGGCTCTCAGAAGGTATTCCTTTTGTGTACAGTGTGTAAAGTGTAAATCCTTTTTCT

TTATAAACTTTAGAGTAGCATGAGAGAATTGTATCATTTGAACAACTAGGCTTCAGCATA

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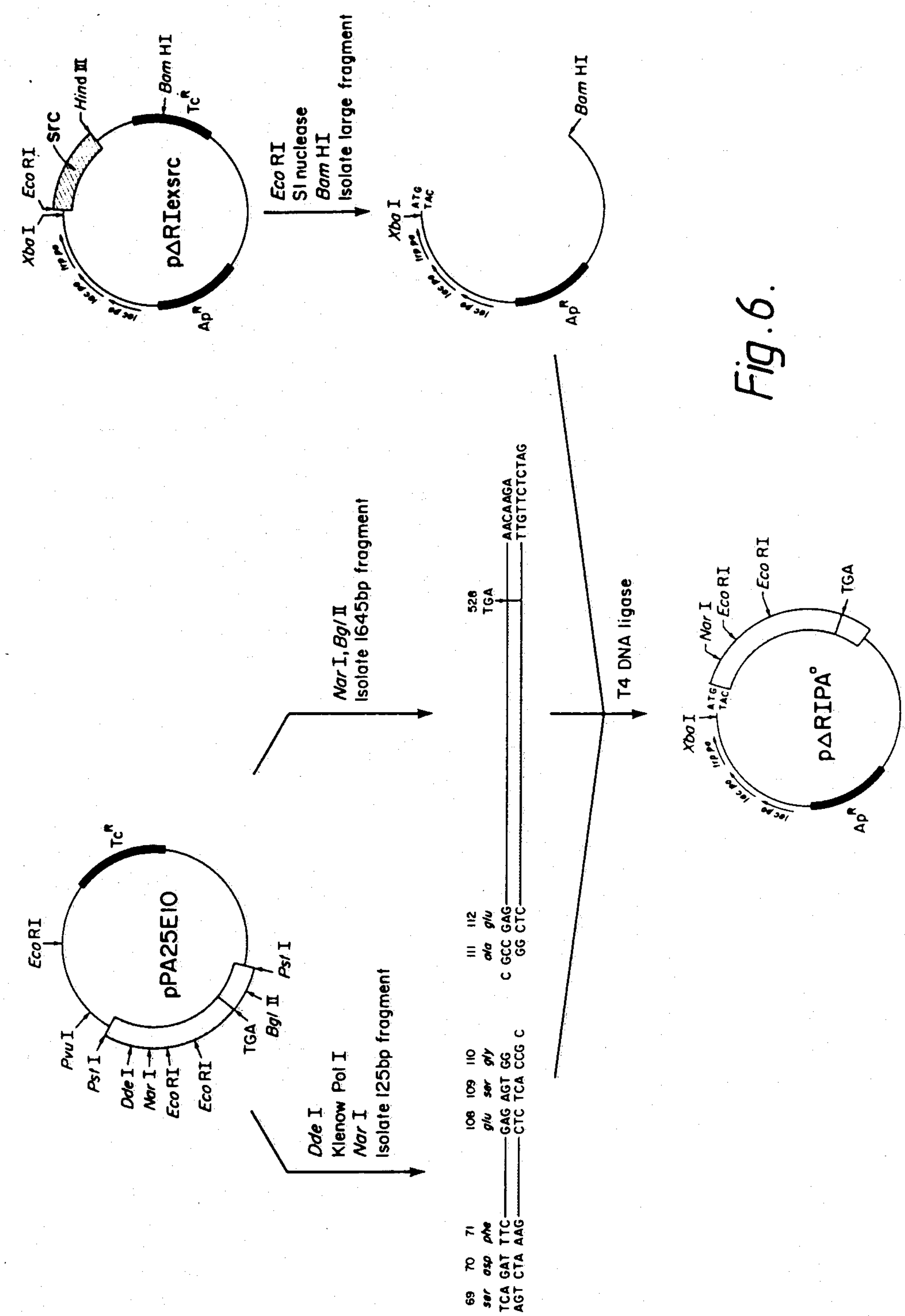
Fig. 5C.



pΔRIexsrc
src
Xba I
Eco RI
Hind III
Bam HI
Tc^R
Ap^R
Eco RI
SI nuclease
Bam HI
Isolate large fragment
Xba I
ATG
TAC
Bam HI
Ap^R
pPA25E10
Eco RI
Tc^R
Pvu I
Pst I
Dde I
Nar I
Eco RI
Eco RI
TGA
Bgl II
Pst I
Nar I, Bgl II
Isolate 1645bp fragment
Dde I
Klenow Pol I
Nar I
Isolate 125bp fragment
528
TGA
AACAAGA
TTGTTCTCTAG
69 70 71
ser asp phe
TCA GAT TTC
AGT CTA AAG
108 109 110
glu ser gly
GAG AGT GG
CTC TCA CCG C
111 112
ala glu
C GCC GAG
GG CTC
T4 DNA ligase
pΔRIPA°
Xba I
ATG
TAC
Nar I
Eco RI
Eco RI
TGA
Ap^R

Fig. 6.

Fig. 7.

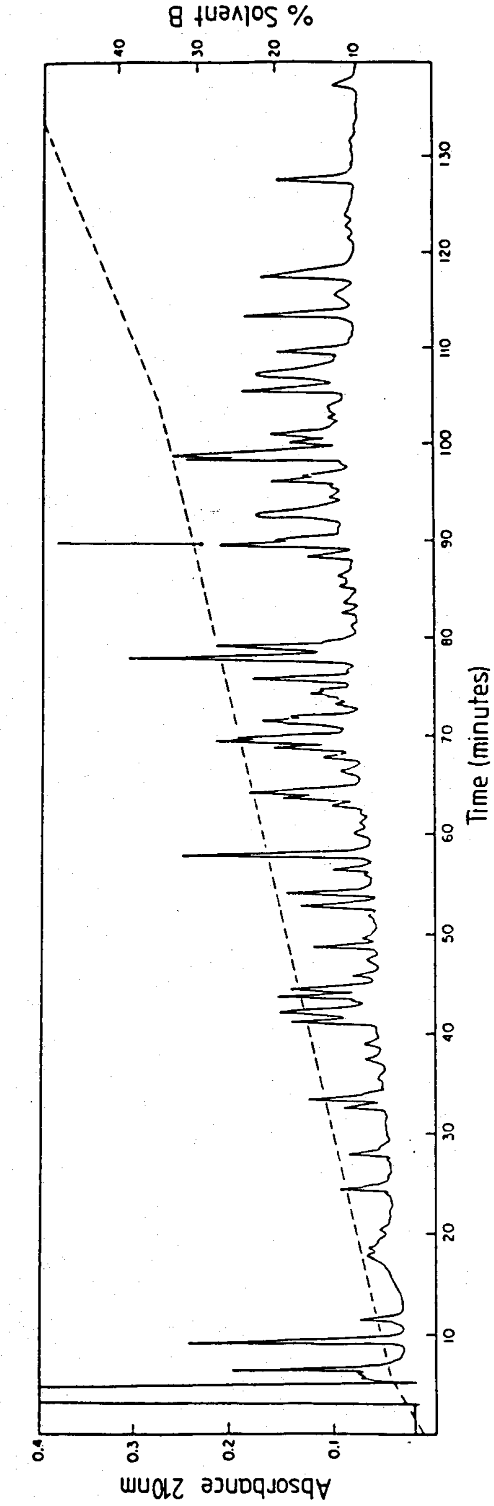
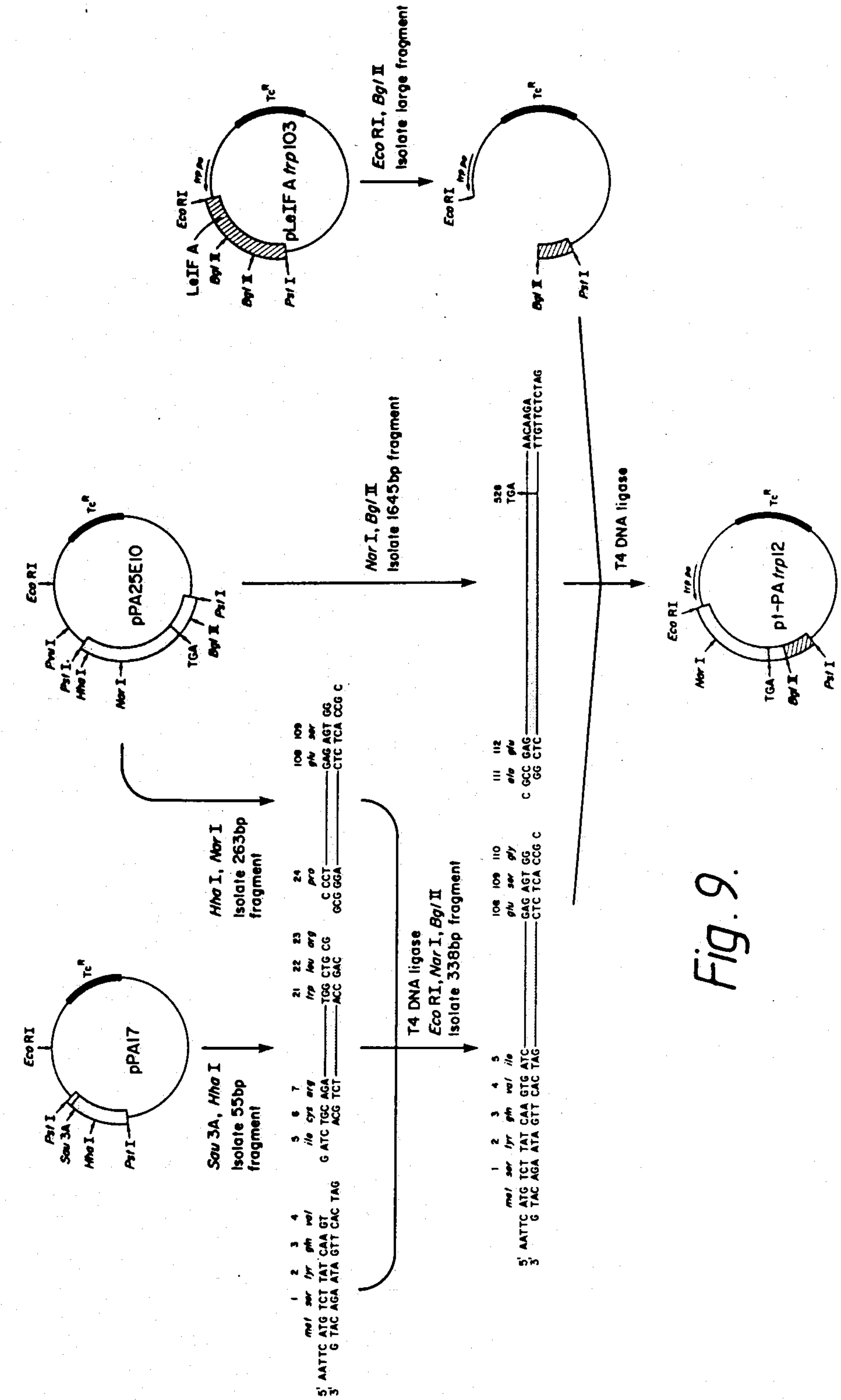

% Solvent B
40
30
20
10
Absorbance 210nm
0.4
0.3
0.2
0.1
Time (minutes)
10
20
30
40
50
60
70
80
90
100
110
120
130

Fig. 8.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EcoRI
LeIF A
Bgl II
Pst I
Tc^R
Eco RI, Bgl II
isolate large fragment
pPA25E10
Nar I
TGA
Nar I, Bgl II
isolate 1645bp fragment
pPA17
Sau 3A
Hha I
Sau 3A, Hha I
isolate 55bp fragment
Hha I, Nar I
isolate 263bp fragment
T4 DNA ligase
Eco RI, Nar I, Bgl II
isolate 338bp fragment
T4 DNA ligase
p1-PA trp12

Fig. 9.

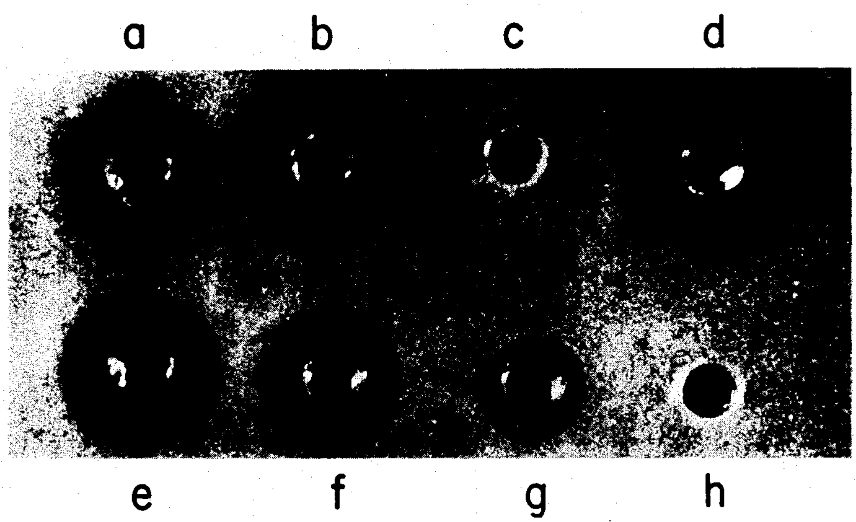

Fig. 10.

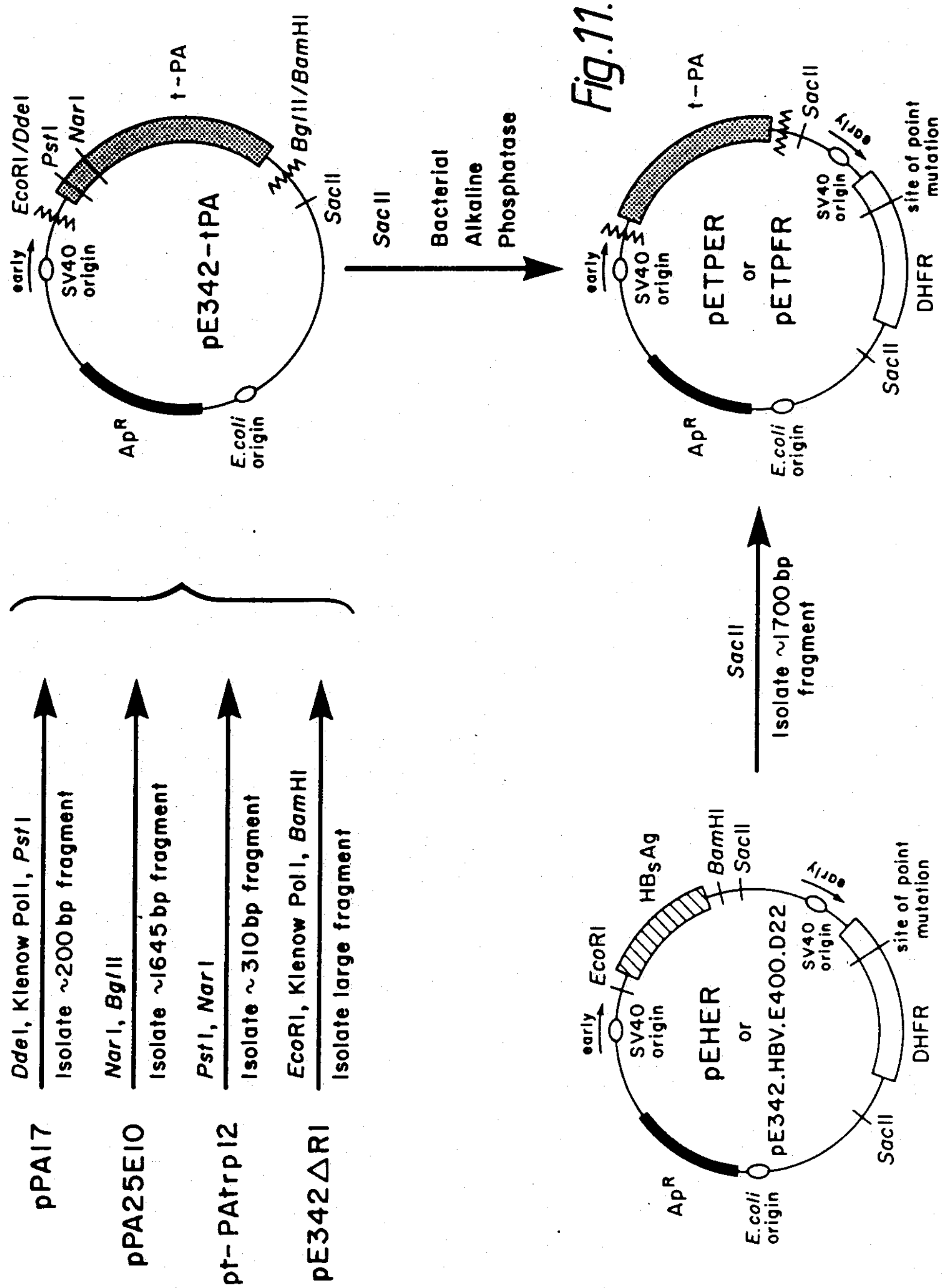
pPA17
DdeI, Klenow PolI, PstI
Isolate ~200 bp fragment
pPA25E10
NarI, BglII
Isolate ~1645 bp fragment
pt-PAtrp12
PstI, NarI
Isolate ~310 bp fragment
pE342ΔRI
EcoRI, Klenow PolI, BamHI
Isolate large fragment
pE342-tPA
early
SV40 origin
EcoRI/DdeI
PstI
NarI
t-PA
BglII/BamHI
SacII
ApR
E.coli origin
SacII
Bacterial Alkaline Phosphatase
pETPER or pETPFR
SV40 origin
site of point mutation
DHFR
pEHER or pE342.HBV.E400.D22
EcoRI
HBsAg
BamHI
SacII
SacII
Isolate ~1700 bp fragment

Fig.11.