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(54) Title: FAP α–SPECIFIC ANTIBODY WITH IMPROVED PRODUCIBILITY

(57) Abstract

Recombinant antibody proteins are provided that specifically bind fibroblast activation protein alpha (FAPα) and comprise framework modifications resulting in the improved producibility in host cells. The invention also relates to the use of said antibodies for diagnostic and therapeutic purposes and methods of producing said antibodies.

Fig. 1

```
1           11          21          31          41
GACATTGTGA TGACCCAATC TCCAGACTCT TTGGCTGTGT CTCTAGGGGA
51          61          71          81          91
GAGGGCCACC ATCAACTGCA AGTCCAGTCA GAGCCTTTTA TATTCTAGAA
101         111         121         131         141
ATCAAAAGAA CTACTTGGCC TGGTATCAGC AGAAACCAGG ACAGCCACCC
151         161         171         181         191
AAACTCCTCA TCTTTTGGGC TAGCACTAGG GAATCTGGGG TACCTGATAG
201         211         221         231         241
GTTCAGTGGC AGTGGGTTTG GGACAGACTT CACCCTCACC ATTAGCAGCC
251         261         271         281         291
TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTTTAGCTAT
301         311         321         331   339
CCGCTCACGT TCGGACAAGG GACCAAGGTG GAAATAAAA
```

Fig. 2

```
1           11          21          31          41
DIVMTQSPDS LAVSLGERAT INCKSSQSLL YSRNQKNYLA WYQQKPGQPP
51          61          71          81          91
KLLIFWASTR ESGVPDRFSG SGFGTDFTLT ISSLQAEDVA VYYCQQYFSY
101         111
PLTFGQGTKV EIK
```

Fig. 3

```
1           11          21          31          41
GACATTGTGA TGACCCAATC TCCAGACTCT TTGGCTGTGT CTCTAGGGGA
51          61          71          81          91
GAGGGCCACC ATCAACTGCA AGTCCAGTCA GAGCCTTTTA TATTCTAGAA
101         111         121         131         141
ATCAAAAGAA CTACTTGGCC TGGTTCCAGC AGAAACCAGG ACAGCCACCC
151         161         171         181         191
AAACTCCTCA TCTTTTGGGC TAGCACTAGG GAATCTGGGG TACCTGATAG
201         211         221         231         241
GTTCAGTGGC AGTGGGTTTG GGACAGACTT CACCCTCACC ATTAGCAGCC
251         261         271         281         291
TGCAGGCTGA AGATGTGGCA GTTTATGACT GTCAACAATA TTTTAGCTAT
301         311         321         331   339
CCGCTCACGT TCGGACAAGG GACCAAGGTG GAAATAAAA
```

Fig. 4

```
1           11          21          31          41
DIVMTQSPDS LAVSLGERAT INCKSSQSLL YSRNQKNYLA WFQQKPGQPP
51          61          71          81          91
KLLIFWASTR ESGVPDRFSG SGFGTDFTLT ISSLQAEDVA VYDCQQYFSY
101         111
PLTFGQGTKV EIK
```

Fig. 5

```
1           11          21          31          41
GACATTGTGA TGACCCAATC TCCAGACTCT TTGGCTGTGT CTCTAGGGGA
51          61          71          81          91
GAGGGCCACC ATCAACTGCA AGTCCAGTCA GAGCCTTTTA TATTCTAGAA
101         111         121         131         141
ATCAAAAGAA CTACTTGGCC TGGTATCAGC AGAAACCAGG ACAGCCACCC
151         161         171         181         191
AAACTCCTCA TCTATTGGGC TAGCACTAGG GAATCTGGGG TACCTGATAG
201         211         221         231         241
GTTCAGTGGC AGTGGGTTTG GGACAGACTT CACCCTCACC ATTAGCAGCC
251         261         271         281         291
TGCAGGCTGA AGATGTGGCA GTTTATTACT GTCAGCAATA TTTTAGCTAT
301         311         321         331   339
CCGCTCACGT TCGGACAAGG GACCAAGGTG GAAATAAAA
```

Fig. 6

```
1           11          21          31          41
DIVMTQSPDS LAVSLGERAT INCKSSQSLL YSRNQKNYLA WYQQKPGQPP
51          61          71          81          91
KLLIYWASTR ESGVPDRFSG SGFGTDFTLT ISSLQAEDVA VYYCQQYFSY
101         111
PLTFGQGTKV EIK
```

Fig. 7

```
1
CAGGTGCAAC TAGTGCAGTC CGGCGCCGAA GTGAAGAAAC CCGGTGCTTC
51
CGTGAAAGTC AGCTGTAAAA CTAGTAGATA CACCTTCACT GAATACACCA
101
TACACTGGGT TAGACAGGCC CCTGGCCAAA GGCTGGAGTG GATAGGAGGT
151
ATTAATCCTA ACAATGGTAT TCCTAACTAC AACCAGAAGT TCAAGGGCCG
201
GGCCACCTTG ACCGTAGGCA AGTCTGCCAG CACCGCCTAC ATGGAACTGT
251
CCAGCCTGCG CTCCGAGGAC ACTGCAGTCT ACTACTGCGC CAGAAGAAGA
301
ATCGCCTATG GTTACGACGA GGGCCATGCT ATGGACTACT GGGGTCAAGG
351                   372
AACCCTTGTC ACCGTCTCCT CA
```

Fig. 8

```
1           11          21          31          41
QVQLVQSGAE VKKPGASVKV SCKTSRYTFT EYTIHWVRQA PGQRLEWIGG
51          61          71          81          91
INPNNGIPNY NQKFKGRATL TVGKSASTAY MELSSLRSED TAVYYCARRR
101         111         121-124
IAYGYDEGHA MDYWGQGTLV TVSS
```

Fig. 9

```
1
CAGGTGCAAC TAGTGCAGTC CGGCGCCGAA GTGAAGAAAC CCGGTGCTTC
51
CGTGAAAGTC AGCTGTAAAA CTAGTAGATA CACCTTCACT GAATACACCA
101
TACACTGGGT TAGACAGGCC CCTGGCCAAA GGCTGGAGTG GATAGGAGGT
151
ATTAATCCTA ACAATGGTAT TCCTAACTAC AACCAGAAGT TCAAGGGCCG
201
GGCCACCTTG ACCGTAGGCA AGTCTGCCAG CACCGCCTAC ATGGAACTGT
251
CCAGCCTGCG CTCCGAGGAC ACTGCAGTCT ACTTCTGCGC CAGAAGAAGA
301
ATCGCCTATG GTTACGACGA GGGCCATGCT ATGGACTACT GGGGTCAAGG
351                   372
AACCCTTGTC ACCGTCTCCT CA
```

Fig. 10

```
1           11          21          31          41
QVQLVQSGAE VKKPGASVKV SCKTSRYTFT EYTIHWVRQA PGQRLEWIGG
51          61          71          81          91
INPNNGIPNY NQKFKGRATL TVGKSASTAY MELSSLRSED TAVYFCARRR
101         111         121-124
IAYGYDEGHA MDYWGQGTLV TVSS
```

Fig. 11

```
1
CAGGTGCAAC TAGTGCAGTC CGGCGCCGAA GTGAAGAAAC CCGGTGCTTC
51
CGTGAAAGTC AGCTGTAAAA CTAGTAGATA CACCTTCACT GAATACACCA
101
TACACTGGGT TAGACAGGCC CCTGGCCAAA GGCTGGAGTG GATAGGAGGT
151
ATTAATCCTA ACAATGGTAT TCCTAACTAC AACCAGAAGT TCAAGGGCCG
201
GGTCACCATC ACCGTAGACA CCTCTGCCAG CACCGCCTAC ATGGAACTGT
251
CCAGCCTGCG CTCCGAGGAC ACTGCAGTCT ACTACTGCGC CAGAAGAAGA
301
ATCGCCTATG GTTACGACGA GGGCCATGCT ATGGACTACT GGGGTCAAGG
351                 372
AACCCTTGTC ACCGTCTCCT CA
```

Fig. 12

```
1           11          21          31          41
QVQLVQSGAE VKKPGASVKV SCKTSRYTFT EYTIHWVRQA PGQRLEWIGG
51          61          71          81          91
INPNNGIPNY NQKFKGRVTI TVDTSASTAY MELSSLRSED TAVYYCARRR
101         111         121-124
IAYGYDEGHA MDYWGQGTLV TVSS
```

Fig. 13

```
1
CAGGTGCAAC TAGTGCAGTC CGGCGCCGAA GTGAAGAAAC CCGGTGCTTC
51
CGTGAAAGTC AGCTGTAAAA CTAGTAGATA CACCTTCACT GAATACACCA
101
TACACTGGGT TAGACAGGCC CCTGGCCAAA GGCTGGAGTG GATAGGAGGT
151
ATTAATCCTA ACAATGGTAT TCCTAACTAC AACCAGAAGT TCAAGGGCCG
201
GGTCACCATC ACCGTAGACA CCTCTGCCAG CACCGCCTAC ATGGAACTGT
251
CCAGCCTGCG CTCCGAGGAC ACTGCAGTCT ACTTCTGCGC CAGAAGAAGA
301
ATCGCCTATG GTTACGACGA GGGCCATGCT ATGGACTACT GGGGTCAAGG
351                  372
AACCCTTGTC ACCGTCTCCT CA
```

Fig. 14

```
1           11          21          31          41
QVQLVQSGAE VKKPGASVKV SCKTSRYTFT EYTIHWVRQA PGQRLEWIGG
51          61          71          81          91
INPNNGIPNY NQKFKGRVTI TVDTSASTAY MELSSLRSED TAVYFCARRR
101         111         121-124
IAYGYDEGHA MDYWGQGTLV TVSS
```

Fig. 15

```
1
CAGGTGCAAC TAGTGCAGTC CGGCGCCGAA GTGAAGAAAC CCGGTGCTTC
51
CGTGAAAGTC AGCTGTAAAA CTAGTGGATA CACCTTCACT GAATACACCA
101
TACACTGGGT TAGACAGGCC CCTGGCCAAA GGCTGGAGTG GATAGGAGGT
151
ATTAATCCTA ACAATGGTAT TCCTAACTAC AACCAGAAGT TCAAGGGCCG
201
GGTCACCATC ACCGTAGACA CCTCTGCCAG CACCGCCTAC ATGGAACTGT
251
CCAGCCTGCG CTCCGAGGAC ACTGCAGTCT ACTACTGCGC CAGAAGAAGA
301
ATCGCCTATG GTTACGACGA GGGCCATGCT ATGGACTACT GGGGTCAAGG
351                  372
AACCCTTGTC ACCGTCTCCT CA
```

Fig. 16

```
1           11          21          31          41
QVQLVQSGAE VKKPGASVKV SCKTSGYTFT EYTIHWVRQA PGQRLEWIGG
51          61          71          81          91
INPNNGIPNY NQKFKGRVTI TVDTSASTAY MELSSLRSED TAVYYCARRR
101         111         121-124
IAYGYDEGHA MDYWGQGTLV TVSS
```

Fig. 17

```
1
DIVMSQSPSS LAVSVGEKVT MSCKSSQSLL YSRNQKNYLA WFQQKPGQSP
51
KLLIFWASTR ESGVPDRFTG SGFGTDFNLT ISSVQAEDLA VYDCQQYFSY
101
PLTFGAGTKL ELKRTVAAPS VFIFPPSDEQ LKSGTASVVC LLNNFYPREA
151
KVQWKVDNAL QSGNSQESVT EQDSKDSTYS LSSTLTLSKA DYEKHKVYAC
201
EVTHQGLSSP VTKSFNRGEC
```

Fig. 18

```
1
VQLQQSGPEL VKPGASVKMS CKTSRYTFTE YTIHWVRQSH GKSLEWIGGI
51
NPNNGIPNYN QKFKGRATLT VGKSSSTAYM ELRSLTSEDS AVYFCARRRI
101
AYGYDEGHAM DYWGQGTSVT VSSASTKGPS VFPLAPSSKS TSGGTAALGC
151
LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS VVTVPSSSLG
201
TQTYICNVNH KPSNTKVDKK VEPKSCDKTH TCPPCPAPEL LGGPSVFLFP
251
PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE
301
QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK TISKAKGQPR
351
EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT
401
PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS
451
PGK
```

Fig. 19

```
340         350         360         370         380
CGTACTGTGG CTGCACCATC TGTCTTCATC TTCCCGCCAT CTGATGAGCA
390         400         410         420         430
GTTGAAATCT GGAACTGCCT CTGTTGTGTG CCTGCTGAAT AACTTCTATC
440         450         460         470         480
CCAGAGAGGC CAAAGTACAG TGGAAGGTGG ATAACGCCCT CCAATCGGGT
490         500         510         520         530
AACTCCCAGG AGAGTGTCAC AGAGCAGGAC AGCAAGGACA GCACCTACAG
540         550         560         570         580
CCTCAGCAGC ACCCTGACGC TGAGCAAAGC AGACTACGAG AAACACAAAG
590         600         610         620         630
TCTACGCCTG CGAAGTCACC CATCAGGGCC TGAGCTCGCC CGTCACAAAG
640         650         660
AGCTTCAACA GGGGAGAGTGT
```

Fig. 20

```
114         124         134         144         154
RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG
164         174         184         194         204
NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK
214-220
SFNRGEC
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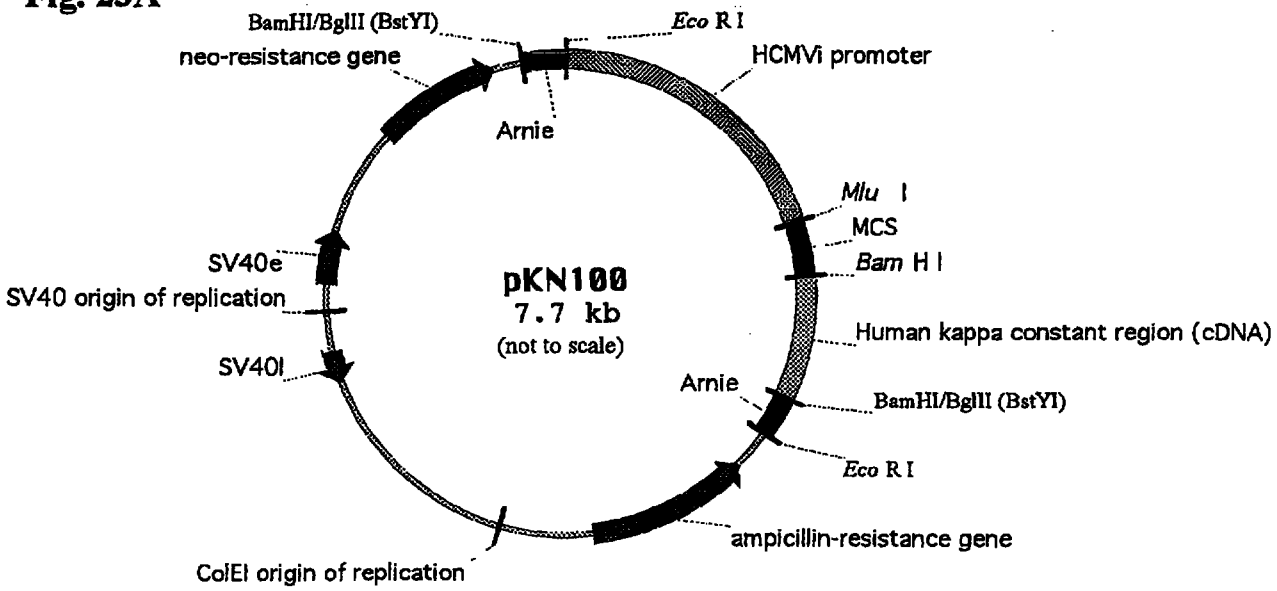
Fig. 21

373
GCCTCCACCA AGGGCCCATC GGTCTTCCCC CTGGCACCCT CCTCCAAGAG
423
CACCTCTGGG GGCACAGCGG CCCTGGGCTG CCTGGTCAAG GACTACTTCC
473
CCGAACCGGT GACGGTGTCG TGGAACTCAG GCGCCCTGAC CAGCGGCGTG
523
CACACCTTCC CGGCTGTCCT ACAGTCCTCA GGACTCTACT CCCTCAGCAG
573
CGTGGTGACC GTGCCCTCCA GCAGCTTGGG CACCCAGACC TACATCTGCA
623
ACGTGAATCA CAAGCCCAGC AACACCAAGG TGGACAAGAA AGTTGAGCCC
673
AAATCTTGTG ACAAAACTCA CACATGCCCA CCGTGCCCAG CACCTGAACT
723
CCTGGGGGGA CCGTCAGTCT TCCTCTTCCC CCCAAAACCC AAGGACACCC
773
TCATGATCTC CCGGACCCCT GAGGTCACAT GCGTGGTGGT GGACGTGAGC
823
CACGAAGACC CTGAGGTCAA GTTCAACTGG TACGTGGACG GCGTGGAGGT
873
GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC AGCACGTACC
923
GGGTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG
973
GAGTACAAGT GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA
1023
AACCATCTCC AAAGCCAAAG GGCAGCCCCG AGAACCACAG GTGTACACCC
1073
TGCCCCCATC CCGGGAGGAG ATGACCAAGA ACCAGGTCAG CCTGACCTGC
1123
CTGGTCAAAG GCTTCTATCC CAGCGACATC GCCGTGGAGT GGGAGAGCAA
1173
TGGGCAGCCG GAGAACAACT ACAAGACCAC GCCTCCCGTG CTGGACTCCG
1223
ACGGCTCCTT CTTCCTCTAC AGCAAGCTCA CCGTGGACAA GAGCAGGTGG
1273
CAGCAGGGGA ACGTCTTCTC ATGCTCCGTG ATGCATGAGG CTCTGCACAA
1323 1362
CCACTACACG CAGAAGAGCC TCTCCCTGTC TCCGGGTAAA

Fig. 22

```
125
ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
175
HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP
225
KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS
275
HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
325
EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC
375
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
425                              454
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
```

Fig. 23A



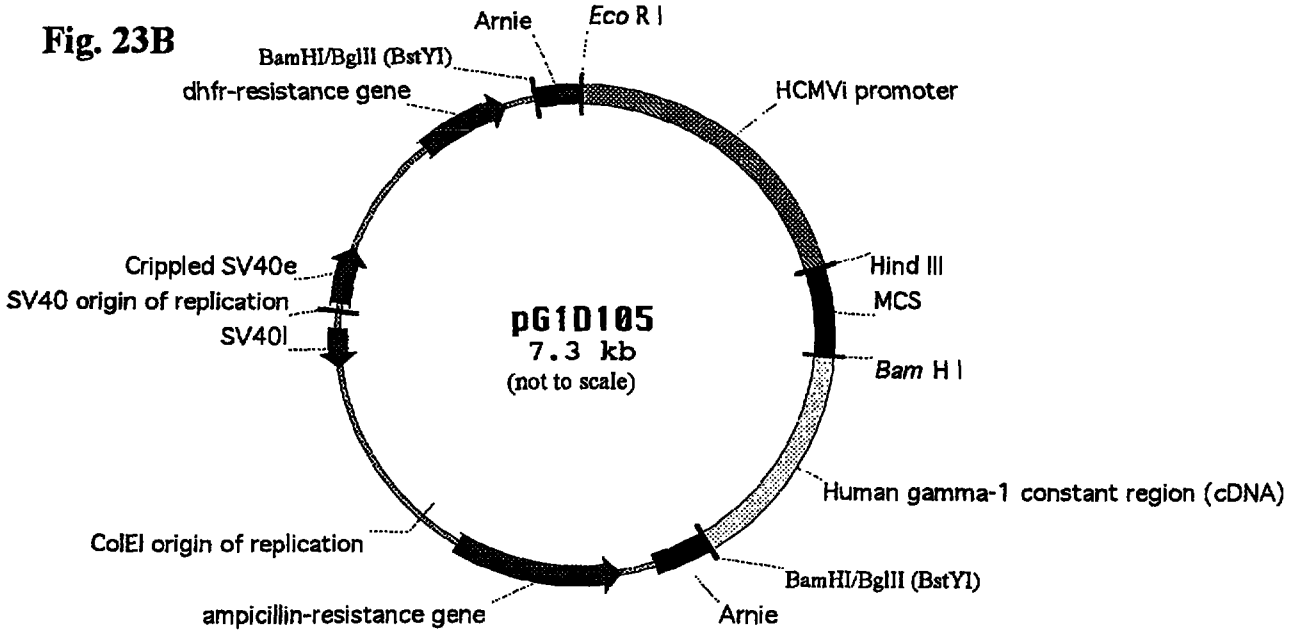
HCMVi enhancer/promoter/intron elements

Multiple cloning site: 5' MluI-HindIII-SalI-XbaI-BamHI 3'

spaC2 termination signal sequence (also known as Arnie)

pSV2neo vector fragment

Fig. 23B



HCMVi enhancer/promoter/intron elements

Multiple cloning site: 5' MluI-HindIII-Sse8387I-PmeI-SalI-XbaI-PacI-BamHI 3'

spaC2 termination signal sequence (also known as Arnie)

pSV2dhfr vector fragment

Fig. 24

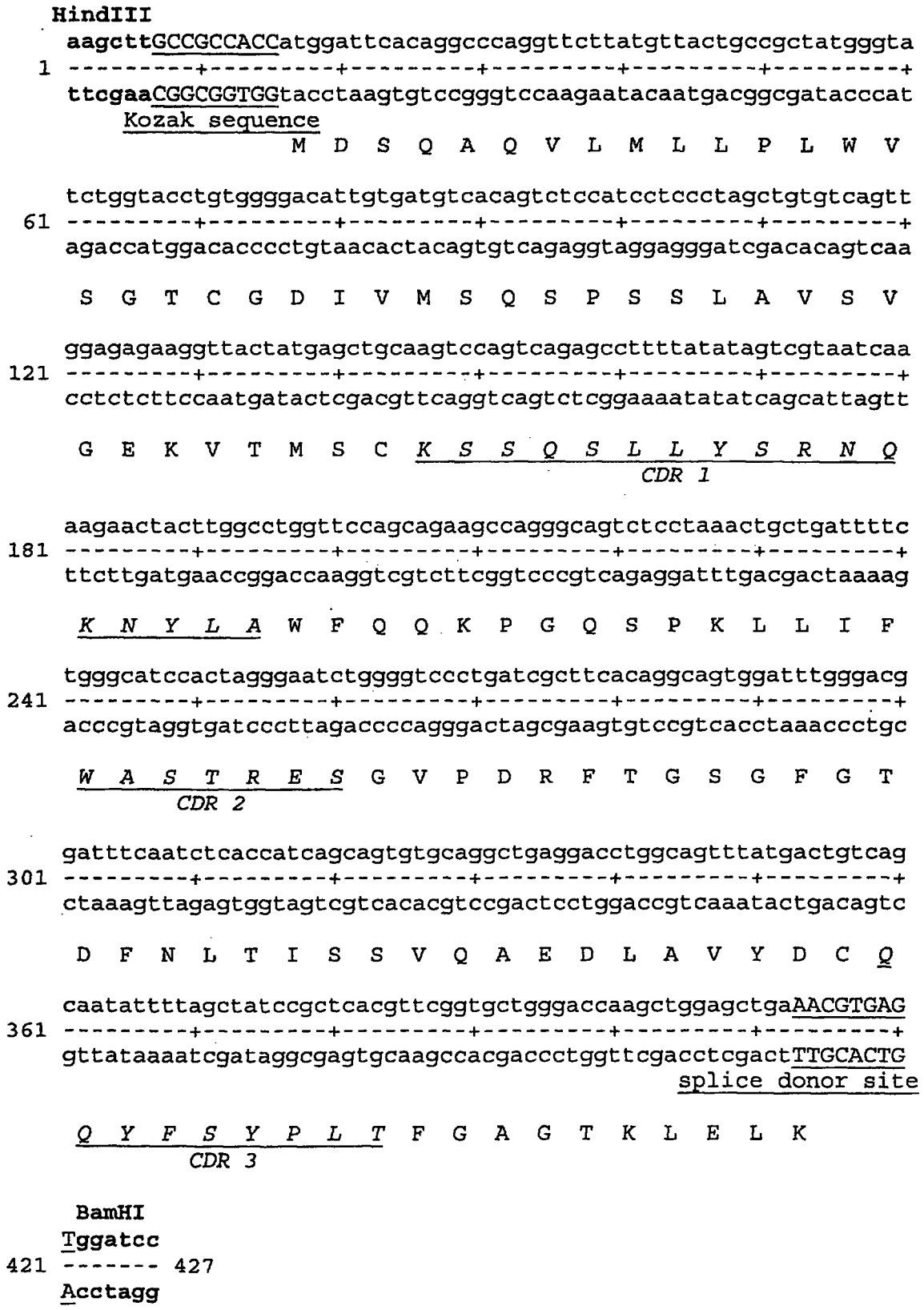

HindIII
aagcttGCCGCCACCatggattcacaggcccaggttcttatgttactgccgctatgggta
ttcgaaCGGCGGTGGtacctaagtgtccgggtccaagaatacaatgacggcgatacccat
Kozak sequence
M D S Q A Q V L M L L P L W V
tctggtacctgtggggacattgtgatgtcacagtctccatcctccctagctgtgtcagtt
agaccatggacacccctgtaacactacagtgtcagaggtaggagggatcgacacagtcaa
S G T C G D I V M S Q S P S S L A V S V
ggagagaaggttactatgagctgcaagtccagtcagagcctttatatagtcgtaatcaa
cctctcttccaatgatactcgacgttcaggtcagtctcggaaaatatatcagcattagtt
G E K V T M S C K S S Q S L L Y S R N Q
CDR 1
aagaactacttggcctggttccagcagaagccagggcagtctcctaaactgctgattttc
ttcttgatgaaccggaccaaggtcgtcttcggtcccgtcagaggatttgacgactaaaag
K N Y L A W F Q Q K P G Q S P K L L I F
tgggcatccactagggaatctggggtccctgatcgcttcacaggcagtggatttgggacg
acccgtaggtgatcccttagaccccagggactagcgaagtgtccgtcacctaaaccctgc
W A S T R E S G V P D R F T G S G F G T
CDR 2
gatttcaatctcaccatcagcagtgtgcaggctgaggacctggcagtttatgactgtcag
ctaaagttagagtggtagtcgtcacacgtccgactcctggaccgtcaaatactgacagtc
D F N L T I S S V Q A E D L A V Y D C Q
caatattttagctatccgctcacgttcggtgctgggaccaagctggagctgaAACGTGAG
gttataaaatcgataggcgagtgcaagccacgaccctggttcgacctcgactTTGCACTG
splice donor site
Q Y F S Y P L T F G A G T K L E L K
CDR 3
BamHI
Tggatcc
421 ------- 427
Acctagg

Fig. 25

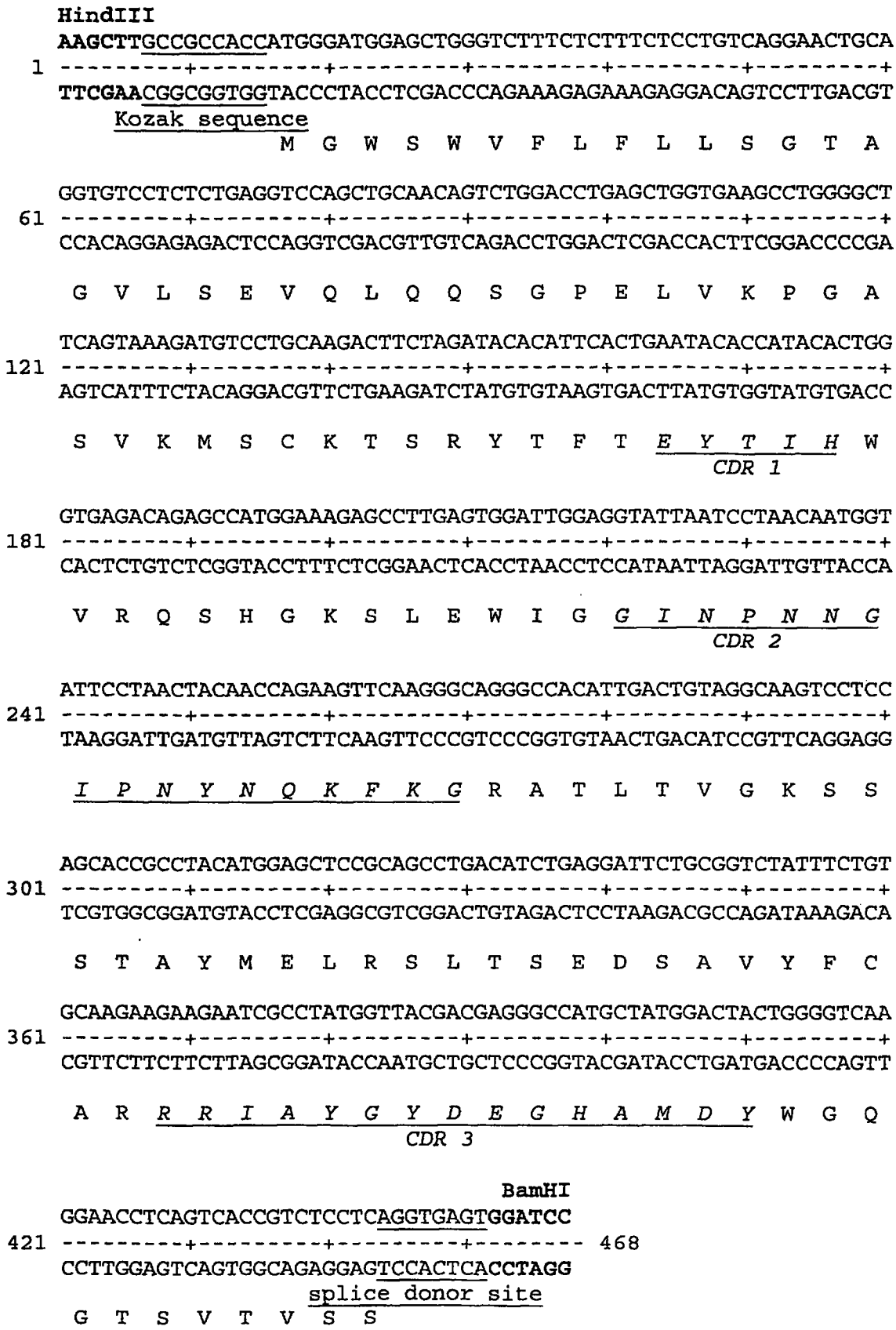

HindIII
AAGCTTGCCGCCACCATGGGATGGAGCTGGGTCTTTCTCTTTCTCCTGTCAGGAACTGCA
TTCGAACGGCGGTGGTACCCTACCTCGACCCAGAAAGAGAAAGAGGACAGTCCTTGACGT
Kozak sequence
M G W S W V F L F L L S G T A
GGTGTCCTCTCTGAGGTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCT
CCACAGGAGAGACTCCAGGTCGACGTTGTCAGACCTGGACTCGACCACTTCGGACCCCGA
G V L S E V Q L Q Q S G P E L V K P G A
TCAGTAAAGATGTCCTGCAAGACTTCTAGATACACATTCACTGAATACACCATACACTGG
AGTCATTTCTACAGGACGTTCTGAAGATCTATGTGTAAGTGACTTATGTGGTATGTGACC
S V K M S C K T S R Y T F T E Y T I H W
CDR 1
GTGAGACAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGGTATTAATCCTAACAATGGT
CACTCTGTCTCGGTACCTTTCTCGGAACTCACCTAACCTCCATAATTAGGATTGTTACCA
V R Q S H G K S L E W I G G I N P N N G
CDR 2
ATTCCTAACTACAACCAGAAGTTCAAGGGCAGGGCCACATTGACTGTAGGCAAGTCCTCC
TAAGGATTGATGTTAGTCTTCAAGTTCCCGTCCCGGTGTAACTGACATCCGTTCAGGAGG
I P N Y N Q K F K G R A T L T V G K S S
AGCACCGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGATTCTGCGGTCTATTTCTGT
TCGTGGCGGATGTACCTCGAGGCGTCGGACTGTAGACTCCTAAGACGCCAGATAAAGACA
S T A Y M E L R S L T S E D S A V Y F C
GCAAGAAGAAGAATCGCCTATGGTTACGACGAGGGCCATGCTATGGACTACTGGGGTCAA
CGTTCTTCTTCTTAGCGGATACCAATGCTGCTCCCGGTACGATACCTGATGACCCCAGTT
A R R R I A Y G Y D E G H A M D Y W G Q
CDR 3
BamHI
GGAACCTCAGTCACCGTCTCCTCAGGTGAGTGGATCC
421 468
CCTTGGAGTCAGTGGCAGAGGAGTCCACTCACCTAGG
splice donor site
G T S V T V S S

Fig. 26 /1

```
                                          Spe I
   1 gaattccagc acactggcgg ccgttACTAG TTATTAATAG TAATCAATTA
  51 CGGGGTCATT AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT
 101 ACGGTAAATG GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC
 151 GTCAATAATG ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT
 201 GACGTCAATG GGTGGAGTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT
 251 CAAGTGTATC ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA
 301 ATGGCCCGCC TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA
                   SnaB I
 351 CTTGGCAGTA CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG
 401 TTTTGGCAGT ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT
 451 TCCAAGTCTC CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA
 501 ATCAACGGGA CTTTCCAAAA TGTCGTAACA ACTCCGCCCC ATTGACGCAA
 551 ATGGGCGGTA GGCGTGTACG GTGGGAGGTC TATATAAGCA GAGCTCGTTT
 601 AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC
                                     Sac II
 651 ATAGAAGACA CCGGGACCGA TCCAGCCTCC GCGGCCGGGA ACGGTGCATT
 701 GGAACGCGGA TTCCCCGTGC CAAGAGTGAC GTAAGTACCG CCTATAGAGT
 751 CTATAGGCCC ACCCCCTTGG CTTCTTATGC ATGCTATACT GTTTTTGGCT
 801 TGGGGTCTAT ACACCCCCGC TTCCTCATGT TATAGGTGAT GGTATAGCTT
 851 AGCCTATAGG TGTGGGTTAT TGACCATTAT TGACCACTCC CCTATTGGTG
 901 ACGATACTTT CCATTACTAA TCCATAACAT GGCTCTTTGC CACAACTCTC
 951 TTTATTGGCT ATATGCCAAT ACACTGTCCT TCAGAGACTG ACACGGACTC
1001 TGTATTTTTA CAGGATGGGG TCTCATTTAT TATTTACAAA TTCACATATA
1051 CAACACCACC GTCCCCAGTG CCCGCAGTTT TTATTAAACA TAACGTGGGA
                                     BspE I
1101 TCTCCACGCG AATCTCGGGT ACGTGTTCCG GACATGGGCT CTTCTCCGGT
1151 AGCGGCGGAG CTTCTACATC CGAGCCCTGC TCCCATGCCT CCAGCGACTC
1201 ATGGTCGCTC GGCAGCTCCT TGCTCCTAAC AGTGGAGGCC AGACTTAGGC
1251 ACAGCACGAT GCCCACCACC ACCAGTGTGC CGCACAAGGC CGTGGCGGTA
```

Fig. 26 /2

```
1301  GGGTATGTGT CTGAAAATGA GCTCggggag cgggcttgca ccgctgacgc
                  Afl II
1351  atttggaaga cttaaggcag cggcagaaga agatgcaggc agctgagttg

1401  ttgtgttctg ataagagtca gaggtaactc ccgttgcggt gctgttaacg

1451  gtggagggca gtgtagtctg agcagtactc gttgctgccg cgcgccgccac

1501  cagacataat agctgacaga ctaacagact gttcctttcc atgggtcttt
                              Mlu I        Hind III
1551  tctgcagtca ccgtccttga cacgcgtctc gggaagcttG CCGCCACCAT
                                                        M
                                                     Kpn I
1601  GGATTCACAG GCCCAGGTTC TTATGTTACT GCCGCTATGG GTATCTGGTA
        D  S  Q    A  Q  V    L  M  L  L    P  L  W    V  S  G
1651  CCTGTGGGGA CATTGTGATG TCACAGTCTC CATCCTCCCT AGCTGTGTCA
      T  C  G  D    I  V  M    S  Q  S    P  S  S  L    A  V  S
1701  GTTGGAGAGA AGGTTACTAT GAGCTGCAAG TCCAGTCAGA GCCTTTTATA
       V  G  E    K  V  T  M    S  C  K  S  S  Q  S  L  L  Y
        XbaI                                  CDR 1
1751  TTCTAGAAAT CAAAAGAACT ACTTGGCCTG GTTCCAGCAG AAGCCAGGGC
        S  R  N  Q  K  N  Y  L  A  W    F  Q  Q    K  P  G

1801  AGTCTCCTAA ACTGCTGATT TTCTGGGCAT CCACTAGGGA ATCTGGGGTC
      Q  S  P  K    L  L  I    F  W  A  S  T  R  E  S  G  V
                                          CDR 2
1851  CCTGATCGCT TCACAGGCAG TGGATTTGGG ACGGATTTCA ATCTCACCAT
       P  D  R    F  T  G  S    G  F  G    T  D  F    N  L  T  I

1901  CAGCAGTGTG CAGGCTGAGG ACCTGGCAGT TTATGACTGT CAGCAATATT
        S  S  V    Q  A  E    D  L  A  V    Y  D  C  Q  Q  Y

1951  TTAGCTATCC GCTCACGTTC GGTGCTGGGA CCAAGCTGGA GCTGAAACGT
      F  S  Y  P  L  T  F    G  A  G    T  K  L  E    L  K  R
            CDR 3
          BamH I
2001  GAGTggatcc ATCTGGGATA AGCATGCTGT TTTCTGTCTG TCCCTAACAT

2051  GCCCTGTGAT TATGCGCAAA CAACACACCC AAGGGCAGAA CTTTGTTACT

2101  TAAACACCAT CCTGTTTGCT TCTTTCCTCA GGAACTGTGG CTGCACCATC
                                       T  V    A  A  P  S
2151  TGTCTTCATC TTCCCGCCAT CTGATGAGCA GTTGAAATCT GGAACTGCCT
        V  F  I    F  P  P    S  D  E  Q    L  K  S    G  T  A
2201  CTGTTGTGTG CCTGCTGAAT AACTTCTATC CCAGAGAGGC CAAAGTACAG
      S  V  V  C    L  L  N    N  F  Y    P  R  E  A    K  V  Q
2251  TGGAAGGTGG ATAACGCCCT CCAATCGGGT AACTCCCAGG AGAGTGTCAC
       W  K  V    D  N  A  L    Q  S  G    N  S  Q    E  S  V  T
2301  AGAGCAGGAC AGCAAGGACA GCACCTACAG CCTCAGCAGC ACCCTGACGC
        E  Q  D    S  K  D    S  T  Y  S    L  S  S    T  L  T
```

Fig. 26 /3

```
2351  TGAGCAAAGC AGACTACGAG AAACACAAAG TCTACGCCTG CGAAGTCACC
        L  S  K  A   D  Y  E   K  H  K   V  Y  A  C   E  V  T
2401  CATCAGGGCC TGAGCTCGCC CGTCACAAAG AGCTTCAACA GGGGAGAGTG
        H  Q  G   L  S  S  P   V  T  K   S  F  N   R  G  E  C
2451  TTAGAGGGAG AAGTGCCCCC ACCTGCTCCT CAGTTCCAGC CTGACCCCCT
          *
2501  CCCATCCTTT GGCCTCTGAC CCTTTTTCCA CAGGGGACCT ACCCCTATTG

2551  CGGTCCTCCA GCTCATCTTT CACCTCACCC CCCTCCTCCT CCTTGGCTTT

2601  AATTATGCTA ATGTTGGAGG AGAATGAATA AATAAAGTGA ATCTTTGCAC

2651  CTGTGGTGGA TCTAATAAAA GATATTTATT TTCATTAGAT ATGTGTGTTG

2701  GTTTTTTGTG TGCAGTGCCT CTATCTGGAG GCCAGGTAGG GCTGGCCTTG

2751  GGGGAGGGGG AGGCCAGAAT GACTCCAAGA GCTACAGGAA GGCAGGTCAG

2801  AGACCCCACT GGACAAACAG TGGCTGGACT CTGCACCATA ACACACAATC

2851  AACAGGGGAG TGAGCTGGAA ATTTGCTAGC GAATTCTTGA AGACGAAAGG

2901  GCCTCGTGAT ACGCCTATTT TTATAGGTTA ATGTCATGAT AATAATGGTT

2951  TCTTAGACGT CAGGTGGCAC TTTTCGGGGA AATGTGCGCG GAACCCCTAT

3001  TTGTTTATTT TTCTAAATAC ATTCAAATAT GTATCCGCTC ATGAGACAAT

3051  AACCCTGATA AATGCTTCAA TAATATTGAA AAAGGAAGAG TATGAGTATT

3101  CAACATTTCC GTGTCGCCCT TATTCCCTTT TTTGCGGCAT TTTGCCTTCC

3151  TGTTTTTGCT CACCCAGAAA CGCTGGTGAA AGTAAAAGAT GCTGAAGATC

3201  AGTTGGGTGC ACGAGTGGGT TACATCGAAC TGGATCTCAA CAGCGGTAAG

3251  ATCCTTGAGA GTTTTCGCCC CGAAGAACGT TTTCCAATGA TGAGCACTTT

3301  TAAAGTTCTG CTATGTGGCG CGGTATTATC CCGTGTTGAC GCCGGGCAAG

3351  AGCAACTCGG TCGCCGCATA CACTATTCTC AGAATGACTT GGTTGAGTAC

3401  TCACCAGTCA CAGAAAAGCA TCTTACGGAT GGCATGACAG TAAGAGAATT

3451  ATGCAGTGCT GCCATAACCA TGAGTGATAA CACTGCGGCC AACTTACTTC
                Pvu I
3501  TGACAACGAT CGGAGGACCG AAGGAGCTAA CCGCTTTTTT GCACAACATG

3551  GGGGATCATG TAACTCGCCT TGATCGTTGG GAACCGGAGC TGAATGAAGC

3601  CATACCAAAC GACGAGCGTG ACACCACGAT GCCTGCAGCA ATGGCAACAA
```

Fig. 26 /4

```
3651  CGTTGCGCAA ACTATTAACT GGCGAACTAC TTACTCTAGC TTCCCGGCAA
3701  CAATTAATAG ACTGGATGGA GGCGGATAAA GTTGCAGGAC CACTTCTGCG
3751  CTCGGCCCTT CCGGCTGGCT GGTTTATTGC TGATAAATCT GGAGCCGGTG
3801  AGCGTGGGTC TCGCGGTATC ATTGCAGCAC TGGGGCCAGA TGGTAAGCCC
3851  TCCCGTATCG TAGTTATCTA CACGACGGGG AGTCAGGCAA CTATGGATGA
3901  ACGAAATAGA CAGATCGCTG AGATAGGTGC CTCACTGATT AAGCATTGGT
3951  AACTGTCAGA CCAAGTTTAC TCATATATAC TTTAGATTGA TTTAAAACTT
4001  CATTTTTAAT TTAAAAGGAT CTAGGTGAAG ATCCTTTTTG ATAATCTCAT
4051  GACCAAAATC CCTTAACGTG AGTTTTCGTT CCACTGAGCG TCAGACCCCG
4101  TAGAAAAGAT CAAAGGATCT TCTTGAGATC CTTTTTTTCT GCGCGTAATC
4151  TGCTGCTTGC AAACAAAAAA ACCACCGCTA CCAGCGGTGG TTTGTTTGCC
4201  GGATCAAGAG CTACCAACTC TTTTTCCGAA GGTAACTGGC TTCAGCAGAG
4251  CGCAGATACC AAATACTGTC CTTCTAGTGT AGCCGTAGTT AGGCCACCAC
4301  TTCAAGAACT CTGTAGCACC GCCTACATAC CTCGCTCTGC TAATCCTGTT
4351  ACCAGTGGCT GCTGCCAGTG GCGATAAGTC GTGTCTTACC GGGTTGGACT
4401  CAAGACGATA GTTACCGGAT AAGGCGCAGC GGTCGGGCTG AACGGGGGGT
4451  TCGTGCACAC AGCCCAGCTT GGAGCGAACG ACCTACACCG AACTGAGATA
4501  CCTACAGCGT GAGCTATGAG AAAGCGCCAC GCTTCCCGAA GGGAGAAAGG
4551  CGGACAGGTA TCCGGTAAGC GGCAGGGTCG GAACAGGAGA GCGCACGAGG
4601  GAGCTTCCAG GGGGAAACGC CTGGTATCTT TATAGTCCTG TCGGGTTTCG
4651  CCACCTCTGA CTTGAGCGTC GATTTTTGTG ATGCTCGTCA GGGGGGCGGA
4701  GCCTATGGAA AAACGCCAGC AACGCGGCCT TTTTACGGTT CCTGGCCTTT
                          BspLU11I
4751  TGCTGGCCTT TTGCTCACAT GTTCTTTCCT GCGTTATCCC CTGATTCTGT
4801  GGATAACCGT ATTACCGCCT TTGAGTGAGC TGATACCGCT CGCCGCAGCC
4851  GAACGACCGA GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA AGAGCGCCTG
4901  ATGCGGTATT TTCTCCTTAC GCATCTGTGC GGTATTTCAC ACCGCATATG
```

Fig. 26 /5

Bst1107I

4951 GTGCACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCA**GTATA**

5001 **C**ACTCCGCTA TCGCTACGTG ACTGGGTCAT GGCTGCGCCC CGACACCCGC

5051 CAACACCCGC TGACGCGCCC TGACGGGCTT GTCTGCTCCC GGCATCCGCT

5101 TACAGACAAG CTGTGACCGT CTCCGGGAGC TGCATGTGTC AGAGGTTTTC

5151 ACCGTCATCA CCGAAACGCG CGAGGCAGCT GTGGAATGTG TGTCAGTTAG

5201 GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG

5251 CATCTCAATT AGTCAGCAAC CAGGCTCCCC AGCAGGCAGA AGTATGCAAA

5301 GCATGCATCT CAATTAGTCA GCAACCATAG TCCCGCCCCT AACTCCGCCC

5351 ATCCCGCCCC TAACTCCGCC CAGTTCCGCC CATTCTCCGC CCCATGGCTG

Sfi I

5401 ACTAATTTTT TTTATTTATG CAGAGGCCGA **GGCCGCCTCG GCC**TCTGAGC

Stu I/Avr II

5451 TATTCCAGAA GTAGTGAGGA GGCTTTTTTG G**AGGCCTAGG** CTTTTGCAAA

5501 AAGCTAGCTT CACGCTGCCG CAAGCACTCA GGGCGCAAGG GCTGCTAAAG

5551 GAAGCGGAAC ACGTAGAAAG CCAGTCCGCA GAAACGGTGC TGACCCCGGA

5601 TGAATGTCAG CTACTGGGCT ATCTGGACAA GGGAAAACGC AAGCGCAAAG

5651 AGAAAGCAGG TAGCTTGCAG TGGGCTTACA TGGCGATAGC TAGACTGGGC

5701 GGTTTTATGG ACAGCAAGCG AACCGGAATT GCCAGCTGGG GCGCCCTCTG

5751 GTAAGGTTGG GAAGCCCTGC AAAGTAAACT GGATGGCTTT CTTGCCGCCA

Bgl II/Bcl I

5801 AGGATCTGAT GGCGCAGGGG ATCA**AGATCT GATCA**AGAGA CAGGATGAGG

5851 ATCGTTTCGC ATGATTGAAC AAGATGGATT GCACGCAGGT TCTCCGGCCG

5901 CTTGGGTGGA GAGGCTATTC GGCTATGACT GGGCACAACA GACAATCGGC

5951 TGCTCTGATG CCGCCGTGTT CCGGCTGTCA GCGCAGGGGC GCCCGGTTCT

6001 TTTTGTCAAG ACCGACCTGT CCGGTGCCCT GAATGAACTG CAGGACGAGG

Msc I

6051 CAGCGCGGCT ATCGTGGC**TG GCCA**CGACGG GCGTTCCTTG CGCAGCTGTG

6101 CTCGACGTTG TCACTGAAGC GGGAAGGGAC TGGCTGCTAT TGGGCGAAGT

6151 GCCGGGGCAG GATCTCCTGT CATCTCACCT TGCTCCTGCC GAGAAAGTAT

6201 CCATCATGGC TGATGCAATG CGGCGGCTGC ATACGCTTGA TCCGGCTACC

Fig. 26 /6

6251 TGCCCATTCG ACCACCAAGC GAAACATCGC ATCGAGCGAG CACGTACTCG

6301 GATGGAAGCC GGTCTTGTCG ATCAGGATGA TCTGGACGAA GAGCATCAGG

6351 GGCTCGCGCC AGCCGAACTG TTCGCCAGGC TCAAGGCGCG CATGCCCGAC

6401 GGCGAGGATC TCGTCGTGAC CCATGGCGAT GCCTGCTTGC CGAATATCAT

6451 GGTGGAAAAT GGCCGCTTTT CTGGATTCAT CGACTGTGGC CGGCTGGGTG

Rsr II

6501 TGGCGGACCG CTATCAGGAC ATAGCGTTGG CTACCCGTGA TATTGCTGAA

6551 GAGCTTGGCG GCGAATGGGC TGACCGCTTC CTCGTGCTTT ACGGTATCGC

6601 CGCTCCCGAT TCGCAGCGCA TCGCCTTCTA TCGCCTTCTT GACGAGTTCT

Nsp V

6651 TCTGAGCGGG ACTCTGGGGT TCGAAATGAC CGACCAAGCG ACGCCCAACC

6701 TGCCATCACG AGATTTCGAT TCCACCGCCG CCTTCTATGA AAGGTTGGGC

6751 TTCGGAATCG TTTTCCGGGA CGCCGGCTGG ATGATCCTCC AGCGCGGGGA

Sma I Nru I

6801 TCTCATGCTG GAGTTCTTCG CCCACCCCGG GCTCGATCCC CTCGCGAGTT

6851 GGTTCAGCTG CTGCCTGAGG CTGGACGACC TCGCGGAGTT CTACCGGCAG

6901 TGCAAATCCG TCGGCATCCA GGAAACCAGC AGCGGCTATC CGCGCATCCA

6951 TGCCCCCGAA CTGCAGGAGT GGGGAGGCAC GATGGCCGCT TTGGTCCCGG

7001 ATCTTTGTGA AGGAACCTTA CTTCTGTGGT GTGACATAAT TGGACAAACT

7051 ACCTACAGAG ATTTAAAGCT CTAAGGTAAA TATAAAATTT TTAAGTGTAT

7101 AATGTGTTAA ACTACTGATT CTAATTGTTT GTGTATTTTA GATTCCAACC

7151 TATGGAACTG ATGAATGGGA GCAGTGGTGG AATGCCTTTA ATGAGGAAAA

7201 CCTGTTTTGC TCAGAAGAAA TGCCATCTAG TGATGATGAG GCTACTGCTG

7251 ACTCTCAACA TTCTACTCCT CCAAAAAAGA AGAGAAAGGT AGAAGACCCC

7301 AAGGACTTTC CTTCAGAATT GCTAAGTTTT TTGAGTCATG CTGTGTTTAG

7351 TAATAGAACT CTTGCTTGCT TTGCTATTTA CACCACAAAG GAAAAAGCTG

7401 CACTGCTATA CAAGAAAATT ATGGAAAAAT ATTCTGTAAC CTTTATAAGT

7451 AGGCATAACA GTTATAATCA TAACATACTG TTTTTTCTTA CTCCACACAG

7501 GCATAGAGTG TCTGCTATTA ATAACTATGC TCAAAAATTG TGTACCTTTA

Fig. 26 /7

```
7551  GCTTTTTAAT TTGTAAAGGG GTTAATAAGG AATATTTGAT GTATAGTGCC

7601  TTGACTAGAG ATCATAATCA GCCATACCAC ATTTGTAGAG GTTTTACTTG

7651  CTTTAAAAAA CCTCCCACAC CTCCCCCTGA ACCTGAAACA TAAAATGAAT
            Mun I
7701  GCAATTGTTG TTGTTAACTT GTTTATTGCA GCTTATAATG GTTACAAATA

7751  AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT TCACTGCATT

7801  CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA TGTCTGGATC

7851  TAATAAAAGA TATTTATTTT CATTAGATAT GTGTGTTGGT TTTTTGTGTG

7901  CAGTGCCTCT ATCTGGAGGC CAGGTAGGGC TGGCCTTGGG GGAGGGGGAG

7951  GCCAGAATGA CTCCAAGAGC TACAGGAAGG CAGGTCAGAG ACCCCACTGG

8001  ACAAACAGTG GCTGGACTCT GCACCATAAC ACACAATCAA CAGGGGAGTG

8051  AGCTGGAAAT TTGCTAGC
```

Fig. 27/1

```
   1 TTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAAT

  61 GGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTT

 121 ATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCT

 181 TCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCC

 241 CTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAA

 301 AGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGG

 361 TAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGT

 421 TCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCG

 481 CATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTAC

 541 GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGC
                          Pvu I
 601 GGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAA

 661 CATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACC
                                                    Fsp I
 721 AAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATT

 781 AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA
```

Fig. 27 /2

```
 841 TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAA

 901 ATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAA

 961 GCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAA

1021 TAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGT

1081 TTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGT

1141 GAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTG

1201 AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT

1261 AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCA

1321 AGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATAC

1381 TGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTAC

1441 ATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT

1501 TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGG

1561 GGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACA

1621 GCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGT

1681 AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTA

1741 TCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC

1801 GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
                          BspLU11I
1861 CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA

1921 CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAG

1981 CGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCT

2041 GTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATA
            Bst1107 I
2101 GTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACAC

2161 CCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGA

2221 CAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAA

2281 CGCGCGAGGCAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC

2341 CATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTT
                                  Sfi I
2401 TTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGG
                    Stu I/Avr II
2461 AGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTAGCTTACAGCTCAGGGCTGCGATT
```

Fig. 27 /3

2521 TCGCGCCAAACTTGACGGCAATCCTAGCGTGAAGGCTGGTAGGATTTTATCCCCGCTGCC

2581 ATCATGGTTCGACCATTGAACTGCATCGTCGCCGTGTCCCAAAATATGGGGATTGGCAAG

2641 AACGGAGACCTACCCTGGCCTCCGCTCAGGAACGAGTTCAAGTACTTCCAAAGAATGACC

2701 ACAACCTCTTCAGTGGAAGGTAAACAGAATCTGGTGATTATGGGTAGGAAAACCTGGTTC

2761 TCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATTAATATAGTTCTCAGTAGAGAA

2821 CTCAAAGAACCACCACGAGGAGCTCATTTTCTTGCCAAAAGTTTGGATGATGCCTTAAGA

2881 CTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGATAGTCGGAGGCAGT

2941 TCTGTTTACCAGGAAGCCATGAATCAACCAGGCCACCTCAGACTCTTTGTGACAAGGATC

3001 ATGCAGGAATTTGAAAGTGACACGTTTTTCCCAGAAATTGATTTGGGGAAATATAAACTT

3061 CTCCCAGAATACCCAGGCGTCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAG

3121 TTTGAAGTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGCTCCCCTC

Bgl II

3181 CTAAAGCTATGCATTTTTATAAGACCATGGGACTTTTGCTGGCTTT**AGATCT**TTGTGAAG

3241 GAACCTTACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAAGCTCT

3301 AAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGT

3361 GTATTTTAGATTCCAACCTATGGAACTGATGAATGGGAGCAGTGGTGGAATGCCTTTAAT

3421 GAGGAAAACCTGTTTTGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTGCTGAC

3481 TCTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGACTTTCCT

3541 TCAGAATTGCTAAGTTTTTTGAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTT

3601 GCTATTTACACCACAAAGGAAAAAGCTGCACTGCTATACAAGAAAATTATGGAAAAATAT

3661 TCTGTAACCTTTATAAGTAGGCATAACAGTTATAATCATAACATACTGTTTTTTCTTACT

3721 CCACACAGGCATAGAGTGTCTGCTATTAATAACTATGCTCAAAAATTGTGTACCTTTAGC

3781 TTTTTAATTTGTAAAGGGGTTAATAAGGAATATTTGATGTATAGTGCCTTGACTAGA**GAT**

BsaB I

3841 **CATAATC**AGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCT

Mun I

3901 CCCCCTGAACCTGAAACATAAAATGAATG**CAATTG**TTGTTGTTAACTTGTTTATTGCAGC

3961 TTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTC

4021 ACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTA

4081 ATAAAAGATATTTATTTTCATTAGATATGTGTGTTGGTTTTTTGTGTGCAGTGCCTCTAT

4141 CTGGAGGCCAGGTAGGGCTGGCCTTGGGGGAGGGGGAGGCCAGAATGACTCCAAGAGCTA

4201 CAGGAAGGCAGGTCAGAGACCCCACTGGACAAACAGTGGCTGGACTCTGCACCATAACAC

Fig. 27 /5

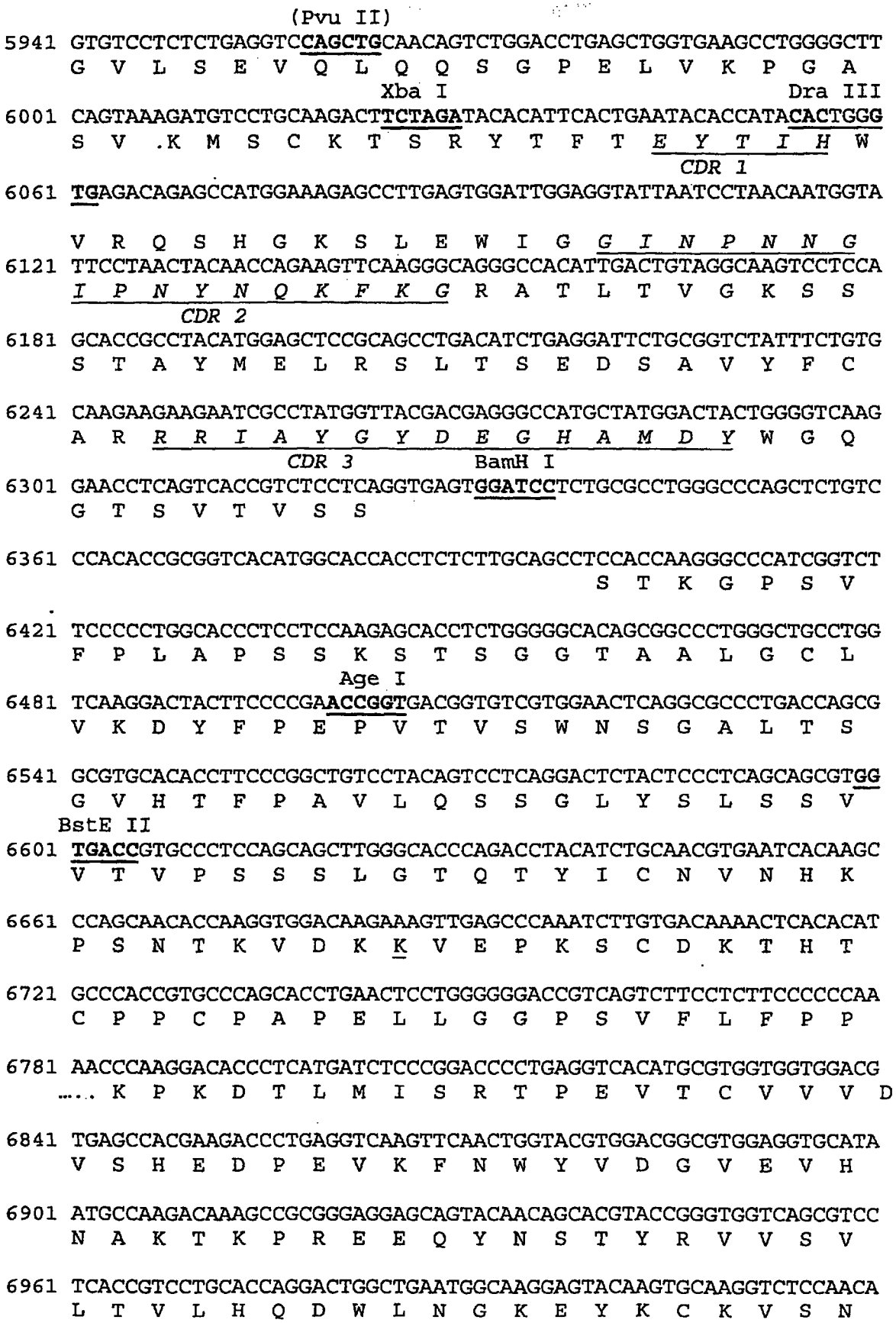

(Pvu II)
5941 GTGTCCTCTCTGAGGTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTT
G V L S E V Q L Q Q S G P E L V K P G A
Xba I
Dra III
6001 CAGTAAAGATGTCCTGCAAGACTTCTAGATACACATTCACTGAATACACCATACACTGGG
S V .K M S C K T S R Y T F T E Y T I H W
CDR 1
6061 TGAGACAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGGTATTAATCCTAACAATGGTA
V R Q S H G K S L E W I G G I N P N N G
6121 TTCCTAACTACAACCAGAAGTTCAAGGGCAGGGCCACATTGACTGTAGGCAAGTCCTCCA
I P N Y N Q K F K G R A T L T V G K S S
CDR 2
6181 GCACCGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGATTCTGCGGTCTATTTCTGTG
S T A Y M E L R S L T S E D S A V Y F C
6241 CAAGAAGAAGAATCGCCTATGGTTACGACGAGGGCCATGCTATGGACTACTGGGGTCAAG
A R R R I A Y G Y D E G H A M D Y W G Q
CDR 3
BamH I
6301 GAACCTCAGTCACCGTCTCCTCAGGTGAGTGGATCCTCTGCGCCTGGGCCCAGCTCTGTC
G T S V T V S S
6361 CCACACCGCGGTCACATGGCACCACCTCTCTTGCAGCCTCCACCAAGGGCCCATCGGTCT
S T K G P S V
6421 TCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGG
F P L A P S S K S T S G G T A A L G C L
Age I
6481 TCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCG
V K D Y F P E P V T V S W N S G A L T S
6541 GCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGG
G V H T F P A V L Q S S G L Y S L S S V
BstE II
6601 TGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGC
V T V P S S S L G T Q T Y I C N V N H K
6661 CCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACAT
P S N T K V D K K V E P K S C D K T H T
6721 GCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAA
C P P C P A P E L L G G P S V F L F P P
6781 AACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACG
..... K P K D T L M I S R T P E V T C V V V D
6841 TGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATA
V S H E D P E V K F N W Y V D G V E V H
6901 ATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCC
N A K T K P R E E Q Y N S T Y R V V S V
6961 TCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACA
L T V L H Q D W L N G K E Y K C K V S N

Fig. 27 /4

```
                                                    EcoR I
4261 ACAATCAACAGGGGAGTGAGCTGGAAATTTGCTAGCGAATTCcagcacactggcggccgt
       Spe I
4321 tACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTT

4381 CCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC

4441 ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACG

4501 TCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT

4561 GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCA
                                                    SnaB I
4621 GTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTAT

4681 TACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACG

4741 GGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCA

4801 ACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCG

4861 TGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAG

4921 ACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGG

4981 CCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTA

5041 TAGAGTCTATAGGCCCACCCCCTTGGCTTCTTATGCATGCTATACTGTTTTTGGCTTGGG
                                                    Bpu1102I
5101 GTCTATACACCCCCGCTTCCTCATGTTATAGGTGATGGTATAGCTTAGCCTATAGGTGTG
                                  Xcm I
5161 GGTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCA

5221 TAACATGGCTCTTTGCCACAACTCTCTTTATTGGCTATATGCCAATACACTGTCCTTCAG

5281 AGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCTCATTTATTATTTACAAATTCA

5341 CATATACAACACCACCGTCCCCAGTGCCCGCAGTTTTTATTAAACATAACGTGGGATCTC
                                  BspE I
5401 CACGCGAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGGTAGCGGCGGAGCTTC

5461 TACATCCGAGCCCTGCTCCCATGCCTCCAGCGACTCATGGTCGCTCGGCAGCTCCTTGCT

5521 CCTAACAGTGGAGGCCAGACTTAGGCACAGCACGATGCCCACCACCACCAGTGTGCCGCA

5581 CAAGGCCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCggggagcgggcttgcaccgc
                                                    (Pvu II)
5641 tgacgcatttggaagacttaaggcagcggcagaagaagatgcaggcagctgagttgttgt

5701 gttctgataagagtcagaggtaactcccgttgcggtgctgttaacggtggagggcagtgt

5761 agtctgagcagtactcgttgctgccgcgcgcgccaccagacataatagctgacagactaa
                                                    Mlu I
5821 cagactgttcctttccatgggtcttttctgcagtcaccgtccttgacACGCGTCTCGGGA
   Hind III
5881 AGCTTGCCGCCACCATGGGATGGAGCTGGGTCTTTCTCTTTCTCCTGTCAGGAACTGCAG
                    M  G  W  S  W  V  F  L  F  L  L  S  G  T  A
```

Fig. 27 /6

```
7021 AAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC
     K  A  L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E

7081 CACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGA
     P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N  Q  V  S  L

7141 CCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGC
     T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G

7201 AGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCC
     Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F

7261 TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCT
     L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C

7321 CCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG
     S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P
                      NgoM I
7381 GTAAATGAGTGCGACGGCCGGCAAGCCCCGCTCCCCGGGCTCTCGCGGTCGCACGAGGAT
     G  K  *
7441 GCTTGGCACGTACCCCCTGTACATACTTCCCGGGCGCCCAGCATGGAAATAAAGCACCGG

7501 ATCTAATAAAAGATATTTATTTTCATTAGATATGTGTGTTGGTTTTTTGTGTGCAGTGCC

7561 TCTATCTGGAGGCCAGGTAGGGCTGGCCTTGGGGGAGGGGGAGGCCAGAATGACTCCAAG

7621 AGCTACAGGAAGGCAGGTCAGAGACCCCACTGGACAAACAGTGGCTGGACTCTGCACCAT

7681 AACACACAATCAACAGGGGAGTGAGCTGGaaatttgctagcgaattaattc 7731
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Fig. 28:

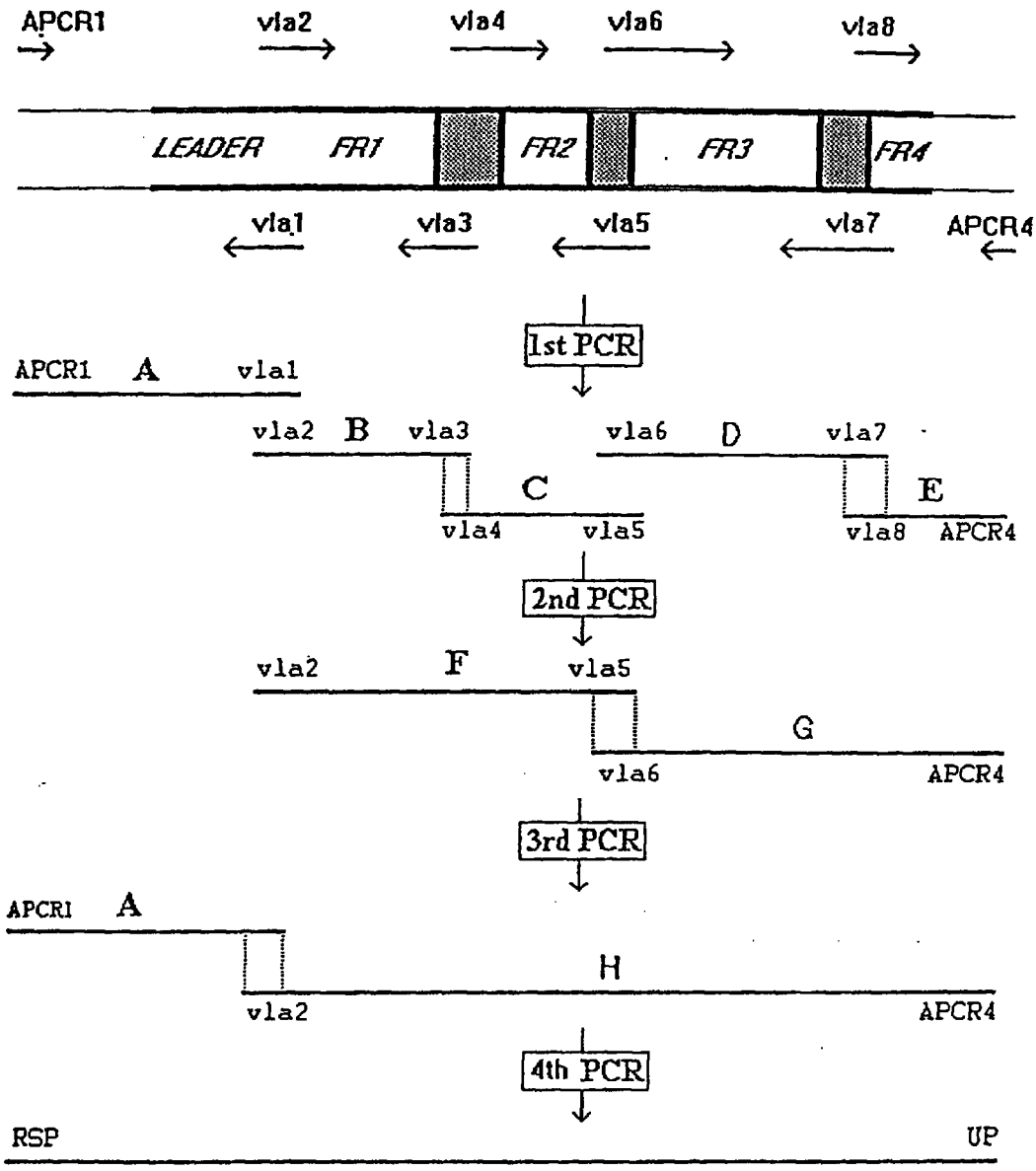

APCR1
vla2
vla4
vla6
vla8
LEADER
FR1
FR2
FR3
FR4
vla1
vla3
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vla7
APCR4
1st PCR
APCR1 A vla1
vla2 B vla3
vla6 D vla7
C
E
vla4 vla5
vla8 APCR4
2nd PCR
vla2 F vla5
G
vla6 APCR4
3rd PCR
APCRI A
H
vla2 APCR4
4th PCR
RSP UP

Fig. 29 /1

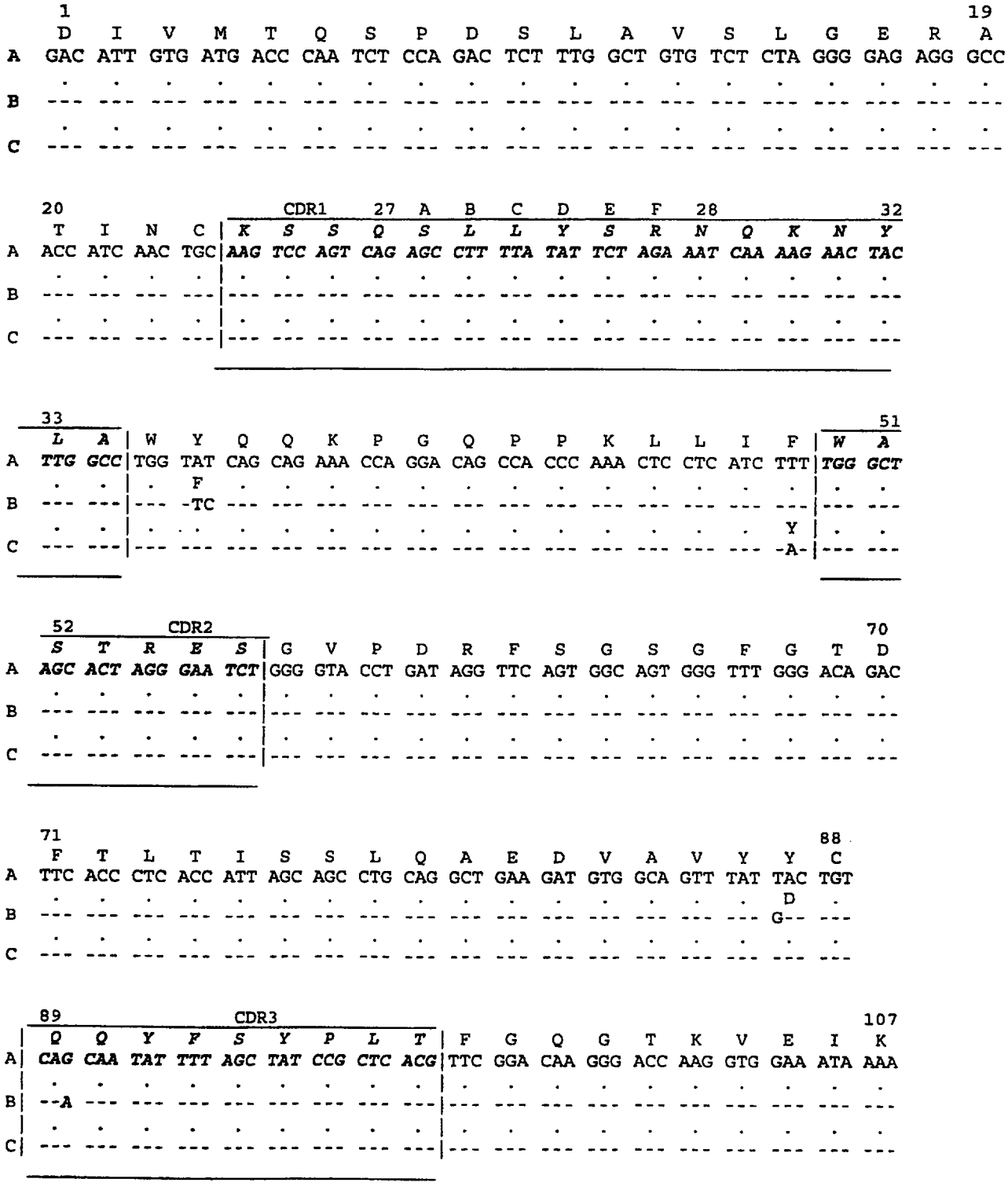

1 19
D I V M T Q S P D S L A V S L G E R A
A GAC ATT GTG ATG ACC CAA TCT CCA GAC TCT TTG GCT GTG TCT CTA GGG GAG AGG GCC
B
C
20 CDR1 27 A B C D E F 28 32
T I N C K S S Q S L L Y S R N Q K N Y
A ACC ATC AAC TGC AAG TCC AGT CAG AGC CTT TTA TAT TCT AGA AAT CAA AAG AAC TAC
B
C
33 51
L A W Y Q Q K P G Q P P K L L I F W A
A TTG GCC TGG TAT CAG CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TTT TGG GCT
B F -TC
C Y -A-
52 CDR2 70
S T R E S G V P D R F S G S G F G T D
A AGC ACT AGG GAA TCT GGG GTA CCT GAT AGG TTC AGT GGC AGT GGG TTT GGG ACA GAC
B
C
71 88
F T L T I S S L Q A E D V A V Y Y C
A TTC ACC CTC ACC ATT AGC AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT TAC TGT
B D G--
C
89 CDR3 107
Q Q Y F S Y P L T F G Q G T K V E I K
A CAG CAA TAT TTT AGC TAT CCG CTC ACG TTC GGA CAA GGG ACC AAG GTG GAA ATA AAA
B --A
C

Fig. 30 /1

```
                                        Spe I
   1  gaattccagc acactggcgg ccgttACTAG TTATTAATAG TAATCAATTA
  51  CGGGGTCATT AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT
 101  ACGGTAAATG GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC
 151  GTCAATAATG ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT
 201  GACGTCAATG GGTGGAGTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT
 251  CAAGTGTATC ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA
 301  ATGGCCCGCC TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA
                    SnaB I
 351  CTTGGCAGTA CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG
 401  TTTTGGCAGT ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT
 451  TCCAAGTCTC CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA
 501  ATCAACGGGA CTTTCCAAAA TGTCGTAACA ACTCCGCCCC ATTGACGCAA
 551  ATGGGCGGTA GGCGTGTACG GTGGGAGGTC TATATAAGCA GAGCTCGTTT
 601  AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC
                                        Sac II
 651  ATAGAAGACA CCGGGACCGA TCCAGCCTCC GCGGCCGGGA ACGGTGCATT
 701  GGAACGCGGA TTCCCCGTGC CAAGAGTGAC GTAAGTACCG CCTATAGAGT
 751  CTATAGGCCC ACCCCCTTGG CTTCTTATGC ATGCTATACT GTTTTTGGCT
 801  TGGGGTCTAT ACACCCCCGC TTCCTCATGT TATAGGTGAT GGTATAGCTT
 851  AGCCTATAGG TGTGGGTTAT TGACCATTAT TGACCACTCC CCTATTGGTG
 901  ACGATACTTT CCATTACTAA TCCATAACAT GGCTCTTTGC CACAACTCTC
 951  TTTATTGGCT ATATGCCAAT ACACTGTCCT TCAGAGACTG ACACGGACTC
1001  TGTATTTTTA CAGGATGGGG TCTCATTTAT TATTTACAAA TTCACATATA
1051  CAACACCACC GTCCCCAGTG CCCGCAGTTT TTATTAAACA TAACGTGGGA
                                  (BspE I)
1101  TCTCCACGCG AATCTCGGGT ACGTGTTCCG GACATGGGCT CTTCTCCGGT
1151  AGCGGCGGAG CTTCTACATC CGAGCCCTGC TCCCATGCCT CCAGCGACTC
1201  ATGGTCGCTC GGCAGCTCCT TGCTCCTAAC AGTGGAGGCC AGACTTAGGC
```

Fig. 30 /2

```
1251 ACAGCACGAT GCCCACCACC ACCAGTGTGC CGCACAAGGC CGTGGCGGTA

1301 GGGTATGTGT CTGAAAATGA GCTCggggag cgggcttgca ccgctgacgc
                Afl II
1351 atttggaaga cttaaggcag cggcagaaga agatgcaggc agctgagttg

1401 ttgtgttctg ataagagtca gaggtaactc ccgttgcggt gctgttaacg

1451 gtggagggca gtgtagtctg agcagtactc gttgctgccg cgcgccgcac

1501 cagacataat agctgacaga ctaacagact gttcctttcc atgggtcttt
                          Mlu I        Hind III
1551 tctgcagtca ccgtccttga cacgcgtctc gggaagcttG CCGCCACCAT
                                                      M
1601 GGAGACAGAC ACACTCCTGC TATGGGTGCT GCTGCTCTGG GTTCCAGGTT
       E T D   T L L   L W V L   L L W   V P G
     (BspE I)
1651 CCTCCGGAGA CATTGTGATG ACCCAATCTC CAGACTCTTT GGCTGTGTCT
     S S G D   I V M   T Q S   P D S L   A V S

1701 CTAGGGGAGA GGGCCACCAT CAACTGCAAG TCCAGTCAGA GCCTTTTATA
      L G E   R A T I   N C  K S S Q  S L L Y
     XbaI                                CDR 1
1751 TTCTAGAAAT CAAAAGAACT ACTTGGCCTG GTATCAGCAG AAACCAGGAC
       S R N  Q K N  Y L A W  Y Q Q  K P G
                                                  KpnI
1801 AGCCACCCAA ACTCCTCATC TTTTGGGCTA GCACTAGGGA ATCTGGGGTA
     Q P P K  L L I  F W A  S T R E  S G V
                          CDR 2
1851 CCTGATAGGT TCAGTGGCAG TGGGTTTGGG ACAGACTTCA CCCTCACCAT
      P D R  F S G S  G F G  T D F  T L T I

1901 TAGCAGCCTG CAGGCTGAAG ATGTGGCAGT TTATTACTGT CAGCAATATT
       S S L  Q A E  D V A V  Y Y C  Q Q Y
1951 TTAGCTATCC GCTCACGTTC GGACAAGGGA CCAAGGTGGA AATAAAACGT
     F S Y P  L T  F  G Q G  T K V E  I K R
              CDR 3
        BamH I
2001 GAGTggatcc ATCTGGGATA AGCATGCTGT TTTCTGTCTG TCCCTAACAT

2051 GCCCTGTGAT TATGCGCAAA CAACACACCC AAGGGCAGAA CTTTGTTACT

2101 TAAACACCAT CCTGTTTGCT TCTTTCCTCA GGAACTGTGG CTGCACCATC
                                    T V  A A P S
2151 TGTCTTCATC TTCCCGCCAT CTGATGAGCA GTTGAAATCT GGAACTGCCT
       V F I  F P P  S D E Q  L K S  G T A
2201 CTGTTGTGTG CCTGCTGAAT AACTTCTATC CCAGAGAGGC CAAAGTACAG
     S V V C  L L N  N F Y  P R E A  K V Q
2251 TGGAAGGTGG ATAACGCCCT CCAATCGGGT AACTCCCAGG AGAGTGTCAC
      W K V  D N A L  Q S G  N S Q  E S V T
2301 AGAGCAGGAC AGCAAGGACA GCACCTACAG CCTCAGCAGC ACCCTGACGC
       E Q D  S K D  S T Y S  L S S  T L T
```

Fig. 30 /3

```
2351  TGAGCAAAGC AGACTACGAG AAACACAAAG TCTACGCCTG CGAAGTCACC
        L  S  K  A   D  Y  E   K  H  K   V  Y  A  C   E  V  T
2401  CATCAGGGCC TGAGCTCGCC CGTCACAAAG AGCTTCAACA GGGGAGAGTG
        H  Q  G   L  S  S  P   V  T  K   S  F  N   R  G  E  C
2451  TTAGAGGGAG AAGTGCCCCC ACCTGCTCCT CAGTTCCAGC CTGACCCCCT
        *                                    Psp5 II
2501  CCCATCCTTT GGCCTCTGAC CCTTTTTCCA CAGGGGACCT ACCCCTATTG

2551  CGGTCCTCCA GCTCATCTTT CACCTCACCC CCCTCCTCCT CCTTGGCTTT

2601  AATTATGCTA ATGTTGGAGG AGAATGAATA AATAAAGTGA ATCTTTGCAC

2651  CTGTGGTGGA TCTAATAAAA GATATTTATT TTCATTAGAT ATGTGTGTTG

2701  GTTTTTTGTG TGCAGTGCCT CTATCTGGAG GCCAGGTAGG GCTGGCCTTG

2751  GGGGAGGGGG AGGCCAGAAT GACTCCAAGA GCTACAGGAA GGCAGGTCAG

2801  AGACCCCACT GGACAAACAG TGGCTGGACT CTGCACCATA ACACACAATC

2851  AACAGGGGAG TGAGCTGGAA ATTTGCTAGC GAATTCTTGA AGACGAAAGG

2901  GCCTCGTGAT ACGCCTATTT TTATAGGTTA ATGTCATGAT AATAATGGTT

2951  TCTTAGACGT CAGGTGGCAC TTTTCGGGGA AATGTGCGCG GAACCCCTAT

3001  TTGTTTATTT TTCTAAATAC ATTCAAATAT GTATCCGCTC ATGAGACAAT

3051  AACCCTGATA AATGCTTCAA TAATATTGAA AAAGGAAGAG TATGAGTATT

3101  CAACATTTCC GTGTCGCCCT TATTCCCTTT TTTGCGGCAT TTTGCCTTCC

3151  TGTTTTTGCT CACCCAGAAA CGCTGGTGAA AGTAAAAGAT GCTGAAGATC

3201  AGTTGGGTGC ACGAGTGGGT TACATCGAAC TGGATCTCAA CAGCGGTAAG

3251  ATCCTTGAGA GTTTTCGCCC CGAAGAACGT TTTCCAATGA TGAGCACTTT

3301  TAAAGTTCTG CTATGTGGCG CGGTATTATC CCGTGTTGAC GCCGGGCAAG

3351  AGCAACTCGG TCGCCGCATA CACTATTCTC AGAATGACTT GGTTGAGTAC

3401  TCACCAGTCA CAGAAAAGCA TCTTACGGAT GGCATGACAG TAAGAGAATT

3451  ATGCAGTGCT GCCATAACCA TGAGTGATAA CACTGCGGCC AACTTACTTC
            Pvu I
3501  TGACAACGAT CGGAGGACCG AAGGAGCTAA CCGCTTTTTT GCACAACATG

3551  GGGGATCATG TAACTCGCCT TGATCGTTGG GAACCGGAGC TGAATGAAGC
```

Fig. 30 /4

```
3601  CATACCAAAC GACGAGCGTG ACACCACGAT GCCTGCAGCA ATGGCAACAA
3651  CGTTGCGCAA ACTATTAACT GGCGAACTAC TTACTCTAGC TTCCCGGCAA
3701  CAATTAATAG ACTGGATGGA GGCGGATAAA GTTGCAGGAC CACTTCTGCG
3751  CTCGGCCCTT CCGGCTGGCT GGTTTATTGC TGATAAATCT GGAGCCGGTG
3801  AGCGTGGGTC TCGCGGTATC ATTGCAGCAC TGGGGCCAGA TGGTAAGCCC
3851  TCCCGTATCG TAGTTATCTA CACGACGGGG AGTCAGGCAA CTATGGATGA
3901  ACGAAATAGA CAGATCGCTG AGATAGGTGC CTCACTGATT AAGCATTGGT
3951  AACTGTCAGA CCAAGTTTAC TCATATATAC TTTAGATTGA TTTAAAACTT
4001  CATTTTTAAT TTAAAAGGAT CTAGGTGAAG ATCCTTTTTG ATAATCTCAT
4051  GACCAAAATC CCTTAACGTG AGTTTTCGTT CCACTGAGCG TCAGACCCCG
4101  TAGAAAAGAT CAAAGGATCT TCTTGAGATC CTTTTTTTCT GCGCGTAATC
4151  TGCTGCTTGC AAACAAAAAA ACCACCGCTA CCAGCGGTGG TTTGTTTGCC
4201  GGATCAAGAG CTACCAACTC TTTTTCCGAA GGTAACTGGC TTCAGCAGAG
4251  CGCAGATACC AAATACTGTC CTTCTAGTGT AGCCGTAGTT AGGCCACCAC
4301  TTCAAGAACT CTGTAGCACC GCCTACATAC CTCGCTCTGC TAATCCTGTT
4351  ACCAGTGGCT GCTGCCAGTG GCGATAAGTC GTGTCTTACC GGGTTGGACT
4401  CAAGACGATA GTTACCGGAT AAGGCGCAGC GGTCGGGCTG AACGGGGGGT
4451  TCGTGCACAC AGCCCAGCTT GGAGCGAACG ACCTACACCG AACTGAGATA
4501  CCTACAGCGT GAGCTATGAG AAAGCGCCAC GCTTCCCGAA GGGAGAAAGG
4551  CGGACAGGTA TCCGGTAAGC GGCAGGGTCG GAACAGGAGA GCGCACGAGG
4601  GAGCTTCCAG GGGGAAACGC CTGGTATCTT TATAGTCCTG TCGGGTTTCG
4651  CCACCTCTGA CTTGAGCGTC GATTTTTGTG ATGCTCGTCA GGGGGGCGGA
4701  GCCTATGGAA AAACGCCAGC AACGCGGCCT TTTTACGGTT CCTGGCCTTT
                          BspLU11I
4751  TGCTGGCCTT TTGCTCACAT GTTCTTTCCT GCGTTATCCC CTGATTCTGT
4801  GGATAACCGT ATTACCGCCT TTGAGTGAGC TGATACCGCT CGCCGCAGCC
```

Fig. 30 /5

```
4851  GAACGACCGA GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA AGAGCGCCTG

4901  ATGCGGTATT TTCTCCTTAC GCATCTGTGC GGTATTTCAC ACCGCATATG
                                                    Bst1107I
4951  GTGCACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAGTATA

5001  CACTCCGCTA TCGCTACGTG ACTGGGTCAT GGCTGCGCCC CGACACCCGC

5051  CAACACCCGC TGACGCGCCC TGACGGGCTT GTCTGCTCCC GGCATCCGCT

5101  TACAGACAAG CTGTGACCGT CTCCGGGAGC TGCATGTGTC AGAGGTTTTC

5151  ACCGTCATCA CCGAAACGCG CGAGGCAGCT GTGGAATGTG TGTCAGTTAG

5201  GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG

5251  CATCTCAATT AGTCAGCAAC CAGGCTCCCC AGCAGGCAGA AGTATGCAAA

5301  GCATGCATCT CAATTAGTCA GCAACCATAG TCCCGCCCCT AACTCCGCCC

5351  ATCCCGCCCC TAACTCCGCC CAGTTCCGCC CATTCTCCGC CCCATGGCTG
                                       Sfi I
5401  ACTAATTTTT TTTATTTATG CAGAGGCCGA GGCCGCCTCG GCCTCTGAGC
                                        Stu I/Avr II
5451  TATTCCAGAA GTAGTGAGGA GGCTTTTTTG GAGGCCTAGG CTTTTGCAAA

5501  AAGCTAGCTT CACGCTGCCG CAAGCACTCA GGGCGCAAGG GCTGCTAAAG

5551  GAAGCGGAAC ACGTAGAAAG CCAGTCCGCA GAAACGGTGC TGACCCCGGA

5601  TGAATGTCAG CTACTGGGCT ATCTGGACAA GGGAAAACGC AAGCGCAAAG

5651  AGAAAGCAGG TAGCTTGCAG TGGGCTTACA TGGCGATAGC TAGACTGGGC

5701  GGTTTTATGG ACAGCAAGCG AACCGGAATT GCCAGCTGGG GCGCCCTCTG

5751  GTAAGGTTGG GAAGCCCTGC AAAGTAAACT GGATGGCTTT CTTGCCGCCA
                                    Bgl II/Bcl I
5801  AGGATCTGAT GGCGCAGGGG ATCAAGATCT GATCAAGAGA CAGGATGAGG

5851  ATCGTTTCGC ATGATTGAAC AAGATGGATT GCACGCAGGT TCTCCGGCCG

5901  CTTGGGTGGA GAGGCTATTC GGCTATGACT GGGCACAACA GACAATCGGC

5951  TGCTCTGATG CCGCCGTGTT CCGGCTGTCA GCGCAGGGGC GCCCGGTTCT

6001  TTTTGTCAAG ACCGACCTGT CCGGTGCCCT GAATGAACTG CAGGACGAGG
                          Msc I
6051  CAGCGCGGCT ATCGTGGCTG GCCACGACGG GCGTTCCTTG CGCAGCTGTG
```

Fig. 30 /6

```
6101  CTCGACGTTG TCACTGAAGC GGGAAGGGAC TGGCTGCTAT TGGGCGAAGT
6151  GCCGGGGCAG GATCTCCTGT CATCTCACCT TGCTCCTGCC GAGAAAGTAT
6201  CCATCATGGC TGATGCAATG CGGCGGCTGC ATACGCTTGA TCCGGCTACC
6251  TGCCCATTCG ACCACCAAGC GAAACATCGC ATCGAGCGAG CACGTACTCG
6301  GATGGAAGCC GGTCTTGTCG ATCAGGATGA TCTGGACGAA GAGCATCAGG
6351  GGCTCGCGCC AGCCGAACTG TTCGCCAGGC TCAAGGCGCG CATGCCCGAC
6401  GGCGAGGATC TCGTCGTGAC CCATGGCGAT GCCTGCTTGC CGAATATCAT
6451  GGTGGAAAAT GGCCGCTTTT CTGGATTCAT CGACTGTGGC CGGCTGGGTG
          Rsr II
6501  TGGCGGACCG CTATCAGGAC ATAGCGTTGG CTACCCGTGA TATTGCTGAA
6551  GAGCTTGGCG GCGAATGGGC TGACCGCTTC CTCGTGCTTT ACGGTATCGC
6601  CGCTCCCGAT TCGCAGCGCA TCGCCTTCTA TCGCCTTCTT GACGAGTTCT
                          Nsp V
6651  TCTGAGCGGG ACTCTGGGGT TCGAAATGAC CGACCAAGCG ACGCCCAACC
6701  TGCCATCACG AGATTTCGAT TCCACCGCCG CCTTCTATGA AAGGTTGGGC
6751  TTCGGAATCG TTTTCCGGGA CGCCGGCTGG ATGATCCTCC AGCGCGGGGA
                                  Sma I                Nru I
6801  TCTCATGCTG GAGTTCTTCG CCCACCCCGG GCTCGATCCC CTCGCGAGTT
6851  GGTTCAGCTG CTGCCTGAGG CTGGACGACC TCGCGGAGTT CTACCGGCAG
6901  TGCAAATCCG TCGGCATCCA GGAAACCAGC AGCGGCTATC CGCGCATCCA
6951  TGCCCCCGAA CTGCAGGAGT GGGGAGGCAC GATGGCCGCT TTGGTCCCGG
7001  ATCTTTGTGA AGGAACCTTA CTTCTGTGGT GTGACATAAT TGGACAAACT
7051  ACCTACAGAG ATTTAAAGCT CTAAGGTAAA TATAAAATTT TTAAGTGTAT
7101  AATGTGTTAA ACTACTGATT CTAATTGTTT GTGTATTTTA GATTCCAACC
7151  TATGGAACTG ATGAATGGGA GCAGTGGTGG AATGCCTTTA ATGAGGAAAA
7201  CCTGTTTTGC TCAGAAGAAA TGCCATCTAG TGATGATGAG GCTACTGCTG
7251  ACTCTCAACA TTCTACTCCT CCAAAAAAGA AGAGAAAGGT AGAAGACCCC
7301  AAGGACTTTC CTTCAGAATT GCTAAGTTTT TTGAGTCATG CTGTGTTTAG
```

Fig. 30 /7

```
7351  TAATAGAACT CTTGCTTGCT TTGCTATTTA CACCACAAAG GAAAAAGCTG

7401  CACTGCTATA CAAGAAAATT ATGGAAAAAT ATTCTGTAAC CTTTATAAGT

7451  AGGCATAACA GTTATAATCA TAACATACTG TTTTTTCTTA CTCCACACAG

7501  GCATAGAGTG TCTGCTATTA ATAACTATGC TCAAAAATTG TGTACCTTTA

7551  GCTTTTTAAT TTGTAAAGGG GTTAATAAGG AATATTTGAT GTATAGTGCC

7601  TTGACTAGAG ATCATAATCA GCCATACCAC ATTTGTAGAG GTTTTACTTG

7651  CTTTAAAAAA CCTCCCACAC CTCCCCCTGA ACCTGAAACA TAAAATGAAT
          Mun I
7701  GCAATTGTTG TTGTTAACTT GTTTATTGCA GCTTATAATG GTTACAAATA

7751  AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT TCACTGCATT

7801  CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA TGTCTGGATC

7851  TAATAAAAGA TATTTATTTT CATTAGATAT GTGTGTTGGT TTTTTGTGTG

7901  CAGTGCCTCT ATCTGGAGGC CAGGTAGGGC TGGCCTTGGG GGAGGGGGAG

7951  GCCAGAATGA CTCCAAGAGC TACAGGAAGG CAGGTCAGAG ACCCCACTGG

8001  ACAAACAGTG GCTGGACTCT GCACCATAAC ACACAATCAA CAGGGGAGTG

8051  AGCTGGAAAT TTGCTAGC
```

Fig. 31

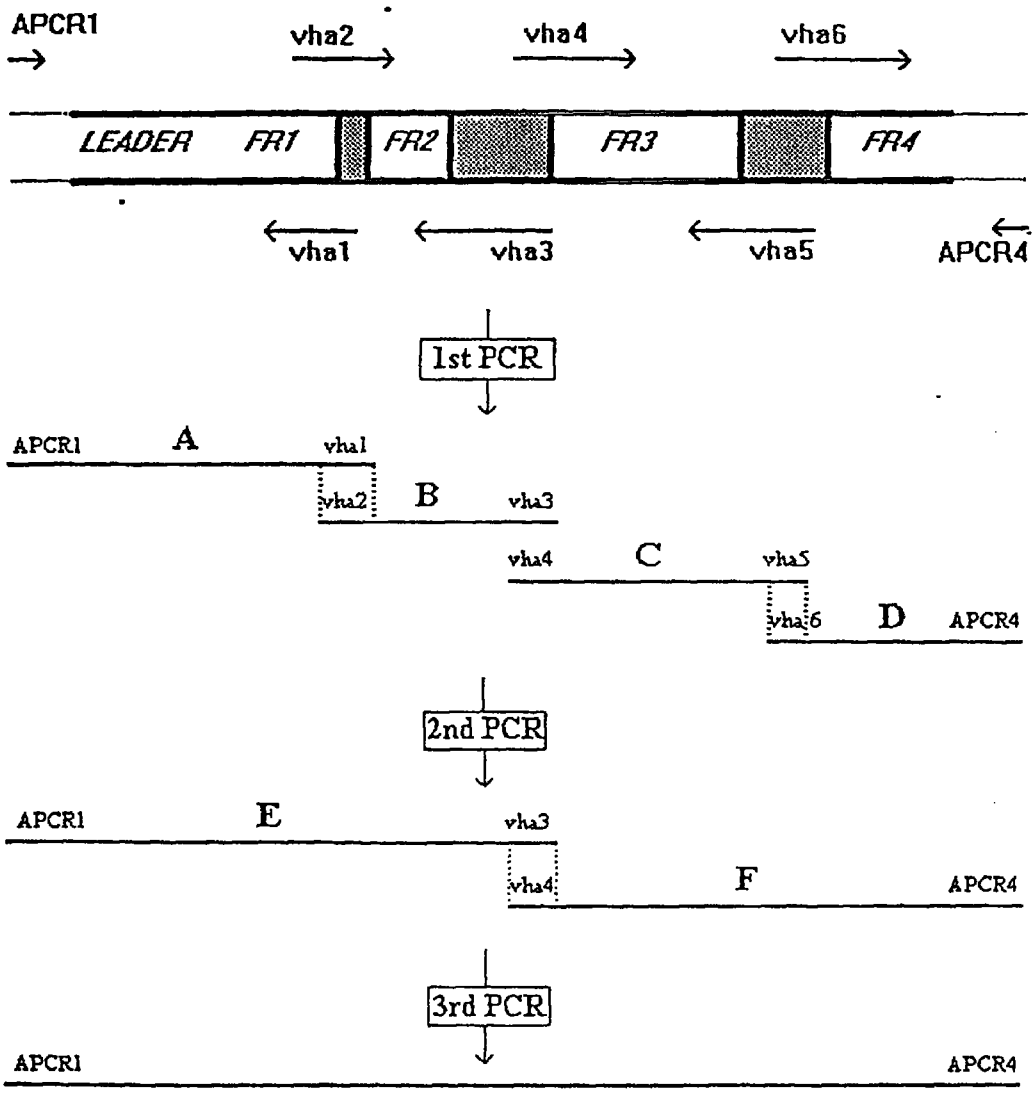

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vha4
vha6
LEADER
FR1
FR2
FR3
FR4
vha1
vha3
vha5
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APCR1
A
vha1
vha2
B
vha3
vha4
C
vha5
vha6
D
APCR4
2nd PCR
APCR1
E
vha3
vha4
F
APCR4
3rd PCR
APCR1
APCR4

Fig. 32 /1

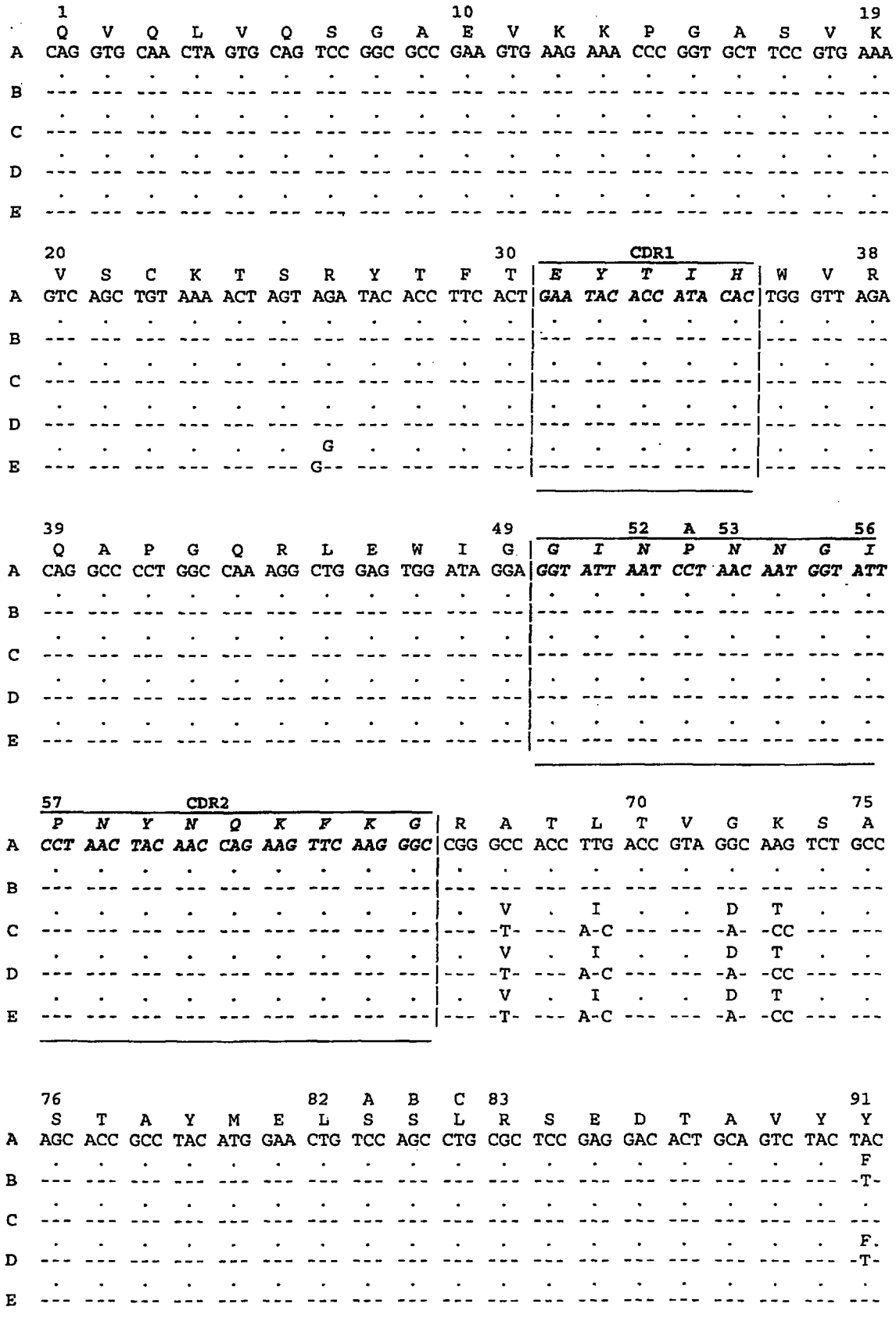

1 10 19
Q V Q L V Q S G A E V K K P G A S V K
A CAG GTG CAA CTA GTG CAG TCC GGC GCC GAA GTG AAG AAA CCC GGT GCT TCC GTG AAA
B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
E --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
20 30 CDR1 38
V S C K T S R Y T F T E Y T I H W V R
A GTC AGC TGT AAA ACT AGT AGA TAC ACC TTC ACT GAA TAC ACC ATA CAC TGG GTT AGA
B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
E --- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- --- ---
39 49 52 A 53 56
Q A P G Q R L E W I G G I N P N N G I
A CAG GCC CCT GGC CAA AGG CTG GAG TGG ATA GGA GGT ATT AAT CCT AAC AAT GGT ATT
B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
E --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
57 CDR2 70 75
P N Y N Q K F K G R A T L T V G K S A
A CCT AAC TAC AAC CAG AAG TTC AAG GGC CGG GCC ACC TTG ACC GTA GGC AAG TCT GCC
B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C --- --- --- --- --- --- --- --- --- --- V --- I --- --- D T --- ---
D --- --- --- --- --- --- --- --- --- --- -T- --- A-C --- --- -A- -CC --- ---
E --- --- --- --- --- --- --- --- --- --- V --- I --- --- D T --- ---
--- --- --- --- --- --- --- --- --- --- -T- --- A-C --- --- -A- -CC --- ---
76 82 A B C 83 91
S T A Y M E L S S L R S E D T A V Y Y
A AGC ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT GCA GTC TAC TAC
B --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T-
C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
D --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T-
E --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

Fig. 32 /2

	92			CDR3					100	A	B	C	D	I	J	K	101		103
	C	A	R	R	R	I	A	Y	G	Y	D	E	G	H	A	M	D	Y	W
A	TGC	GCC	AGA	AGA	AGA	ATC	GCC	TAT	GGT	TAC	GAC	GAG	GGC	CAT	GCT	ATG	GAC	TAC	TGG
B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	104									113
	G	Q	G	T	L	V	T	V	S	S
A	GGT	CAA	GGA	ACC	CTT	GTC	ACC	GTC	TCC	TCA
B	---	---	---	---	---	---	---	---	---	---
C	---	---	---	---	---	---	---	---	---	---
D	---	---	---	---	---	---	---	---	---	---
E	---	---	---	---	---	---	---	---	---	---

Fig. 33 /1

```
   1 TTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAAT
  61 GGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTT
 121 ATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCT
 181 TCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCC
 241 CTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAA
 301 AGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGG
 361 TAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGT
 421 TCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCG
 481 CATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTAC
 541 GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGC
                          Pvu I
 601 GGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAA
 661 CATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACC
                                                    Fsp I
 721 AAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATT
 781 AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA
```

Fig. 33 /2

```
 841 TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAA

 901 ATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAA

 961 GCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAA

1021 TAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGT

1081 TTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGT

1141 GAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTG

1201 AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT

1261 AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCA

1321 AGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATAC

1381 TGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTAC

1441 ATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT

1501 TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGG

1561 GGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACA

1621 GCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGT

1681 AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTA

1741 TCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC

1801 GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
                          BspLU11I
1861 CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA

1921 CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAG

1981 CGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCT

2041 GTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATA
            Bst1107 I
2101 GTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACAC

2161 CCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGA

2221 CAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAA

2281 CGCGCGAGGCAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC

2341 CATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTT
                                  Sfi I
2401 TTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGG
                    Stu I/Avr II
2461 AGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTAGCTTACAGCTCAGGGCTGCGATT
```

Fig. 33 /3

```
2521 TCGCGCCAAACTTGACGGCAATCCTAGCGTGAAGGCTGGTAGGATTTTATCCCCGCTGCC

2581 ATCATGGTTCGACCATTGAACTGCATCGTCGCCGTGTCCCAAAATATGGGGATTGGCAAG

2641 AACGGAGACCTACCCTGGCCTCCGCTCAGGAACGAGTTCAAGTACTTCCAAAGAATGACC

2701 ACAACCTCTTCAGTGGAAGGTAAACAGAATCTGGTGATTATGGGTAGGAAAACCTGGTTC

2761 TCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATTAATATAGTTCTCAGTAGAGAA

2821 CTCAAAGAACCACCACGAGGAGCTCATTTTCTTGCCAAAAGTTTGGATGATGCCTTAAGA

2881 CTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGATAGTCGGAGGCAGT

2941 TCTGTTTACCAGGAAGCCATGAATCAACCAGGCCACCTCAGACTCTTTGTGACAAGGATC

3001 ATGCAGGAATTTGAAAGTGACACGTTTTTCCCAGAAATTGATTTGGGGAAATATAAACTT

3061 CTCCCAGAATACCCAGGCGTCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAG

3121 TTTGAAGTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGCTCCCCTC
                                                    Bgl II
3181 CTAAAGCTATGCATTTTTATAAGACCATGGGACTTTTGCTGGCTTTAGATCTTTGTGAAG

3241 GAACCTTACTTCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAAGCTCT

3301 AAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGT

3361 GTATTTTAGATTCCAACCTATGGAACTGATGAATGGGAGCAGTGGTGGAATGCCTTTAAT

3421 GAGGAAAACCTGTTTTGCTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTGCTGAC

3481 TCTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGACTTTCCT

3541 TCAGAATTGCTAAGTTTTTTGAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTT

3601 GCTATTTACACCACAAAGGAAAAAGCTGCACTGCTATACAAGAAAATTATGGAAAAATAT

3661 TCTGTAACCTTTATAAGTAGGCATAACAGTTATAATCATAACATACTGTTTTTTCTTACT

3721 CCACACAGGCATAGAGTGTCTGCTATTAATAACTATGCTCAAAAATTGTGTACCTTTAGC

3781 TTTTTAATTTGTAAAGGGGTTAATAAGGAATATTTGATGTATAGTGCCTTGACTAGAGAT
     BsaB I
3841 CATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCT
                                          Mun I
3901 CCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGC

3961 TTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTC

4021 ACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTA

4081 ATAAAAGATATTTATTTTCATTAGATATGTGTGTTGGTTTTTTGTGTGCAGTGCCTCTAT

4141 CTGGAGGCCAGGTAGGGCTGGCCTTGGGGGAGGGGGAGGCCAGAATGACTCCAAGAGCTA
```

Fig. 33 /4

```
4201 CAGGAAGGCAGGTCAGAGACCCCACTGGACAAACAGTGGCTGGACTCTGCACCATAACAC
                                                EcoR I
4261 ACAATCAACAGGGGAGTGAGCTGGAAATTTGCTAGCGAATTCcagcacactggcggccgt
       (Spe I)
4321 tACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTT

4381 CCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC

4441 ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACG

4501 TCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT

4561 GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCA
                                                SnaB I
4621 GTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTAT

4681 TACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACG

4741 GGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCA

4801 ACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCG

4861 TGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAG

4921 ACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGG

4981 CCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTA

5041 TAGAGTCTATAGGCCCACCCCCTTGGCTTCTTATGCATGCTATACTGTTTTTGGCTTGGG
                                                Bpu1102I
5101 GTCTATACACCCCCGCTTCCTCATGTTATAGGTGATGGTATAGCTTAGCCTATAGGTGTG
                                  Xcm I
5161 GGTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCA

5221 TAACATGGCTCTTTGCCACAACTCTCTTTATTGGCTATATGCCAATACACTGTCCTTCAG

5281 AGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCTCATTTATTATTTACAAATTCA

5341 CATATACAACACCACCGTCCCCAGTGCCCGCAGTTTTTATTAAACATAACGTGGGATCTC
                                  BspE I
5401 CACGCGAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGGTAGCGGCGGAGCTTC

5461 TACATCCGAGCCCTGCTCCCATGCCTCCAGCGACTCATGGTCGCTCGGCAGCTCCTTGCT

5521 CCTAACAGTGGAGGCCAGACTTAGGCACAGCACGATGCCCACCACCACCAGTGTGCCGCA

5581 CAAGGCCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCggggagcgggcttgcaccgc
                                                (Pvu II)
5641 tgacgcatttggaagacttaaggcagcggcagaagaagatgcaggcagctgagttgttgt

5701 gttctgataagagtcagaggtaactcccgttgcggtgctgttaacggtggagggcagtgt

5761 agtctgagcagtactcgttgctgccgcgcgcgccaccagacataatagctgacagactaa
                                                Mlu I
5821 cagactgttcctttccatgggtcttttctgcagtcaccgtccttgacACGCGTCTCGGGA
```

Fig. 33 /5

```
   Hind III
5881 AGCTTGCCGCCACCATGGACTGGACCTGGCGCGTGTTTTGCCTGCTCGCCGTGGCTCCTG
                    M  D  W  T  W  R  V  F  C  L  L  A  V  A  P

5941 GGGCCCACAGCCAGGTGCAACTGGTGCAGTCCGGCGCCGAAGTGAAGAAACCCGGTGCTT
     G  A  H  S  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  A
              (Pvu II)  (Spe I)
6001 CCGTGAAAGTCAGCTGTAAAACTAGTAGATACACCTTCACTGAATACACCATACACTGGG
     S  V  K  V  S  C  K  T  S  R  Y  T  F  T  E  Y  T  I  H  W
                      Msc I                              CDR 1
6061 TTAGACAGGCCCCTGGCCAAAGGCTGGAGTGGATAGGAGGTATTAATCCTAACAATGGTA
     V  R  Q  A  P  G  Q  R  L  E  W  I  G  G  I  N  P  N  N  G

6121 TTCCTAACTACAACCAGAAGTTCAAGGGCCGGGCCACCTTGACCGTAGGCAAGTCTGCCA
     I  P  N  Y  N  Q  K  F  K  G  R  A  T  L  T  V  G  K  S  A
           CDR 2
6181 GCACCGCCTACATGGAACTGTCCAGCCTGCGCTCCGAGGACACTGCAGTCTACTACTGCG
     S  T  A  Y  M  E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C

6241 CCAGAAGAAGAATCGCCTATGGTTACGACGAGGGCCATGCTATGGACTACTGGGGTCAAG
     A  R  R  R  I  A  Y  G  Y  D  E  G  H  A  M  D  Y  W  G  Q
                       CDR 3          BamH I
6301 GAACCCTTGTCACCGTCTCCTCAGGTGAGTGGATCCTCTGCGCCTGGGCCCAGCTCTGTC
     G  T  L  V  T  V  S  S

6361 CCACACCGCGGTCACATGGCACCACCTCTCTTGCAGCCTCCACCAAGGGCCCATCGGTCT
                                          S  T  K  G  P  S  V

6421 TCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGG
     F  P  L  A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L
                          Age I
6481 TCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCG
     V  K  D  Y  F  P  E  P  V  T  V  S  W  N  S  G  A  L  T  S

6541 GCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGG
     G  V  H  T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S  S  V
   BstE II
6601 TGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGC
     V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K

6661 CCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACAT
     P  S  N  T  K  V  D  K  K  V  E  P  K  S  C  D  K  T  H  T

6721 GCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAA
     C  P  P  C  P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P

6781 AACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACG
     K  P  K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D

6841 TGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATA
     V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H

6901 ATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCC
     N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V
```

Fig. 33 /6

```
6961 TCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACA
     L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N

7021 AAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC
     K  A  L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E

7081 CACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGA
     P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N  Q  V  S  L

7141 CCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGC
     T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G

7201 AGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCC
     Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F

7261 TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCT
     L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C

7321 CCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG
     S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P
                    NgoM I
7381 GTAAATGAGTGCGACGGCCGGCAAGCCCCGCTCCCCGGGCTCTCGCGGTCGCACGAGGAT
     G  K  *
7441 GCTTGGCACGTACCCCCTGTACATACTTCCCGGGCGCCCAGCATGGAAATAAAGCACCGG

7501 ATCTAATAAAAGATATTTATTTTCATTAGATATGTGTGTTGGTTTTTTGTGTGCAGTGCC

7561 TCTATCTGGAGGCCAGGTAGGGCTGGCCTTGGGGGAGGGGGAGGCCAGAATGACTCCAAG

7621 AGCTACAGGAAGGCAGGTCAGAGACCCCACTGGACAAACAGTGGCTGGACTCTGCACCAT

7681 AACACACAATCAACAGGGGAGTGAGCTGGaaatttgctagcgaattaattc 7731
```

Fig. 34 A

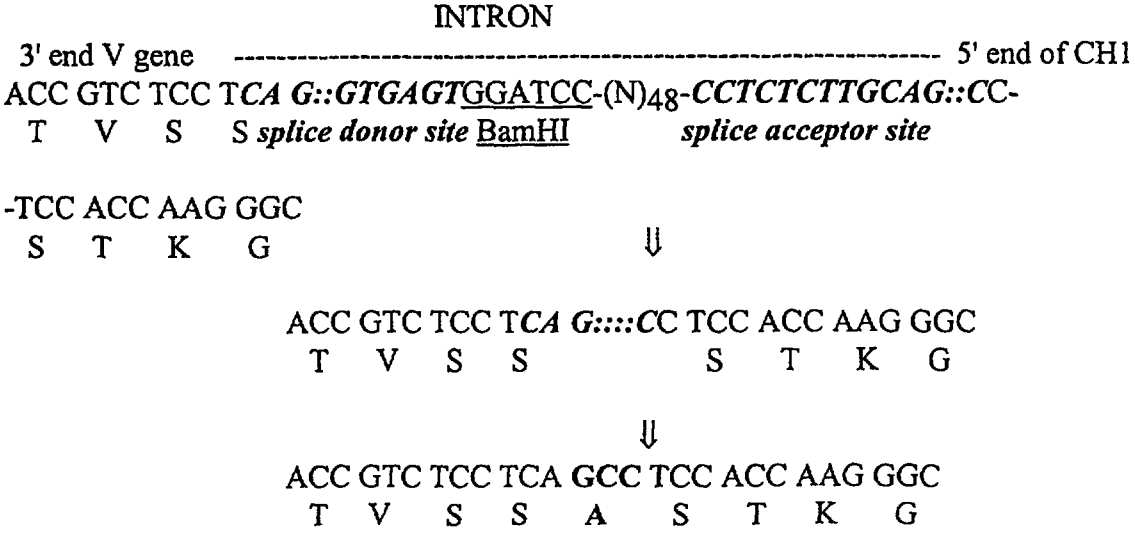


Fig. 34 B

INTRON

3' end V gene ---------- 5' end Kappa constant

GAA ATA AA*A C::GTGAGT*GGATCC-$(N)_{108}$-*CTTCTTTCCTCAG::GA*-

E I K *splice donor site*BamHI *splice acceptor site*

-ACT GTG GCT GCA

T V A A

⇓

GAA ATA AA*A C::::GA* ACT GTG GCT GCA

E I K T V A A

⇓

GAA ATA AAA **CGA** ACT GTG GCT GCA

E I K **R** T V A A

Fig. 35

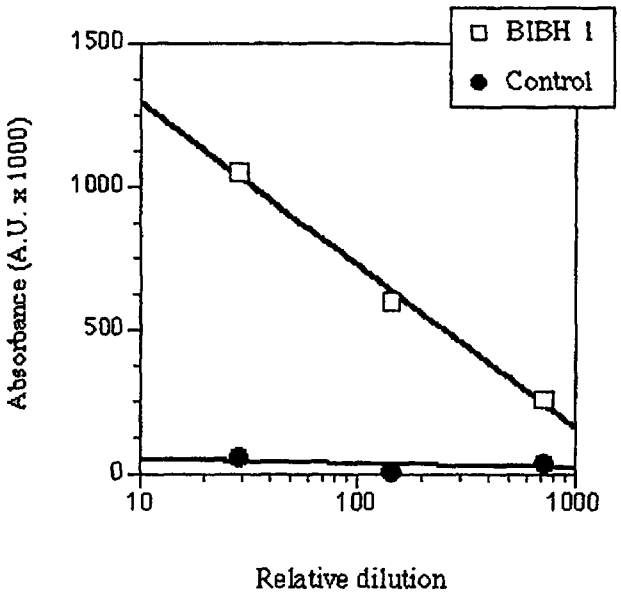


Fig. 36

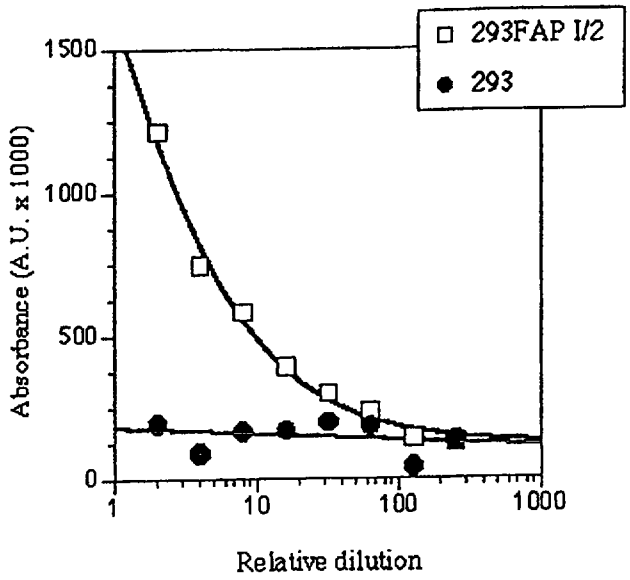
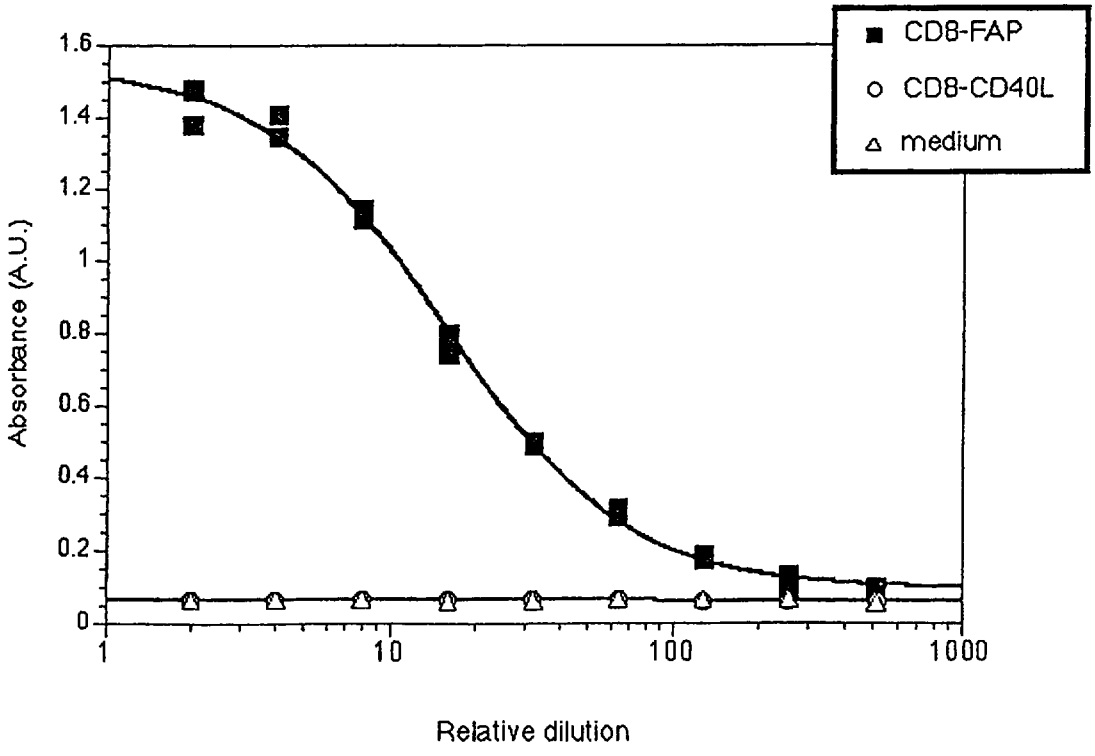

293FAP I/2
293
1500
1000
500
0
Absorbance (A.U. x 1000)
1
10
100
1000
Relative dilution

Fig. 37


CD8-FAP
CD8-CD40L
medium
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (A.U.)
1
10
100
1000
Relative dilution

FAPα-specific antibody with improved producibility

Field of the invention

The present invention relates to antibody proteins that specifically bind fibroblast activation protein alpha (FAPα). The invention also relates to the use of said antibodies for diagnostic and therapeutic purposes and methods of producing said antibodies.

Background of the invention

The invasive growth of epithelial cancers is associated with a number of characteristic cellular and molecular changes in the supporting stroma. A highly consistent molecular trait of the reactive stroma of many types of epithelial cancer is induction of the fibroblast activation protein alpha (from now on referred to as FAP), a cell surface molecule of reactive stromal fibroblasts originally identified with monoclonal antibody F19 (Garin-Chesa P., Old L. J. and Rettig W. J. (1990) Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc. Natl. Acad. Sci.* **87**: 7235). Since the FAP antigen is selectively expressed in the stroma of a range of epithelial carcinomas, independent of location and histological type, an FAP-targeting concept has been developed for imaging, diagnosis and treatment of epithelial cancers and certain other conditions. For this purpose a monoclonal antibody termed F19 that specifically binds to FAP was developed and described in US Patents 5,059,523 and WO 93/05804, which are hereby incorporated by reference in their entirety.

One serious problem that arises when using non-human antibodies for applications *in vivo* in humans is that they quickly raise a human anti-non-human response that reduces the efficacy of the antibody in patients and impairs continued administration. Humanization of non-human antibodies is commonly achieved in one of two ways: (1) by constructing non-human/human chimeric antibodies, wherein the non-human variable regions are joined to human constant regions (Boulianne G. L., Hozumi N. and Shulman, M .J. (1984) Production of

functional chimeric mouse/human antibody *Nature* **312**: 643) or (2) by grafting the complementarity determining regions (CDRs) from the non-human variable regions to human variable regions and then joining these "reshaped human" variable regions to human constant regions (Riechmann L., Clark M., Waldmann H. and Winter G. (1988) Reshaping human antibodies for therapy. *Nature* **332**: 323). Chimeric antibodies, although significantly better than mouse antibodies, can still elicit an anti-mouse response in humans (LoBuglio A. F., Wheeler R. H., Trang J., Haynes A., Rogers K., Harvey E. B., Sun L., Ghrayeb J. and Khazaeli M. B. (1989) Mouse/human chimeric monoclonal antibody in man: Kinetics and immune response. *Proc. Natl. Acad. Sci.* **86**: 4220). CDR-grafted or reshaped human antibodies contain little or no protein sequences that can be identified as being derived from mouse antibodies. Although an antibody humanised by CDR-grafting may still be able to elicit some immune reactions, such as an anti-allotype or an anti-idiotypic response, as seen even with natural human antibodies, the CDR-grafted antibody will be significantly less immunogenic than a mouse antibody thus enabling a more prolonged treatment of patients.

Another serious limitation relating to the commercial use of antibodies for diagnosis, imaging and therapy is their producibility in large amounts. In many instances recombinant expression of native, chimeric and/or CDR-grafted antibodies in cell culture systems is poor. Factors contributing to poor producibility may include the choice of leader sequences and the choice of host cells for production as well as improper folding and reduced secretion. Improper folding can lead to poor assembly of heavy and light chains or a transport incompetent conformation that forbids secretion of one or both chains. It is generally accepted that the L-chain confers the ability of secretion of the assembled protein. In some instances multiple or even single substitutions can result in the increased producibility of antibodies.

Because of the clinical importance of specific immunological targeting *in vitro* and *in vivo* of specific disease-related antigens for diagnosis and therapy in humans, there is a growing need for antibodies that combine the features of antigen specificity, low immunogenicity and high producibility.

Therefore, the problem underlying the present invention was to provide antibody proteins that combine the properties of specific binding to FAP, low immunogenicity in humans, and high producibility in recombinant systems.

Disclosure of the invention

The technical problem is solved by the embodiments characterized in the claims.

The present invention provides new antibody proteins having the complementary determining regions of the monoclonal antibody F19 (ATCC Accession No. HB 8269), said new antibody proteins specifically binding to fibroblast activation protein (FAP), characterised in that they have framework modifications resulting in the improved producibility in host cells as compared to a chimeric antibody having the variable regions of F19 and foreign constant regions.

As used herein, an "antibody protein" is a protein with the antigen binding specificity of a monoclonal antibody.

"Complementarity determining regions of a monoclonal antibody" are understood to be those amino acid sequences involved in specific antigen binding according to Kabat (Kabat E. A., Wu T. T., Perry H. M., Gottesman K. S. and Foeller C. (1991) *Sequences of Proteins of Immunological Interest* (5th Ed.). NIH Publication No. 91-3242. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD.) in connection with Chothia and Lesk (Chothia and Lesk (1987) *J. Mol. Biol.* 196:901-917).

As used herein, the term "framework modifications" refers to the exchange, deletion or addition of single or multiple amino acids in the variable regions surrounding the individual complementarity determining regions. Framework modifications may have an impact on the immunogenicity, producibility or binding specificity of an antibody protein.

"Fibroblast activation protein (FAP)", also designated fibroblast activation protein alpha (FAPα), is a membrane-bound glycoprotein belonging to the serine protease gene family (WO 97/34927). No shed or secreted form of FAP is known. FAP can be characterized by its binding to the monoclonal antibody F19 (F19 is obtainable from the hybridoma cell line with the accession No. HB 8269 deposited at the ATCC).

The term ""fibroblast activation protein specific binding" of an antibody protein is defined herein by its ability to specifically recognise and bind FAP-expressing human cells. The binding specificity of the proteins of the invention can be determined by standard methods for the evaluation of binding specificity such as described in an exemplary fashion in example 6, 8 and example 12.

The term "chimeric antibody" refers to an antibody protein having the light and heavy chain variable regions as described in figures 17 and 18 and foreign constant regions. "Foreign constant regions" as defined herein are constant regions which are different from the constant regions of F19. For comparing an antibody protein of the invention to a chimeric antibody it is to be understood that such a chimeric antibody must contain the same constant regions as said antibody protein. For the purpose of demonstration and comparison alone the human constant heavy and light chains as described in Figures 19 to 22 are used in an exemplary fashion.

To provide the antibody proteins of the present invention, the nucleic acid sequences of the heavy and light chain genes of the murine antibody designated F19 were determined from RNA extracted from F19 hybridoma cells (ATCC Accession No. HB 8269).

In one embodiment the present invention relates to antibody proteins having the complementary determining regions of the monoclonal antibody F19 (ATCC Accession No. HB 8269), said new antibody proteins specifically binding to fibroblast activation protein (FAP), characterized in that they have framework modifications resulting in the improved producibility in host cells as compared to a chi-

meric antibody having the variable regions of F19 and foreign constant regions, wherein said antibody protein is derived from the murine antibody designated F19 (ATCC Accession No. HB 8269).

To generate humanised FAP-specific antibody proteins a chimeric antibody was constructed, having variable regions of the light and heavy chains of F19 and human light and heavy constant regions, respectively. The construction and production of chimeric mouse/human antibodies is well known (Boulianne et al. (1984), referenced above) and demonstrated in an exemplary fashion in examples 1 and 2.

The variable regions of the antibody proteins of the present invention are typically linked to at least a portion of the immunoglobulin constant region (F_C), typically that of a human immunoglobulin. Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably immortalized B cells (see Kabat et al., *supra*, and WO 87/02671). Hence the antibody proteins of the invention may contain all or only a portion of the constant region as long as they exhibit specific binding to the FAP antigen. The choice of the type and extent of the constant region depends on whether effector functions like complement fixation or antibody dependent cellular toxicity are desired, and on the desired pharmacological properties of the antibody protein. The antibody protein of the invention will typically be a tetramer consisting of two light chain/heavy chain pairs, but may also be dimeric, i.e. consisting of a light chain/heavy chain pair, e.g. a Fab or Fv fragment.

Therefore, in a further embodiment the invention relates to antibody proteins according to the invention, characterised in that they have a variable light chain region and a variable heavy chain region, each joined to a human constant region. In particular, the variable region of the light chain was joined to a human kappa constant region and the variable region of the heavy chain was joined to a human gamma-1 constant region. Other human constant regions for humanising light and heavy chains are also available to the expert.

Therefore, in one particular embodiment the antibody proteins of the invention contain a human kappa constant region.

Also, in another particular embodiment the antibody proteins of the invention contain a human gamma-1 constant region.

One particular "chimeric F19 antibody" protein (cF19) consists of the light and heavy chain variable and constant regions described in Figures 17 to 22. cF19 demonstrates specific binding and high avidity to the FAP antigen. As demonstrated in example 2, the expression of cF19 in COS cells (cells derived from the kidney of an African green monkey) is poor, ranging from about 10 to 60 ng/ml, which is at least 10 fold less than most antibodies.

In an attempt to increase expression levels of cF19, the leader sequence of the F19 V_L region was changed by substitution of proline to leucine at position –9. This single change in amino acid in the leader sequence resulted in at least doubling the amount of chimeric antibody produced in COS cells . For the expression of this particular chimeric antibody in COS cells the following mutated leader sequence of the light chain: MDSQAQVLMLLLLWVSGTCG, and the following leader sequence of the heavy chain: MGWSWVFLFLLSGTAGVLS were used.

According to the invention the term "improved producibility" in host cells refers to the substantial improvement of expression levels and/or purified antibody yields when compared with the expression levels and/or antibody yields of a chimeric antibody without framework modifications as defined above. Two particular but not limiting examples for demonstrating improved producibility are exemplified for the COS cell expression system (in examples 2 and 5) and for the CHO cell expression system (in example 10 and 11).

While the mutation of the leader sequence only leads to a doubling of the expression yield of the chimeric F19 antibody, a substantial improvement as defined herein refers to an improvement in expression level and/or purification yield of at least a factor of 10.

In a preferred embodiment, the invention refers to antibody proteins, characterised in that their expression levels in crude media samples as determined by ELISA and/or purified antibody yields exceed the expression levels and/or purification yields of the chimeric antibodies without framework modifications by at least a factor of 10.

In more preferred embodiment, the invention refers to antibody proteins, characterised in that their expression levels in crude media samples as determined by ELISA and/or purified antibody yields exceed the expression levels and/or purification yields of the chimeric antibodies without framework modifications by at least a factor of 20.

In a most preferred embodiment, antibody proteins, characterised in that their expression levels in crude media samples as determined by ELISA and/or purified antibody yields exceed the expression levels and/or purification yields of the chimeric antibodies without framework modifications by at least a factor of 100.

Improved producibility of the recombinant antibody proteins of the invention can be demonstrated for eukaryotic cells in general as shown for COS and CHO (Chinese hamster ovary derived cells) eukaryotic cells (see examples 5 and 11). In a further embodiment, the present invention relates to recombinant antibody proteins characterised in that they display improved producibility in eukaryotic cells.

In a preferred embodiment the present invention relates to antibody proteins, wherein said eukaryotic cell is a Chinese hamster ovary cell (CHO cell).

It was unexpectedly found that certain framework modifications of the light chain variable regions determine the improved producibility of the antibody proteins of the invention. Three versions of reshaped light chain variable regions, designated version A, B, and C, as described in Figures 1 to 6, were prepared.

Light chain variable region versions A, B, and C demonstrate substantially improved producibility in CHO cells (see example 11). While light chain variable region versions A and C differ from light chain variable region version B by only two common amino acid residues they display an even further substantial improvement in producibility. There is at least another 10 fold difference in antibody secretion levels between the human reshaped F19 light chain version B and versions A or C. Reshaped human F19 light chain version A and B only differ in their amino acid sequences by two residues at positions 36 (Tyr to Phe mutation) and 87 (Tyr to Asp mutation) (nomenclature according to Kabat). This negative effect on the secretory capability of antibodies containing the light chain variable region version B could have been indirect if the Tyr to Asp and Tyr to Phe mutations, considered individually or together, merely caused improper folding of the protein. But this is unlikely to be the case since antigen binding assays show that immunoglobulins containing F19 light chain version B have similar avidities to those paired with F19 light chain version A or C, suggesting that they were not grossly misfolded.

Residue 87 in reshaped human F19 light chain version B seems particularly responsible for the reduction of secretion when compared to versions A and C.

In a preferred embodiment, the present invention relates to antibody proteins according to the invention, wherein the amino acid in Kabat position 87 of the light chain region is not asparagine.

In a more preferred embodiment, the invention relates to antibody proteins according to the invention, wherein the amino acid in Kabat position 87 of the light chain region is selected from aromatic or aliphatic amino acids.

In a most preferred embodiment, the present invention relates to antibody proteins according to the invention, wherein the aromatic amino acid in Kabat position 87 of the light chain region is a tyrosine or phenylalanine.

In a further embodiment, the present invention also pertains to antibody proteins according to the invention, wherein the amino acid in Kabat position 36 of the light chain region is selected from aromatic amino acids.

In a particular embodiment the invention relates to the specific antibody proteins that may be prepared from the individually disclosed reshaped variable regions of the light and heavy chains.

Especially light chain variable region versions A and C are particularly suitable to practice the invention because of their exceptionally high producibility, while retaining full FAP-binding specificity and achieving low immunogenicity. This holds especially true when compared to the chimeric antibody having the variable regions of F19 and the same constant regions but also when compared to light chain version B.

Therefore, in one embodiment the present invention relates to antibody proteins that contain the variable region of the light chain as set forth in SEQ ID NO: 2.

In a further embodiment the invention also relates to antibody proteins, characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1.

In one embodiment the present invention relates to antibody proteins that contain the variable region of the light chain as set forth in SEQ ID NO: 6.

In a further embodiment the invention also relates to antibody proteins characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 5.

The present invention also discloses several different variable regions of the heavy chain that work particularly well with the variable regions of the light chain versions A and C in terms of improved producibility.

In one embodiment the invention relates to antibody proteins containing a variable region of the heavy chain as set forth in any one of SEQ ID NOs: 8, 10, 12, 14.

In another embodiment the invention relates to antibody proteins characterised in that the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in any one of SEQ ID NOs: 7, 9, 11, 13.

In a very particular embodiment the invention relates to antibody proteins containing the variable region of the light chain as set forth in SEQ ID NO: 2 and the variable region of the heavy chain as set forth in SEQ ID NO: 12. Most preferably, this antibody protein additionally contains the constant region of the light chain as set forth in SEQ ID NO: 20 and the constant region of the heavy chain as set forth in SEQ ID NO: 22.

Thus a further aspect of the present invention is an antibody protein containing an amino acid sequence as set forth in SEQ ID NO: 2. More preferably, such an antibody protein further contains an amino acid sequence as set forth in SEQ ID NO: 12. More preferably, said antibody protein further contains an amino acid sequence as set forth in SEQ ID NO: 20 and an amino acid sequence as set forth in SEQ ID NO: 22. A further aspect of the invention is an antibody protein as described in this paragraph which is conjugated to a radioisotope, preferably ^{131}I, ^{125}I, ^{186}Re, ^{188}Re, or ^{90}Y. An additional aspect of the present invention is a DNA molecule coding for an antibody protein as described in this paragraph. A further aspect of the invention is a host cell carrying such a DNA molecule. Accordingly, a further aspect of the invention is a method of producing an antibody protein as described in this paragraph, said method comprising the steps of cultivating such a host cell under conditions where said antibody protein is expressed by said host cell, and isolating said protein. A further aspect of the invention is a pharmaceutical composition comprising an antibody protein of the present invention and a pharmaceutically acceptable carrier. In a further particular embodiment the invention relates to antibody proteins characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1 and

the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 11.

In a further particular embodiment the invention relates to antibody proteins containing the variable region of the light chain as set forth in SEQ ID NO: 2 and the variable region of the heavy chain as set forth in SEQ ID NO: 8.

In a further particular embodiment the invention relates to antibody proteins characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1 and the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 7.

Humanization of the variable region of a murine antibody may be achieved employing methods known in the art. EP 0239400 discloses grafting of the CDRs of a murine variable region into the framework of a human variable region. WO 90/07861 discloses methods of reshaping a CDR-grafted variable region by introducing additional framework modifications. WO 92/11018 discloses methods of producing humanized Ig combining donor CDRs with an acceptor framework that has a high homology to the donor framework. WO 92/05274 discloses the preparation of framework mutated antibodies starting from a murine antibody. Further prior art references related to humanization of murine monoclonal antibodies are EP 0368684; EP 0438310; WO 92/07075, or WO 92/22653. Thus, the expert can produce the antibodies of the present invention starting from the publicly available murine monoclonal antibody F19 and employing techniques known in the art, e.g. from WO 92/05274. DNA molecules coding for the antibody proteins of the present invention may of course also be obtained by state-of-the-art synthetic procedures, e.g. by chemical synthesis of appropriate oligonucleotides and subsequent ligation and amplification procedures (see e.g. Frank et al. (1987) *Methods Enzymol.* **154**: 221-249).

In a further aspect, the present invention relates to nucleic acid molecules containing the coding information for the antibody proteins according to the invention as disclosed above. Preferably, a nucleic acid molecule according to the pre-

sent invention is a nucleic acid molecule containing a nucleotide sequence selected from SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, or 15.

A further aspect of the present invention is a recombinant DNA vector containing the nucleotide sequence of any one of the above-mentioned nucleic acids, especially when said nucleotide sequence is operationally linked to an expression control sequence as in expression vectors. Preferred is a recombinant DNA vector, said vector being an expression vector.

A further aspect of the present invention is a host cell carrying a vector as described, especially an expression vector. Such a host cell can be a prokaryotic or eukaryotic cell. Preferably, such a host cell is an eukaryotic cell, a yeast cell, or a mammalian cell. More preferably, said host cell is a CHO (Chinese hamster ovary) cell or a COS cell.

Accordingly, a still further aspect of the present invention is a method of producing antibody proteins according to the invention. Such a method comprises the steps of:

(a) cultivating a host cell as described above under conditions where said antibody protein is expressed by said host cell, and
(b) isolating said antibody protein.

Mammalian host cells, preferably CHO or COS cells are preferred. Host cells for producing the antibody proteins of the invention may be transfected with a single vector containing the expression units for both, the light and the heavy chain (see e.g. WO 94/11523). In one particular embodiment the method of producing antibody proteins according to the invention pertains to host cells, wherein said host cells are cotransfected with two plasmids carrying the expression units for the light and heavy chains respectively (See e.g. EP 0481790).

The antibody proteins of the invention provide a highly specific tool for targeting therapeutic agents to the FAP antigen. Therefore, in a further aspect, the invention relates to antibody proteins according to the invention, wherein said antibody protein is conjugated to a therapeutic agent. Of the many therapeutic

agents known in the art, therapeutic agents selected from the group consisting of radioisotopes, toxins, toxoids, inflammatogenic agents, enzymes, antisense molecules, peptides, cytokines, and chemotherapeutic agents are preferred. Among the radioisotopes, gamma, beta and alpha-emitting radioisotopes may be used as a therapeutic agent. ß-emitting radioisotopes are preferred as therapeutic radioisotopes. ^{186}Rhenium, ^{188}Rhenium, ^{131}Iodine and ^{90}Yttrium have been proven to be particularly useful ß-emitting isotopes to achieve localized irradiation and destruction of malignant tumor cells. Therefore, radioisotopes selected from the group consisting of ^{186}Rhenium, ^{188}Rhenium, ^{131}Iodine and ^{90}Yttrium are particularly preferred as therapeutic agents conjugated to the antibody proteins of the invention. For example, for the radioiodination of an antibody of the invention, a method as disclosed in WO 93/05804 may be employed.

A further aspect of the present invention pertains to antibody proteins according to the invention, characterised in that they are labelled. Such an FAP-specific labelled antibody allows for the localisation and/or detection of the FAP antigen *in vitro* and/or *in vivo*. A label is defined as a marker that may be directly or indirectly detectable. An indirect marker is defined as a marker that cannot be detected by itself but needs a further directly detectable marker specific for the indirect marker. Preferred labels for practicing the invention are detectable markers. From the large variety of detectable markers, a detectable marker selected from the group consisting of enzymes, dyes, radioisotopes, digoxygenin, and biotin is most preferred.

A further aspect of the present invention relates to antibody proteins according to the invention, characterised in that they are conjugated to an imageable agent. A large variety of imageable agents, especially radioisotopes, are available from the state of the art. For practising the invention gamma-emitting isotopes are more preferred. Most preferred is ^{125}Iodine.

One aspect of the present invention relates to pharmaceutical compositions containing an antibody protein according to the present invention as described above and a pharmaceutically acceptable carrier. Such pharmaceutical compositions are useful for treating tumors, wherein said tumors are associated with acti-

vated stromal fibroblasts. There are two possible effector principles for an anti-tumor stroma immunotherapy that may act synergistically: (a) An unmodified (unconjugated, `naked`) antibody according to the invention may induce immune destruction or inflammatory reactions in the tumor stroma while (b) an antibody conjugated to a therapeutic agent, such as for example, a radioisotope or other toxic substance, may achieve localized irradiation and destruction of the malignant tumor cells. Accordingly, a further aspect of the present invention is the use of an antibody protein as described for the manufacture of a pharmaceutical composition, especially for the treatment of tumors.

One further embodiment are pharmaceutical compositions containing an antibody protein according to the invention conjugated to a therapeutic agent as described above and a pharmaceutically acceptable carrier useful for treating tumors, wherein said tumors are associated with activated stromal fibroblasts. Another embodiment pertains to pharmaceutical compositions containing an antibody protein according to the present invention conjugated to an imageable agent as described above and a pharmaceutically acceptable carrier useful for imaging the presence of activated stromal fibroblasts in a healing wound, inflamed skin or a tumor, in a human patient. A most preferred embodiment relates to the pharmaceutical compositions mentioned above, wherein said tumors are tumors selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, invasive bladder cancers, pancreatic cancers and cancers metastatic of the brain.

In an animal or human body, it can prove advantageous to apply the pharmaceutical compositions as described above via an intravenous or other route, e.g. systemically, locally or topically to the tissue or organ of interest, depending on the type and origin of the disease or problem treated, e.g. a tumor. For example, a systemic mode of action is desired when different organs or organ systems are in need of treatment as in e.g. systemic autoimmune diseases, or allergies, or transplantations of foreign organs or tissues, or tumors that are diffuse or difficult to localise. A local mode of action would be considered when only local manifestations of neoplastic or immunologic action are expected, such as, for example local tumors.

The antibody proteins of the present invention may be applied by different routes of application known to the expert, notably intravenous injection or direct injection into target tissues. For systemic application, the intravenous, intravascular, intramuscular, intraarterial, intraperitoneal, oral, or intrathecal routes are preferred. A more local application can be effected subcutaneously, intracutaneously, intracardially, intralobally, intramedullarly, intrapulmonarily or directly in or near the tissue to be treated (connective-, bone-, muscle-, nerve-, epithelial tissue). Depending on the desired duration and effectiveness of the treatment, pharmaceutical antibody compositions may be administered once or several times, also intermittently, for instance on a daily basis for several days, weeks or months and in different dosages.

For preparing suitable antibody preparations for the applications described above, the expert may use known injectable, physiologically acceptable sterile solutions. For preparing a ready-to-use solution for parenteral injection or infusion, aqueous isotonic solutions, such as e.g. saline or corresponding plasma protein solutions are readily available. The pharmaceutical compositions may be present as lyophylisates or dry preparations, which can be reconstituted with a known injectable solution directly before use under sterile conditions, e.g. as a kit of parts. The final preparation of the antibody compositions of the present invention are prepared for injection, infusion or perfusion by mixing purified antibodies according to the invention with a sterile physiologically acceptable solution, that may be supplemented with known carrier substances or/and additives (e.g. serum albumin, dextrose, sodium bisulfite, EDTA).

The amount of the antibody applied depends on the nature of the disease. In cancer patients, the applied dose of a 'naked' antibody may be between 0.1 and 100 mg/m^2, preferably between 5 and 50 mg/m^2 per application. For radiolabeled antibodies, e.g. with iodine-131, the maximally tolerated dose (MTD) has to be determined which must not be exceeded in therapeutic settings. Application of radiolabeled antibody to cancer patients may then be carried out by repeated (monthly or weekly) intravenous infusion of a dose which is below the MTD (See e.g. Welt et al. (1994) *J. Clin. Oncol.* **12**: 1193-1203).

Furthermore, one aspect of the present invention relates to the use of the antibody proteins according to the invention for the treatment of cancer. In a preferred embodiment the present invention relates to the use of antibody proteins according to the invention conjugated to a therapeutic agent as described above for the treatment of cancer. In another preferred embodiment the present invention relates to the use of antibody proteins according to the invention conjugated to an imageable agent for imaging activated stromal fibroblasts. In a further preferred embodiment the present invention relates to the use of labelled antibody proteins according to the invention for detecting the presence of activated stromal fibroblasts in a sample.

One aspect of the invention relates to a method of treating tumors, wherein the tumor is associated with activated stromal fibroblasts capable of specifically forming a complex with antibody proteins according to the invention, present as naked/unmodified antibodies, modified antibody proteins, such as e.g. fusion proteins, or antibody proteins conjugated to a therapeutic agent, which comprises contacting the tumor with an effective amount of said antibodies. In a preferred embodiment the present invention relates to a method of treating tumors as mentioned above, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, invasive bladder cancers, pancreatic cancers and metastatic cancers of the brain. The method of treating tumors as described above may be effected *in vitro or in vivo*.

A further aspect of the invention relates to a method of detecting the presence of activated stromal fibroblasts in wound healing, inflammation or in tumors, characterised in that

(a) a sample, possibly containing activated stromal fibroblasts, is contacted with an antibody protein according to the invention under conditions suitable for the formation of a complex between said antibody and antigen,

(b) detecting the presence of said complex, thereby detecting the presence of activated stromal fibroblasts in wound healing, inflammation or a tumor.

In a preferred embodiment, the present invention relates to a method of detecting the presence of activated stromal fibroblasts in a tumor, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain. Most preferred antibody proteins of the invention are those which are characterised in that they are labelled as mentioned above.

A further aspect of the invention relates to a method of imaging the presence of activated stromal fibroblasts in a healing wound, inflamed tissue (rheumatoid arthritis and cirrhosis are also positive) or a tumor, in a human patient, characterised in that

(a) an antibody protein according to the present invention conjugated to an imageable agent is administered to a human patient under conditions suitable for the formation of an antibody-antigen complex,

(b) imaging any complex formed in this manner,

(c) thereby imaging the presence of activated stromal fibroblasts in a human patient.

In a preferred embodiment the present invention relates to a method of imaging the presence of activated stromal fibroblasts as described above in tumors, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain.

In a further aspect the present invention relates to a method of detecting tumor-stroma, characterised in that

(a) a suitable sample is contacted with an antibody protein according to the present invention, under conditions suitable for the formation of an antibody-antigen complex,

(b) detecting the presence of any complex so formed,

(c) relating the presence of said complex to the presence of tumor-stroma.

Antibody proteins for practicing the invention are preferably labelled with a detectable marker.

In a further aspect the present invention relates to a method of imaging tumor-stroma in a human patient, which comprises

(a) administering to the patient an antibody according to the invention conjugated to an imageable agent as described above under conditions suitable for the formation of an antibody-antigen complex,

(b) imaging any complex so formed, and thereby imaging the presence of tumor-stroma in a human patient.

Figure legends

Fig. 1. *DNA sequence of F19 human reshaped light chain variable region version A (hF19L$_A$) SEQ ID NO:1.*

Fig. 2. *Amino acid sequence of F19 human reshaped light chain variable region version A (hF19L$_A$) SEQ ID NO: 2.*

Fig. 3. *DNA sequence of F19 human reshaped light chain variable region version B (hF19L$_B$) SEQ ID NO: 3.* Nucleotides differing from version A are underlined and in bold type.

Fig. 4. *Amino acid sequence of F19 human reshaped light chain variable region version B (hF19L$_B$) SEQ ID NO: 4.* Amino acids differing from version A are underlined and in bold type.

Fig. 5. *DNA sequence of F19 human reshaped light chain variable region version C ($hF19L_C$) SEQ ID NO:5.* Nucleotides differing from version A are underlined and in bold type.

Fig. 6. *Amino acid sequence of F19 human reshaped light chain variable region version C ($hF19L_C$) SEQ ID NO: 6.* Amino acids differing from version A are underlined and in bold type.

Fig. 7. *DNA sequence of F19 human reshaped variable region heavy chain version A ($hF19H_A$) SEQ ID NO: 7.*

Fig. 8. *Amino acid sequence of F19 human reshaped heavy chain variable region version A ($hF19H_A$) SEQ ID NO: 8*

Fig. 9. *DNA sequence of F19 human reshaped heavy chain variable region version B ($hF19H_B$) SEQ ID NO: 9.* Nucleotides differing from version A are underlined and in bold type.

Fig. 10. *Amino acid sequence of F19 human reshaped heavy chain variable region version B ($hF19H_B$) SEQ ID NO: 10.* Amino acids differing from version A are underlined and in bold type.

Fig. 11. *DNA sequence of F19 human reshaped heavy chain variable region version C ($hF19H_C$) SEQ ID NO: 11.* Nucleotides differing from version A are underlined and in bold type.

Fig. 12. *Amino acid sequence of F19 human reshaped heavy chain variable region version C ($hF19H_C$) SEQ ID NO: 12.* Amino acids differing from version A are underlined and in bold type.

Fig. 13. *DNA sequence of F19 human reshaped heavy chain variable region version D (hF19H$_D$) SEQ ID NO: 13.* Nucleotides differing from version A are underlined and in bold type.

Fig. 14. *Amino acid sequence of F19 human reshaped heavy chain variable region version D (hF19H$_D$) SEQ ID NO: 14.* Amino acids differing from version A are underlined and in bold type.

Fig. 15. *DNA sequence of F19 human reshaped heavy chain variable region version E (hF19H$_E$) SEQ ID NO: 15.* Nucleotides differing from version A are underlined and in bold type.

Fig. 16. *Amino acid sequence of F19 human reshaped heavy chain variable region version E (hF19H$_E$) SEQ ID NO: 16.* Amino acids differing from version A are underlined and in bold type

Fig. 17. *Amino acid sequence of F19 chimeric light chain variable region (chF19LC) SEQ ID NO: 17.*

Fig. 18. *Amino acid sequence of F19 chimeric heavy chain variable region (chF19HC) SEQ ID NO: 18.*

Fig. 19. *DNA sequence of human kappa light constant chain SEQ ID NO: 19.*

Fig. 20. *Amino acid sequence of human light constant chain SEQ ID NO: 20.*

Fig. 21. *DNA sequence of human heavy constant chain SEQ ID NO: 21.*

Fig. 22. *Amino acid sequence of human heavy constant chain SEQ ID NO: 22.*

Fig. 23. *Mammalian cell expression vectors used to produce chimeric and reshaped human antibodies with human kappa light chains and human gamma-1 heavy chains.*

A. Light chain expression vector: pKN100

B. Heavy chain expression vector: pG1D105

Fig 24. *DNA and amino acid sequences of mouse F19 light chain variable region as modified for use in the construction of chimeric F19 light chain.* Restriction sites are indicated by bold letters. The Kozak sequence, CDRs 1 to 3 and the splice donor site are underlined.

Fig 25. *DNA and amino acid sequences of mouse F19 heavy chain variable region as modified for use in the construction of chimeric F19 heavy chain.* Restriction sites are indicated by bold letters. The Kozak sequence and the splice donor site are underlined.

Fig. 26. *DNA sequence of F19 chimeric antibody cloned into pKN100 mammalian expression vector.* Restriction sites are indicated by bold letters and underlined. CDRs 1 to 3 and the splice donor site are underlined. This is the DNA sequence of the mouse F19 light chain inside the pKN100 eukaryotic expression vector. This vector has a cDNA version of the human kappa constant region gene (allotype Km(3)) terminated by a strong artificial termination sequence. In addition, the Neo selection gene is also terminated by this artificial sequence and is also in the same orientation as the kappa light chain expression cassette.

The essential components of the pKN100 eukaryotic expression vector are:

1 - 6 = EcoRI site

7 - 1571 = HCMVi promoter/enhancer

583 - 587 = TATAA box

610 = Start of transcription

728 - 736 = Splice donor site
731 = Beginning of intron
1557 = End of intron
1544 - 1558 = Splice acceptor site
1590 - 1598 = Kozak sequence
1599 - 1658 = peptide leader sequence
1659 - 1997 = mouse F19 light chain
1996 - 2004 = splice donor site
2011 - 2657 = cDNA copy of human Kappa constant region (Km(3)) gene
2664 - 2880 = Artificial spaC2 termination sequence
2887 - 7845 = This is the pSV2neo vector DNA fragment comprising of the Amp-resistance gene (in the opposite orientation), the ColEI and SV40 origins of replication and the Neo-resistance gene (in the same orientation as the HCMVi-KCT cassette)
7852 - 8068 = Artificial spaC2 termination signal

This sequence ends immediately upstream of the EcoRI site (position 1-6) at the beginning of the sequence. As a vector this DNA sequence would be circular.

Fig. 27. *DNA sequence of F19 chimeric antibody cloned into pg1d105 mammalian expression vector.* Restriction sites are indicated by bold letters and underlined. CDRs 1 to 3 and the splice donor site are underlined. This is the DNA sequence of the eukaryotic expression vector pG1D105 containing the mouse F19 heavy chain variable region. This vector contains a cDNA version of the human gamma-1 constant region (allotype G1m (non-a, z, -x) also known as Gm1(17) allotype).
The essential components of the construct are:

1 - 2501 = pBR322 based sequence including Ampicillin resistance

gene and ColEI origin plus the SV40 origin and the crippled SV40 early promoter

2502 - 3226 = dhfr gene

3233 - 4073 = SV40 poly A sequence etc.

4074 - 4079 = ligated BamHI and BglII site (BstYI)

4080 - 4302 = SPA site plus C2 termination signal

4303 - 5867 = HCMVi promoter

5879 - 5885 = unique HindIII restriction site for cloning of immunoglobulin variable genes

5886 - 5894 = Kozak sequence

5895 - 5951 = signal peptide

5952 - 6323 = mouse F19 heavy chain

6323 - 6330 = splice donor site

6331 - 6336 = unique BamHI restriction site for cloning of immunoglobulin variable genes

6337 - 7388 = cDNA copy of human gamma-1 constant regions preceded by a 62 bp intron

7389 - 7709 = Arnie termination sequence

The human gamma-1 constant region used in this construct has a G1m (non-a, z, -x) also known as Gm1(17) allotype which is defined by a glutamic acid (E) residue at position 356 (according to Eu numbering), a methionine (M) residue at position 358 (according to Eu numbering) and a lysine (K) residue at position 214 (according to Eu numbering). These three residues are underlined in the sequence above.

Fig. 28. *PCR-based method for the construction of human reshaped F19 light chain.* This figure provides a schematic overview of the strategy of construction. The dotted lines indicate a complementary sequence of at least 21 bases between the primers.

Fig. 29. *Nucleotide and deduced amino acid sequences of reshaped human F19 light chain variable regions version A, B and C.* Nucleotide and deduced amino acid sequences are aligned and compared with that of version A, dashes indicate nucleotide identity, dots indicate amino acid identity with this sequence. Amino acids are numbered according to Kabat *et al.* (1991). The locations of CDRs are indicated in boxes.

Fig. 30. *DNA sequence of F19 L_A (human reshaped light chain version A) cloned into pKN100 mammalian expression vector.* Restriction sites are indicated by bold letters and underlined. CDRs 1 to 3 and the splice donor site are underlined. This is the DNA sequence of the reshaped F19 light chain version A cloned into pKN100 eukaryotic expression vector. This vector has a cDNA version of the human kappa constant region gene (allotype Km(3)) terminated by a strong artificial termination sequence. In addition, the Neo selection gene is also terminated by this artificial sequence and is also in the same orientation as the kappa light chain expression cassette.

The components of the vector are:

7 - 1571 = HCMVi promoter/enhancer
583 - 587 = TATAA box.
610 = Start of transcription.
728 - 736 = Splice donor site.
731 = Beginning of intron.
1557 = End of intron.
1544 - 1558 = Splice acceptor site.
1590 - 1598 = Kozak sequence
1599 - 1658 = peptide leader sequence
1659 - 1997 = reshaped F19 light chain version A
1996 - 2004 = splice donor site
2011 - 2657 = cDNA copy of human kappa constant region (Km(3)) gene.
2664 - 2880 = Artificial spaC2 termination sequence.

2887 - 7845 = This is the pSV2neo vector DNA fragment comprising of the Amp-resistance gene (in the opposite orientation), the ColEI and SV40 origins of replication and the Neo-resistance gene (in the same orientation as the HCMVi-KCT cassette).

7852 - 8068 = Artificial spaC2 termination signal.

This sequence ends immediately upstream of the EcoRI site (position 1-6) at the beginning of the sequence below. As a vector this DNA sequence would be circular.

Fig. 31. *PCR-based method for the construction of human reshaped F19 heavy chain.* This figure provides a schematic overview of the strategy of construction. The dotted lines indicate a complementary sequence of at least 21 bases between the primers.

Fig. 32. *Nucleotide and deduced amino acid sequences of reshaped human F19 heavy chain variable region versions a to e.* Nucleotide and deduced amino acid sequences are aligned and compared with that of version A, dashes indicate nucleotide identity, dots indicate amino acid identity with this sequence. Amino acids are numbered according to Kabat *et al.* (1991). The location of CDRs is indicated by boxes.

Fig. 33. *DNA sequence of F19Ha (human reshaped heavy chain version a) cloned into pg1d105 mammalian expression vector.* Restriction sites are indicated by bold letters and underlined. CDRs 1 to 3 and the splice donor site are underlined. This is the DNA sequence of the eukaryotic expression vector pG1D105 containing the reshaped version A of F19 heavy chain variable region. This vector contains a cDNA version of the human gamma-1 constant region (allotype G1m (non-a, z, -x) also known as Gm1(17) allotype).

The essential components of the construct are:

1 - 2501 = pBR322 based sequence including Ampicillin resistance gene and ColEI origin plus the SV40 origin and the crippled SV40 early promoter
2502 - 3226 = dhfr gene
3233 - 4073 = SV40 poly A sequence etc.
4080 - 4302 = SPA site plus C2 termination signal
4303 - 5867 = HCMVi promoter/enhancer
5879 - 5885 = unique HindIII restriction site for cloning of immunoglobulin variable genes
5886 - 5894 = Kozak sequence
5895 - 5951 = signal peptide
5952 - 6323 = reshaped F19 heavy chain version A
6323 - 6330 = splice donor site
6331 - 6336 = unique BamHI restriction site for cloning of immunoglobulin variable genes
6337 - 7388 = cDNA copy of human gamma-1 constant regions preceded by a 62 bp intron
7389 - 7709 = Arnie termination sequence

The human gamma-1 constant region used in this construct has a G1m (non-a, z, -x) also known as Gm1(17) allotype which is defined by a glutamic acid (E) residue at position 356 (according to Eu numbering), a methionine (M) residue at position 358 (according to Eu numbering) and a lysine (K) residue at position 214 (according to Eu numbering). These three residues are underlined in the sequence above.

Fig. 34. *Heavy (panel A) and light (panel B) chains RNA splicing events taking place during antibody F19 expression in mammalian cells* – schematic overview.

A. Heavy chain RNA splicing

B. Kappa light chain RNA splicing

Fig. 35. *Concentration dependence of L_AH_C supernatant binding to CD8-FAP.*

Fig. 36. *Binding of biotinylated L_AH_C to human FAP.*

Fig. 37. *CD8-FAP carries the F19 epitope as detected with cF19.*

Examples

Example 1: Construction of mouse - human chimeric genes

The chimeric F19 (cF19) antibody was designed to have the mouse F19 V_L and V_H regions linked to human kappa and gamma-1 constant regions, respectively. PCR primers were used to modify the 5'- and 3'- sequences flanking the cDNA sequences coding for the mouse F19 V_L and V_H regions (Table 1). PCR primers specific for F19 light chain V-region were designed. These adapted mouse F19 variable regions were then subcloned into mammalian cell expression vectors already containing the human kappa (pKN100 vector) or gamma-1 (pG1D105 vector) constant regions (Figure 23). These vectors employ the human cytomegalovirus (HCMV) promoter/enhancer to efficiently transcribe the light and heavy chains. The vectors also contain the SV40 origin of replication to permit efficient DNA replication and subsequent protein expression in *COS* cells. The expression vectors were designed to have the variable regions inserted as HindIII-BamHI DNA fragments. PCR primers were designed to introduce these restrictions sites at the 5'- (HindIII) and 3'- (BamHI) ends of the cDNAs coding for the V-regions. In addition the PCR primers were designed to introduce the Kozak sequence (GCCGCCACC) at the 5'-ends of both the light and heavy chain cDNAs to allow efficient

translation (Kozak M.: At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. (1987) *J. Mol. Biol.* **196**: 947), and to introduce splice donor sites at the 3'-ends of both the light and heavy chain cDNAs for the variable regions to be spliced to the constant regions. The PCR primers used in the construction of the chimeric F19 light and heavy chains are shown in Table 1. The DNA and amino acid sequences of the mouse F19 V_L and V_H regions as adapted for use in the construction of chimeric F19 light and heavy chains are shown in Figures 24 and 25. The DNA sequences of mouse F19 light and heavy chains cloned into the eukaryotic expression vectors pKN100 and pG1D105, respectively, are shown in Figures 26 and 27.

TABLE 1: PCR primers for the construction of chimeric F19 antibody.

A. Light chain variable region

1. Primer for the construction of the 5'-end (37mer)

5' CAGA **AAGCTT** GCCGCCACC ATG GAT TCA CAG GCC CAG 3'

HindIII Kozak sequence M D S Q A Q

2. Primer for the construction of the 3'-end (35mer)

5' CCGA **GGATCC** ACTCACG TTT CAG CTC CAG CTT GGT 3'

BamHI Splice donor site

B. Heavy chain variable region

1. Primer for the construction of the 5'-end (37mer)

5' CAGA **AAGCTT** GCCGCCACC ATG GGA TGG AGC TGG GTC 3'

HindIII Kozak sequence M G W S W V

2. Primer for the construction of the 3'-end (35mer)

5' CCGA **GGATCC** ACTCACC TGA GGA GAC GGT GAC TGA 3'

BamHI Splice donor site

Example 2: **Expression and binding activity of chimeric F19 antibody**

The two plasmid DNAs coding for the chimeric F19 light and heavy chains (see example 1) were co-transfected into *COS* cells to look for transient expression of chimeric F19 antibody as described below. After incubation for 72 hours, the medium was collected, centrifuged to remove cellular debris, and analysed by ELISA for the production of a human IgG1-like antibody. The *COS* cell supernatant containing the chimeric F19 antibody was analysed for its ability to bind to HT 1080 cells (see example 13) expressing the FAP antigen on their surface.

Transfection of *COS* cells using electroporation

The mammalian expression vectors pg1d105 and pKN100 containing the chimeric or reshaped human heavy and light chains versions, respectively, were tested in *COS* cells to look for transient expression of F19 antibodies. COS7 cells were passaged routinely in DMEM (Gibco BRL cat. #41966) containing penicillin (50 IU/ml), streptomycin (50 µg/ml), L-glutamine and 10% heat-inactivated gamma globulin-free foetal calf serum (FCS, Harlan Sera-Lab cat. # D0001). The DNA was introduced into the *COS* cells by electroporation using the Gene Pulsar apparatus (BioRad). DNA (10µg of each vector) was added to a 0.8 ml aliquot of $1x10^7$ cells/ml in Phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free). A pulse was delivered at 1,900 volts, 25 µF capacitance. After a 10 min recovery period at ambient temperature the electroporated cells were added to 8 ml of DMEM containing 5% FCS. After incubation at 37°C for 72 hours, the medium was collected, centrifuged to remove cellular debris, and stored under sterile conditions at 4°C for short periods of time, or at -20°C for longer periods.

ELISA method for measuring assembled IgG1/kappa antibody concentrations in *COS* cell supernatants

Samples of antibodies produced in transfected *COS* cells were assayed by ELISA to determine how much chimeric or reshaped human antibody had been produced. For the detection of antibody, plates were coated with goat anti-human IgG (Fcγ fragment specific) antibody (Jackson ImmunoResearch Laboratories Inc., #109-005-098). The samples from *COS* cells were serially diluted and added to each well. After incubation for 1 h at 37°C and washing, horseradish peroxidase conjugated goat anti-human kappa light chain (Sigma, A-7164) was added. After incubation for 30 minutes at 37°C and washing, K-blue substrate (a mixture of 3,3',5,5' tetramethylbenzidine and hydrogen peroxide, Bionostics Limited, #KB175) was added for 30 minutes at room temperature. The reaction was stopped using Red Stop solution (Bionostics Limited, #RS20) and the optical density read on a microplate reader at 650 nm. Purified human IgG1/Kappa antibody (Sigma, I-3889) of known concentration was used as a standard.

The expression of chimeric F19 antibody in *COS* cells was poor (Table 2), between 10 and 60 ng/ml, which is at least 10 fold less than most antibodies.

In an attempt to increase expression levels of the chimeric F19 antibody, the leader sequence of F19 V_L region was changed by substitution of leucine to proline at position –9. This single change in amino acid in the leader sequence resulted in at least doubling the amount of chimeric antibody produced in COS cells.

Cell binding results show that chimeric F19 binds specifically and with the expected avidity to the FAP target.

TABLE 2: Chimeric F19 antibody concentrations in *COS* cell supernatants(These are the results of three independent transfections)

Transfected Antibody components		Human γ1/K
Heavy chain	Kappa light chain	[in µg/ml]
cF19	cF19 (F19 leader sequence)	0.060
cF19	cF19 (mutated leader sequence)	0.212
cF19	cF19 (F19 leader sequence)	0.056
cF19	cF19 (mutated leader sequence)	0.108
cF19	cF19 (F19 leader sequence)	0.011
cF19	cF19 (mutated leader sequence)	0.087

Example 3: **Construction of the reshaped human F19 light chain versions A to C (L_A - L_B)**

The construction of the first version of reshaped human F19 V_L region (L_A) was carried out using overlapping PCR fragments in a method similar to that described by Daugherty B. L., DeMartino J. A., Law M. F., Kawka D. W., Singer I. I. and Mark G. E. (1991) Polymerase chain reaction (PCR) facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins. *Nucl. Acids Res.* **19**: 2471. Ten oligonucleotides were synthesised that consisted of five primer pairs, APCR1-vla1, vla2-vla3, vla4-vla5, vla6-vla7, and vla8-APCR4 (Table 3 and Figure 28). There was an overlapping sequence of at least 21 bases between adjacent pairs (Figure 28). APCR1 and APCR4 hybridised to the flanking pUC19 vector sequences. The mutagenic primers were designed such that their 5' end immediately followed the wobble position of a codon. This strategy was used to counteract the gratuitous addition of one nucleotide to the 3' end of the strand complementary to the mutagenic primer by the DNA polymerase during PCR (Sharrocks A. D. and Shaw P. E. (1992) Improved primer design for PCR-based, site-directed mutagenesis. *Nucl. Acids Res.* **20**: 1147). The appropriate primer pairs (0.2 µM of each) were combined with 10ng of version "B" of reshaped human L25V_L region cDNA, and 1 unit of AmpliTaq (Perkin Elmer Cetus) DNA polymerase in 50µl of PCR buffer containing 10

mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM dNTPs, and 1.5 mM $MgCl_2$. This was overlaid with mineral oil and PCR was performed for 25 cycles, each cycle consisting of a denaturation step at 94°C for 1 min, a primer annealing step at 55°C for 1 min, and an extension step at 72°C for 2 minutes. This was followed by a single cycle consisting of a further elongation step at 72°C for 10 minutes followed by cooling to 4°C. The ramp time between the primer-annealing and extension steps was 2.5 minutes. The PCR products of the five reactions (A, B, C, D and E) were then purified by gel electrophoresis followed by DNA elution using Wizard PCR preps (Promega). PCR products A, B, C, D, and E were assembled by their complementarity to one another. In the second set of PCR reactions, PCR products B and C, and D and E, (50 ng of each) were added to 50 µl PCR reactions (as described above) each containing 1 unit of AmpliTaq (Perkin Elmer Cetus) DNA polymerase. The reactions were cycled for 20 cycles as described above with the exception that the annealing temperature was raised to 60°C. In the third set of PCR reactions, PCR products F and G were PCR-amplified using 1 µl of each prior PCR reaction and the appropriate pair of PCR primers (vla2-vla5 or vla6-APCR4). The PCR reactions contained 1 unit of AmpliTaq DNA polymerase in 50 µl PCR reaction (as described above) and were amplified for 25 cycles as in the first stage. In the fourth set of PCR reactions, the PCR product H was PCR-amplified using 1 µl of each prior PCR reaction and the vla2-APCR4 pair of PCR primers. Finally, PCR products A and H were assembled by their own complementarity in a two step-PCR reaction similar to that described above using RSP and UP as the terminal primers. The fully assembled fragment representing the entire reshaped human F19 V_L region including a leader sequence was digested with HindIII and BamHI and cloned into pUC19 for sequencing. A clone having the correct DNA sequence was designated reshF19La (Figure 29) and was then subcloned into the eukaryotic expression vector pKN100. The DNA sequence of reshF19La cloned into pKN100 is shown in Figure 30.

The second version of reshaped human F19 V_L region (L_B) was constructed using the same scheme as that described for La but where vla4 and vla7 primers were substituted by vlb4 and vlb7 respectively (Table 3). The DNA sequence of L_B is shown in Figure 29.

The third version of reshaped human F19 V_L region (L_C) was constructed using the QuikChange™ site-directed mutagenesis kit from Stratagene. The QuikChange site-directed mutagenesis method was performed according to the manufacturer's instructions, using reshF19La in pKN100 vector as double stranded DNA template. The mutagenic oligonucleotide primers F19Lc-sense and F19Lc-antisense (Table 3) for use in this protocol were designed according to the manufacturer's instructions. Briefly, both the mutagenic primers contained the desired point mutation (codon TTT at Kabat residue position 49 (Phe) changed to TAT coding for Tyr) and annealed to the same sequence on opposite strands of L_A in pKN100 vector. The point mutation was verified by DNA sequencing the entire V_L region. The DNA sequence of L_C is shown in Figure 29. To eliminate the possibility that random mutations occurred in the pKN100 during the PCR reaction, the V_L region was cut out of the pKN100 vector as an HindIII/BamHI fragment and re-subcloned into an unmodified pKN100 vector cut with the same two restriction enzymes beforehand.

TABLE 3: PCR primers for the construction of reshaped human F19 light chain variable regions

1. Primers for the synthesis of version "A"

F19vla1 (36 mer):
5' GTCATCACAATGTCTCCGGAGGAACCTGGAACCCAG 3'

F19vla2 (29 mer):
5' CTCCGGAGACATTGTGATGACCCAATCTC 3'

F19vla3 (45 mer):
5' GAATATAAAAGGCTCTGACTGGACTTGCAGTTGATGGTGGCCCTC 3'

F19vla4 (72 mer):

5' CAGTCAGAGCCTTTTATATTCTAGAAATCAAAAGAACTACTTGGCCTGGTAT CAGCAGAAACCAGGACAGCC 3'

F19vla5 (44 mer):

5' ACCCCAGATTCCCTAGTGCTAGCCCAAAAGATGAGGAGTTTGGG 3'

F19vla6 (67 mer):

5' TAGCACTAGGGAATCTGGGGTACCTGATAGGTTCAGTGGCAGTGGGTTTG GGACAGACTTCACCCTC 3'

F19vla7 (53 mer):

5' GTCCCTTGTCCGAACGTGAGCGGATAGCTAAAATATTGCTGACAGTAA TAAAC 3'

F19vla8 (33 mer):

5' GCTCACGTTCGGACAAGGGACCAAGGTGGAAAT 3'

2. Primers for the synthesis of version "B"

F19vlb4 (72 mer):

5' CAGTCAGAGCCTTTTATATTCTAGAAATCAAAAGAACTACTTGGCCTGG TTCCAGCAGAAACCAGGACAGCC 3'

F19vlb7 (57 mer):

5' GTCCCTTGTCCGAACGTGAGCGGATAGCTAAAATATTGCTGACAGTCATA AACTGCC 3'

3. Primers for the synthesis of version "C"

F19Lc-sense (34 mer):

5' CCCAAACTCCTCATCTATTGGGCTAGCACTAGGG 3'

F19Lc-antisense (34 mer):
5' CCCTAGTGCTAGCCCAATAGATGAGGAGTTTGGG 3'

4. Primers hybridizing to the flanking PUC19 vector sequences

APCR1 (17 mer, sense primer):	5' TACGCAAACCGCCTCTC 3'
APCR4 (18 mer, anti-sense primer):	5' GAGTGCACCATATGCGGT 3'
RSP (-24) (16 mer, sense primer):	5' AACAGCTATGACCATG 3'
UP (-40) (17 mer, anti-sense primer):	5' GTTTTCCCAGTCACGAC 3'

Example 4: **Construction of the reshaped human F19 heavy chain versions A to E (H_A - H_E)**

Version "A" of reshaped human F19 V_H regions (H_A) was constructed using the same PCR methods as described for the construction of version "A" of reshaped human F19 V_L region (L_A) (Figure 31). The template DNA was version "A" of reshaped human 226 V_H (Léger O. J. P., Yednock T. A., Tanner L., Horner H. C., Hines D. K., Keen S., Saldanha J., Jones T., Fritz L. C. and Bendig M. M. (1997). Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis. *Hum. Antibod.* **8**: 3). Six PCR primers were designed and synthesized for the construction of version "A" of reshaped human F19 V_H region (Table 4). PCR products A, B, C, and D were obtained using APCR1-Vha1, Vha2-Vha3, Vha4-Vha5 and Vha6-APCR4 as PCR primer pairs, respectively. The PCR conditions were essentially as described for the construction of reshaped human F19 V_L region. A clone having the correct DNA sequence was designated reshF19Ha (Figure 32) and was then subcloned into the eukaryotic expression vector pG1D105. The DNA sequence of reshF19Ha cloned into pG1D105 is shown in Figure 33.

The third version of reshaped human F19 V_H region (H_C) was constructed using the same scheme as that described for H_A but where Vha4 primer was substituted by Vhc4 (Table 4). The DNA sequence of H_C is shown in Figure 32. The second (H_B) and fourth (H_D) version of reshaped human F19 V_H region were constructed based on the PCR-mutagenesis methods of Kamman et al. (Kamman M., Laufs J., Schell J. and Gronenborn B. (1989) Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). *Nucl. Acids Res.* **17**: 5404). For H_B and H_D, a mutagenic primer F19VHbd6 (Tyr-91 to Phe-91, Table 4) was used paired with APCR4 in PCR reactions with H_A and H_C as the template DNA, respectively. The PCR products VHb and VHd were restriction enzyme digested with PstI and BamHI and subcloned into reshF19Ha and reshF19Hc, respectively, previously digested with the same two restriction enzymes. The DNA sequences of H_B and H_D are shown in Figure 32.

Version "E" of reshaped human F19 V_H region (H_E) was constructed based on the PCR-mutagenesis methods of Kamman *et al.* (1989) already mentioned above:

For reshF19He mutagenic primer F19MsclHe (Table 5) was used paired with primer F19V_HHindIII (Table 5) in PCR reactions with H_C cloned in pg1d105 mammalian expression vector as the template DNA. The appropriate primer pairs (0.2 μM of each) were combined with 10 ng of cDNA of version "A" of reshaped human 226 V_H region in 100 μl of PCR buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8) 2 mM $MgSO_4$, 0.1% Triton X-100 and 200 μM dNTPs. Reaction mixtures were overlaid with mineral oil and kept at 94°C for 5 minutes. Then 1 unit of Deep Vent DNA polymerase (New England Biolabs) was added ("Hot Start" PCR; Chou Q., Russell M., Birch D., Raymond J. and Bloch W. (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucl. Acids Res.* **20:** 1717) and PCR was performed for 25 cycles on a TRIO-Thermoblock Thermal Cycler (Biometra, Göttingen, Germany). Each cycle consisting of a denaturation step at 94°C for 1 min, a primer-annealing step at 70°C for 1 min, and an extension step at 72°C for 2 minutes. This was followed by a single cycle consisting of a further elongation step at

72°C for 10 minutes followed by cooling at 4°C. The PCR products were then extracted and purified from a TAE 1.4% standard agarose gel using a QIAquick™ gel extraction kit, following the protocol supplied by the manufacturer (QIAGEN Ltd., UK). The PCR product V_He was then restriction enzyme digested with MscI and HindIII and ligated into reshF19Hc cloned in pg1d105 previously digested with the same two restriction enzymes. The MscI restriction recognition site is unique to all the reshaped human F19 V_H region versions and is not present in the pg1d105 expression vector. The HindIII restriction recognition site is a unique site in pg1d105 for cloning of V_H immunoglobulin genes. Electroporation-competent XL-1 Blue E. coli cells were transformed with 1 µl of the ligated DNA and plated on agarose plates containing Ampicillin. Colonies were then screened for the presence and correct size of inserts by direct PCR on colonies (Güssow D. and Clackson T. (1989) Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucl. Acids Res.* **17:** 4000) with primers HCMi and Hucγ1 hybridising to the flanking pg1d105 vector sequences (Table 5). DNA from positive colonies was prepared using a Plasmid Midi kit, following the protocol supplied by the manufacturer (QIAGEN Ltd., UK). DNA sequencing was performed by the dideoxy chain termination method (Sanger F., Nicklen S. and Coulson A. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74:** 5463) directly from circular vector DNA using conventional heat denaturation (Andersen A., Pettersson A. and Kieldsen T. (1992) A fast and simple technique for sequencing plasmid DNA with sequenase using heat denaturation. *Biotechniques* **13:** 678) and Sequenase 2.0 (USB, Cleveland, OH). The DNA sequence of reshF19He is shown in Figure 32.

TABLE 4: PCR primers for the construction of reshaped human F19 heavy chain variable regions versions A to D.

1. Primers for the synthesis of version "A"

F19vha1 (47mer):
5' GTGTATTCAGTGAAGGTGTATCTACTAGTTTTACAGCTGACTTTCAC 3'

F19vha2 (53 mer):
5' TAGTAGATACACCTTCACTGAATACACCATACACTGGGTTAGACAGG CCCCTG 3'

F19vha3 (71 mer):
5' CCCTTGAACTTCTGGTTGTAGTTAGGAATACCATTGTTAGGATTAATACC TCCTATCCACTCCAGCCTTTG 3'

F19vha4 (71 mer):
5' TAACTACAACCAGAAGTTCAAGGGCCGGGCCACCTTGACCGTAGGCAA GTCTGCCAGCACCGCCTACATGG 3'

F19vha5 (63 mer):
5' GCATGGCCCTCGTCGTAACCATAGGCGATTCTTCTTCTGGCGCAGTAGT AGACTGCAGTGTCC 3'

F19vha6 (48 mer):
5' CTATGGTTACGACGAGGGCCATGCTATGGACTACTGGGGTCAAGGAAC 3'

2. Primers for the synthesis of version "c"

F19vhc4 (71 mer):
5' TAACTACAACCAGAAGTTCAAGGGCCGGGTCACCATCACCGTAGACA CCTCTGCCAGCACCGCCTACATGG 3'

3. Primers for the synthesis of version "B" and "D"

F19vhbd6 (27 mer):

5' GGACACTGCAGTCTACTTCTGCGCCAG 3'

4. Primers hybridizing to the flanking PUC19 vector sequences

APCR1 (17 mer, sense primer): 5' TACGCAAACCGCCTCTC 3'
APCR4 (18 mer, anti-sense primer): 5' GAGTGCACCATATGCGGT 3'

TABLE 5: PCR primer for the construction of reshaped human F19 heavy chain variable regions version E

1. Primer for the synthesis of version "E"

F19MscIHe (65 mer, anti-sense):

5' CCTT*TGGCCA*GGGGCCTGTCTAACCCAGTGTATGGTGTATTCAGTGAAGGTG
MscI
TATCCACTAGTTTCCACTAGTTT 3'

2. Primers hybridizing to the flanking pg1d105 mammalian expression vector sequences

HCMi (28 mer, sense): 5' GTCACCGTCCTTGACACGCGTCTCGGGA 3'
Hucγ1 (17 mer, anti-sense): 5' TTGGAGGAGGGTGCCAG 3'

Example 5: **Reshaped human F19 antibody concentrations in *COS* cells supernatants**

COS cells were transfected with one pair of a series of reshaped human F19 antibody constructs and the human antibody concentration was measured using the

IgG1/Kappa ELISA as described in example 2.

TABLE 6: Reshaped human F19 antibody concentrations in *COS* cell supernatants

Transfected Antibody components		Human γ1/K
Heavy chain	Kappa light chain	Concentration [µg/ml]
H_A	L_A	2.50
H_A	L_B	0.18
H_B	L_A	1.25
H_B	L_B	0.10
H_D	L_A	1.15
H_D	L_B	0.18
H_A	L_A	1.50
H_A	L_C	1.56
H_C	L_A	1.47
H_C	L_C	1.97
cF19	L_A	1.54
cF19	L_B	0.07
cF19	L_C	2.14

TABLE 7: Reshaped human F19 antibody concentrations in *COS* cell supernatants

Transfected Antibody components		Human γ1/K
Heavy chain	Kappa light chain	concentration [µg/ml]
H_A	L_A	2.00
H_A	L_C	2.50
H_C	L_A	2.90
H_C	L_C	3.00
H_E	L_A	2.80
H_E	L_C	3.50

RNA splicing events required for the expression of immunoglobulin genes in mammalian cells

Both mammalian expression vectors pKN100 and pg1d105 have an intron between the variable and the constant regions which is removed during the process of gene expression to give rise to an messenger RNA. The splicing event which consists of a DNA recombination between the heavy or light chain splice donor sites and the immunoglobulin splice acceptor site is described in Figure 34.

Example 6: **Flow cytometric analysis of the binding of cF19 and L_AH_C to FAP-expressing human cells**

The ability of L_AH_C to bind to both recombinant and endogenously expressed FAP on cell surface was tested.

The example was conducted to determine the binding of L_AH_C to cellular FAP. Both naturally FAP expressing MF-SH human tumour cells (Shirasuma, K, et al., Cancer 1985, 55:2521-32) and FAP-transfected human tumour cell lines were used as cellular targets. L_AH_C was studied in cytofluorometric assays evaluating direct binding to target cells as well as by the inhibitory effect on the binding of either murine F19 or chimeric cF19 anti-FAP antibodies.

Antibodies and cell lines used were F19 (murine monoclonal anti-human FAP antibody, IgG1 subclass), mIgG (murine immunoglobulin, IgG class), cF19 (chimeric monoclonal anti-human FAP antibody, IgG1 subclass), L_AH_C (reshaped monoclonal anti-human FAP antibody, IgG1 subclass), hIgG1 (human immunoglobulin, IgG1 subclass), MF-SH (human malignant fibrous histiocytoma cell line), HT-1080 (human fibrosarcoma cell line), HT-1080FAP clone 33 (HT-1080 cell line transfected with cDNA encoding human FAP). Antibodies were biotinylated as described in examples 8 and 12.

Direct binding of L_AH_C to FAP on the surface of human tumour cell lines

$5x10^5$ cells of the tumour cell line under investigation were incubated with the indicated concentration of test or control antibody in a total volume of 0.2 ml phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) for 30 min on ice. Subsequently, cells were washed twice with 2 ml of PBS, resuspended in 0.2 ml of PBS supplemented with 1% BSA, a 1:20 dilution of mouse anti-human IgG FITC-labelled [Dianova] as secondary reagent was added) and incubated for another 30 min on ice.

Alternatively, $5x10^5$ cells of the tumour cell line under investigation were incubated with the indicated concentration of biotin-labelled cF19 in a total volume of 0.2 ml PBS supplemented with 1% BSA for 30 min on ice. Subsequently, cells were washed twice with 2 ml of PBS, resuspended in 0.2 ml of PBS supplemented with 1% BSA, and incubated for another 30 min on ice with 1:40 dilution of streptavidin-FITC (Dianova) as secondary reagent.

Cells were again washed twice with 2 ml of PBS, resuspended in a total volume of 0.5 ml of PBS supplemented with 1% paraformaldehyde (PFA) and kept on ice. Single cell fluorescence was determined cytofluorometrically by analysing the cellular green fluorescence at 488nm in an EPICS XL (Coulter) fluorescence-activated cell analyzer.

Competition of L_AH_C for binding of biotinylated cF19 to cell-surface FAP on FAP-expressing human cell lines

$5x10^5$ cells of the tumour cell line under investigation were incubated with the indicated amounts of unlabelled test or control antibody added together with 1 µg/ml biotin-labelled cF19 antibody. Subsequently, cells were washed twice with 2 ml of PBS, resuspended in 0.2 ml of PBS supplemented with 1% BSA, 1:40 diluted streptavidin-FITC (Dianova) as secondary reagent and incubated for another 30 min on ice.

Cells were then washed twice with 2 ml of PBS, resuspended in a total volume of 0.5 ml PBS supplemented with 1% PFA and kept on ice. Single cell fluorescence was determined cytofluorometrically by analysing the cellular green fluorescence at 488nm in an EPICS XL (Coulter) fluorescence-activated cell analyzer.

Both, cF19 and L_AH_C bind in a concentration dependent manner specifically to FAP-transfected HT-1080FAP clone33 human tumour cells (Table 8). No binding to FAP-negative HT-1080 cells was detectable (Table 9). Both cF19 and L_AH_C bound in a concentration dependent manner to human MF-SH cells endogenously expressing FAP (Table 10).

Biotinylated cF19 bound to human HT-1080FAP clone 33 (Table 11) in a concentration dependent manner. No binding was detectable to FAP-negative HT-1080 cells (Table 12).

Binding of biotinylated cF19 to HT-1080FAP clone 33 cells was inhibited by both unlabelled cF19 and unlabelled L_AH_C (Table 13).

Chimeric anti-human FAP monoclonal antibody cF19 as well as reshaped human anti-human FAP monoclonal antibody L_AH_C (example 10) were shown to bind directly to FAP expressed on human cell lines either endogenously expressing this protein or transfected with cDNA encoding for it. This binding was shown to be concentration dependent. Binding of biotinylated cF19 could be inhibited by both unlabelled cF19 and unlabelled L_AH_C.

Using cytofluorometric technology, direct binding as well as inhibition of specifically binding reagents showed specificity of chimeric cF19 and reshaped L_AH_C human monoclonal antibodies to cell surface expressed FAP.

Table 8: Binding of anti-FAP antibodies to HT-1080FAP clone 33 cells

Concentration of antibody	Mean fluorescence intensity		
[ng/ml]	hIgG1	cF19	L_AH_C
500.0	0.12	6.65	2.76
100.0	0.12	1.63	0.66
20.0	0.12	0.43	0.22
4.0	0.12	0.17	0.15
0.8	0.12	0.14	0.13

Table 9: Binding of anti-FAP antibodies to non-transfected HT-1080 cells

Concentration of antibody	Mean fluorescence intensity		
[ng/ml]	hIgG1	cF19	L_AH_C
500.0	0.11	0.11	0.12
100.0	0.11	0.11	0.11
20.0	0.11	0.11	0.12
4.0	0.11	0.11	0.12
0.8	0.11	0.11	0.11

Table 10: Binding of anti-FAP antibodies to MF-SH cells

Concentration of antibody	Mean fluorescence intensity		
[ng/ml]	hIgG1	cF19	L_AH_C
4000	0.6	3.6	2.8
2000	n.d.	3.3	2.5
1000	n.d.	2.4	1.9
500	n.d.	1.8	1.3

n.d.: not done

Table 11: Binding of biotinylated cF19 antibody to HT-1080FAP clone 33 cells

Concentration of antibody	Mean fluorescence intensity	
[ng/ml]	Biotinylated hIgG1	Biotinylated cF19
5,000.0	0.2	36.5
1,000.0	0.2	18.1
200.0	0.2	4.5
40.0	0.2	1.3
8.0	0.2	0.5
1.6	0.3	0.3

Table 12: Binding of biotinylated cF19 antibody to non-transfected HT-1080 cells

Concentration of antibody	Mean fluorescence intensity	
[ng/ml]	Biotinylated hIgG1	Biotinylated cF19
5,000.0	0.1	0.1
1,000.0	0.1	0.1
200.0	0.1	0.1
40.0	0.1	0.1
8.0	0.1	0.1
1.6	0.1	0.1

Table 13: Competition of anti-FAP antibodies with the binding of biotinylated cF19 to HT-1080FAP clone 33 cells

	Concentration of competitor antibody	Mean fluorescence concentration
Competitor antibody	[µg/ml]	
No	0.00	11.2
hIgG1	1.00	9.0
hIgG1	3.16	11.3
hIgG1	10.00	9.8
hIgG1	31.66	10.3
cF19	1.00	7.5
cF19	3.16	4.8
cF19	10.00	1.3
cF19	31.66	1.2
L_AH_C	1.00	8.0
L_AH_C	3.16	5.5
L_AH_C	10.00	2.9
L_AH_C	31.66	1.7

Biotinylated cF19 was used at a concentration of 1 µg/ml in all tests shown in the Table 13.

Example 7: **In vitro immune effector functions of monoclonal antibody L_AH_C**

This experiment was conducted to determine the potential of the monoclonal antibody (mAb) L_AH_C with specificity for fibroblast activation antigen (FAP) to lyse FAP-expressing targets in the presence of human complement or human mononuclear leukocytes, respectively.

In particular, the ability of L_AH_C to mediate cytotoxic effects against HT-1080FAP clone 33 cells, which expressed human FAP on the surface, was studied. Cytotoxicity was determined in vitro using the following approach: ^{51}Cr -labelled target cells were incubated in the presence of L_AH_C with human serum as source of complement or human MNC (peripheral blood mononuclear cells) as effectors. Release of ^{51}Cr was measured as measure of target-cell lysis.

Antibodies and cell lines used were L_AH_C (reshaped human anti-human FAP IgG1 antibody), hIgG1 (human IgG1 isotype control), 3S193 (murine monoclonal anti-Lewis^y IgG3 antibody), mIgG (murine IgG control), HT-1080 (human fibrosarcoma), HT-1080FAP clone 33, (HT1080 transfected with cDNA encoding human FAP), MCF-7 (human breast adenocarcinoma cell line).

Complement-mediated lysis of target cells by L_AH_C

Tumour cells were radiolabelled by incubation in RPMI1640 medium with 100 µCi ^{51}Cr (NEN) at 37° C for one hour. Subsequently, cells were washed twice in ^{51}Cr -free medium and resuspended at a concentration of $2x10^5$ cells per ml.

Human serum as source of complement was freshly prepared from blood of different volunteers. Blood was taken by puncturing the arm vein, remained at room temperature for one hour to allow clotting to occur, and was kept at 4° C over night. Serum was separated by centrifugation and taken off from the sediment.

The antibody under study was diluted from the stock solution to the appropriate concentration in RPMI1640 cell culture medium.

$1x10^5$ radiolabelled tumor cells of the indicated cell line were incubated for 2 h at 37° C in an incubator (95% air and 5% CO_2) in the presence of different concentrations of test or control antibody and 25% (v/v) human serum as the source of human complement. Incubations were performed in U-shaped 96-well plates in a total volume of 200 µl RPMI1640 and done in triplicate. After the incubation period, plates were centrifuged, 100 µl supernatant was removed and radioactivity was counted in a gamma-counter. The total amount of incorporated radioactivity was determined by measuring 10^4 target cells. Spontaneous release was defined as activity released from the target cells in the absence of both antibody and complement during the described incubation period.

Specific lysis was calculated as follows:

$$\text{specific lysis (in \%)} = \frac{\text{[activity sample]} - \text{[activity spontaneous release]}}{\text{[maximum activity]} - \text{[activity spontaneous release]}} \times 100$$

Antibody-dependent cellular cytotoxicity (ADCC) of L_AH_C

Tumour cells were radiolabelled by incubation in RPMI1640 medium with 100 µCi ^{51}Cr at 37°C for one hour. Subsequently, cells were washed twice in ^{51}Cr -free medium and resuspended at a concentration of $2x10^5$ cells per ml.

MNC (peripheral blood mononuclear cells) were prepared from peripheral blood taken by puncturing the arm vein of healthy human volunteers. Clotting was prevented by the addition of 20% citrate buffer. MNC from 4 ml of this blood preparation were purified by centrifugation (30 min at 400 G and room temperature) on 3 ml of lymphocyte preparation medium (Boehringer Mannheim, Germany). MNC (peripheral blood mononuclear cells) were taken off from the gradient, washed three times and diluted with RPMI1640 to the appropriate concentration. Lymphocyte activated killer (LAK) cells were derived from MNC (peripheral blood mononuclear cells) by incubation for 5 days at 37° C in an 95% air and 5% CO_2 incubator at an initial density of $1.3x10^6$ cells per ml in the presence of 100 U recombinant human Interleukin-2 (IL-2). The antibody under study was diluted from the stock solution to the appropriate concentration in RPMI1640 cell culture medium.

$1x10^4$ radiolabelled tumour cells of the indicated cell line were incubated for 5 h at 37°C and 5% CO_2 in the presence of different concentrations of test or control antibody and MNC. MNC were added in amounts to reach the indicated effector:target cell ratio. Incubation was performed in U-shaped 96-well plates in a total volume of 200 µl RPMI1640 and done in duplicate.

After the incubation period, plates were centrifuged, 100 µl of the supernatant were taken off and radioactivity was determined in a gamma-counter. The total amount of incorporated radioactivity was determined by measuring 10^4 target cells. Spontaneous release was defined as activity released from the target cells in the absence of both antibody and effector cells during the described incubation period.

Specific lysis was calculated as follows:

$$\text{specific lysis (in \%)} = \frac{\text{[activity sample]} - \text{[activity spontaneous release]}}{\text{[maximum activity]} - \text{[activity spontaneous release]}} \times 100$$

Antibody-mediated complement lysis of tumor cells

No L_AH_C -specific complement-mediated lysis (above that seen with an isotype control) was observed in HT-1080FAP clone 33 cells treated with L_AH_C at concentrations up to 50 µg/ml (Table 14, Table 15a)

Lytic activity of human serum used as source of complement was shown by lysis of MCF-7 human breast carcinoma cells in the presence of 12.5 µg/ml 3S193, a murine monoclonal anti-Lewis^y antibody with known complement activating ability (Table 15b)

Antibody-mediated cellular lysis of tumor cells

In the presence of L_AH_C at concentrations up to 10 µg/ml, no ADCC (antibody-dependent cellular toxicity) mediated by human MNC (Table 16) or human LAK cells (lymphokine activated killer cell, Table 17) of L_AH_C on HT-1080FAP clone 33 as measured by lysis was detectable above that seen with an isotype control at an effector:target ratio of 50:1.

In appropriate in vitro assays with either human complement or with human MNC - as effector mechanisms, human anti-FAP monoclonal antibody L_AH_C revealed no - detectable cytotoxic effects above isotype controls on FAP-expressing tumor cell line HT-1080FAP clone 33.

Table 14: Specific complement lysis (in %) of HT-1080FAP clone 33 tumor cell targets mediated by L_AH_C

Source of human serum:	HT-1080 clone 33:	
Concentration of antibody	hIgG1 isotype control	L_AH_C
A 50 µg/ml	5	4
A 10 µg/ml	5	3
B 50 µg/ml	7	5
B 10 µg/ml	6	5
0 µg/ml	0	0

Incubation: 2 hours at 37°C, 25% serum from human volunteers A or B, respectively, as source of complement.

Table 15a: Specific complement lysis (in %) of HT-1080FAP clone 33 tumor cell targets mediated by human anti-FAP monoclonal antibody L_AH_C

Source of human serum:	HT1080clone 33:	
concentration of antibody	hIgG1	L_AH_C
A 10.00 µg/ml	2	1
A 2.50 µg/ml	2	2
A 0.60 µg/ml	1	1
A 0.15 µg/ml	1	2
A 0.00 µg/ml	2	2
B 10.00 µg/ml	2	2
B 2.50 µg/ml	2	2
B 0.60 µg/ml	2	2
B 0.15 µg/ml	2	2
B 0.00 µg/ml	2	2
C 10.00 µg/ml	2	2
C 2.50 µg/ml	1	1
C 0.60 µg/ml	1	1
C 0.15 µg/ml	2	1
C 0.00 µg/ml	3	3

Incubation: 2 hours at 37°C, 25% serum from human volunteers A, B or C, respectively, as source of complement.

Table 15b: Specific complement lysis (in %) of MCF-7 tumour cell targets mediated by murine anti-Lewis^y monoclonal antibody 3S193

Source of human serum:	MCF-7:	
concentration of antibody	mIgG	3S193
A 10.00 µg/ml	0	21
A 2.50 µg/ml	1	21
A 0.60 µg/ml	0	21
A 0.15 µg/ml	1	18
A 0.00 µg/ml	0	0
B 10.00 µg/ml	1	13
B 2.50 µg/ml	0	17
B 0.60 µg/ml	1	18
B 0.15 µg/ml	1	15
B 0.00 µg/ml	0	0
C 10.00 µg/ml	1	22
C 2.50 µg/ml	0	23
C 0.60 µg/ml	1	26
C 0.15 µg/ml	1	20
C 0.00 µg/ml	1	1

Incubation: 2 hours at 37° C, 25% serum from human volunteers A, B or C, as source of complement.

Table 16: ADCC (antibody-dependant cellular cytotoxicity) (specific lysis in %) of HT-1080FAP clone 33 target cells by human MNC (peripheral blood mononuclear cells) mediated by L_AH_C.

HT-1080FAP clone 33:

Concentration of antibody:	HT-1080FAP clone 33:	
[in µg/ml]	hIgG1	L_AH_C
10.000	2	2
2.500	2	2
0.625	2	2
0.156	3	3
0.000	3	3

Incubation: 5 hours at 37°C, 10^4 target cells and an effector:target cell ration of 50:1.

Table 17: **ADCC (antibody-dependent cellular cytotoxicity, specific lysis in %) of HT-1080FAP clone 33 target cells by LAK cells (lymphokine activated killer cells) mediated by L_AH_C.**

Concentration of antibody:	HT-1080FAP clone 33:	
[in µg/ml]	hIgG1	L_AH_C
10.000	12	14
2.500	14	17
0.625	14	21
0.156	15	21
0.000	14	14

Incubation: 5 hours at 37°C, 10^4 target cells and an effector:target cell ration of 50:1.

Example 8: **Immunohistochemical analysis of monoclonal antibody L_AH_C binding to normal and neoplastic human tissues**

This experiment was performed to determine the binding characteristics of the humanized mAb L_AH_C to normal and neoplastic human tissues.

The following antibodies were used: L_AH_C, cF19, and the negative control hIgG1 were directly biotinylated according to methods of the state of the art and used at concentrations of 2.5 to 0.25 mg/ml in 2% BSA/PBS (bovine serum albumin in phosphate-buffered saline). Murine mAb F19 was used as tissue culture supernatant of the F19 hybridoma, at dilutions of 1:5 to 1:10 in 2% BSA/PBS.

The following reagents were used for immunochemical assays: Streptavidin peroxidase complex (Vector Labs, Burlingame, CA, USA), Avidin-biotin peroxidase complex (Vector Labs.), Biotinylated horse anti-mouse (Vector Labs.), DAB (diaminobenzidine, Sigma Chemical Co. St. Louis, MO, USA), Harris' hematoxylin.

Fresh frozen tissue samples examined included the following: Normal colon, breast, lung, stomach, pancreas, skin, larynx, urinary bladder, smooth and skeletal

muscle. Among the tumors tested were carcinomas from breast, colon, lung, esophagus, uterus, ovary, pancreas, stomach, and head and neck.

An indirect immunoperoxidase method was carried out according to state of the art methods (Garin-Chesa P, Old LJ, Rettig WJ: Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci USA* (1990) **87**:7235-7239) on five micrometer thickness fresh frozen sections. DAB was used as a substrate for the final reaction product. The sections were counterstained with Harris' hematoxylin and examined for antigen expression.

L_AH_C expression in normal human tissues

The normal tissues tested were negative for L_AH_C expression, except for the normal pancreas in which a subset of positive endocrine cells in the islets of Langerhans (A cells) were identified with L_AH_C, cF19 and F19. (Table 18). No immunoreactivity was observed with the hIgG1 (human immunoglobulin IgG1 subclass) used as a negative control.

L_AH_C expression in tumors

In the tumor samples, L_AH_C, cF19 and F19 showed an indistinguishable pattern of expression in the tumor stromal fibroblasts. A strong and homogeneous expression was found in the majority of the cases examined, especially in the cancer samples derived from breast, colon, lung, pancreas and in the squamous cell carcinomas (SQCC) of the head and neck tested (Table 19). No immunoreactivity was observed with the hIgG1 used as negative control.

L_AH_C, cF19 and F19 showed immunoreactivity with the tumor stromal fibroblasts in the epithelial cancer samples tested. No L_AH_C or F19 immunoreactivity was seen with either the fibrocytes of the normal organ mesenchyme or the parenchymal cells of normal adult organs. Anti-FAP immunoreactivity was only observed in a subset of endocrine cells in the pancreatic islets, presumably glucagon-producing A cells, and in

four of nine uterine samples tested, representing subsets of stromal fibroblasts in these tissues.

Immunohistochemical analysis of L_AH_C in normal human tissues and FAP-expressing human carcinomas showed indistinguishable patterns of binding for L_AH_C, cF19 and murine mAb F19.

Table 18: Immunoreactivity of mAbs L_AH_C, cF19 and F19 with normal human tissues

Tissue type	No.	BIBH 1	cF19	F19
Breast	4			
-Epithelial cells ducts / acini		-	-	-
-Myoepithelial cells		-	-	-
-Connective tissue		-	-	-
-Blood vessels		-	-	-
Colon	6			
-Crypts of Lieberkühn		-	-	-
-Connective tissue		-	-	-
-Lymphoid tissue		-	-	-
-Smooth muscle		-	-	-
-Blood vessels		-	-	-
-Myenteric plexus		-	-	-
Lung	4			
Bronchus:				
-Bronchial epithelium		-	-	-
-Hyaline cartilage		-	-	-
-Connective tissue		-	-	-
-Mucous glands		-	-	-
Alveolus:		-	-	-
-Pneumocytes (type I/II)		-	-	-
-Alveolar phagocytes		-	-	-
-Alveolar capillaries		-	-	-

Stomach -Surface epithelium -Gastric glands: -Chief cells -Parietal (oxyntic) cells -Mucous cells -Neuroendocrine cells -Connective tissue -Blood vessels -Smooth muscle	3	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -
Esophagus -Surface epithelium -Connective tissue	1	- -	- -	- -
Small intestine -Epithelium of villi&crypts -Connective tissue -Smooth muscle -Blood vessels -Lymphoid tissue	1	- - - - -	- - - - -	- - - -
Urinary bladder -Urothelium -Connective tissue -Smooth muscle -Blood vessels	2	- - - -	- - - -	- - - -
Pancreas -Duct epithelium -Acinar epithelium -Islets of Langerhans: -B-cells -A-cells -D-cells -Connective tissue -Blood vessels -Nerves	3	- - - - +* - - - -	- - - - +* - - - -	- - - - +* - - - -
Larynx -Squamous epithelium -Mucous glands -Connective tissue -Hyaline cartilage -Blood vessels -Skeletal muscle	1	- - - - - -	- - - - - -	- - - - - -
Lymph node	1			

-Lymphoid cells		-	-	-
-Lymph sinuses		-	-	-
-Connective tissue		-	-	-
-Blood vessels		-	-	-
Spleen	1			
-Red & white pulp		-	-	-
-Sinuses		-	-	-
-Connective tissue		-	-	-
Liver	1			
-Hepatocytes		-	-	-
-Bile ducts		-	-	-
-Portal triad		-	-	-
Thyroid gland	2			
-Follicular epithelium		-	-	-
-Parafollicular cells		-	-	-
-Connective tissue		-	-	-
Prostate gland	1			
-Glandular epithelium		-	-	-
-Stroma		-	-	-
Testicle	1			
-Seminiferous tubules		-	-	-
-Stroma		-	-	-
Ovary	3			
-Follicles		-	-	-
-Stroma		-	-	-
Uterine cervix	1			
-Epithelium		-	-	-
-Stroma		-	-	-
Uterus	9			
-Endometrium:				
glands		-	-	-
stroma		+#	+#	+#
blood vessels		-	-	-
-Myometrium		-	-	-
Cerebral cortex	1			
-Neurons		-	-	-
-Neuroglial cells		-	-	-
-Blood vessels		-	-	-
Cerebellum	1			
-Molecular layer		-	-	-
-Granular cell layer		-	-	-
-Purkinje cells		-	-	-
-Blood vessels		-	-	-
Skin	3			
-Squamous epithelium		-	-	-
-Melanocytes		-	-	-

-Skin appendages		-	-	-
-Connective tissue		-	-	-
-Blood vessels		-	-	-

Acetone-fixed frozen sections were tested by the avidin-biotin complex immunoperoxidase procedure.
No, Number of tissue samples derived from different individuals tested.
* Identification of A-cells, based on morphology and location within the islets.
# Positive immunoreactivity in the stroma in 4 out of 9 samples tested. The positive samples represent early (x2) and intermediate (x2) phase proliferative endometrium.

Example 9: **Species specificity of L_AH_C binding in tissue sections**

This experiment was conducted to assess the reactivity of L_AH_C with tissues from mouse, rat, rabbit and cynomolgus monkeys by immunohistochemical methods.

Also used in these tests were cF19 and human IgG1 (hIgG1) as negative controls. The reagents used for immunohistochemistry were Streptavidin peroxidase complex (Vector Labs., Burlingame, CA, USA), DAB (Sigma Chemical Co., St. Louis, MO, USA) and Harris' hematoxylin.

The following fresh frozen tissue samples from mouse, rat, rabbit and cynomolgus were tested: Brain, liver, lung, kidney, stomach, pancreas, intestine, thymus, skin, muscle, heart, spleen, ovary, uterus and testes. As positive control, sections from normal human pancreas and a breast carcinoma sample were included in every assay.

<u>Immunohistochemistry</u>

An indirect immunoperoxidase method was carried out as described in the state of the art (Garin-Chesa P, Old LJ, Rettig WJ: Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci USA* (1990) **87**:7235-7239) on five micrometer thickness fresh frozen sections. The antibodies L_AH_C, cF19 and hIgG1 (at 1 µg/ml) were biotinylated according to the state of the art and were detected with streptavidin peroxidase complex. DAB was used as a

substrate for the final reaction product. The sections were counterstained with Harris' hematoxylin and examined for antigen expression.

The normal tissues tested did not react with either L_AH_C or cF19 in the experiments (Table 1).

The normal human pancreas used as positive control showed L_AH_C and cF19 binding in a subset of endocrine cells in the islets of Langerhans as previously described for F19. In addition, binding of L_AH_C and cF19 was seen in the tumor stromal fibroblasts in the breast carcinoma sample.

Immunohistochemical analysis of normal tissues from mouse, rat, rabbit and cynomolgus failed to detect any binding of either L_AH_C or cF19, in the experiments performed.

Table 20: Binding of L_AH_C to tissue sections of non-human species, as determined by immunohistochemistry.

Organ / Tissue type	Mouse	Rat	Rabbit	Cynomolgus
Brain –Cerebral cortex	-	-	-	
-Cerebellum	-	-	-	-
Liver –Hepatocytes	-	-	-	-
-Portal triad	-	-	-	-
Lung –Bronchi	-	-	-	-
-Alveoli	-	-	-	-
Kidney –Glomeruli	-	-	-	-
-Tubular epithelium	-	-	-	-
Stomach –Glandular epithelium				
-Smooth muscle	-	-	-	-
	-	-	-	-
Pancreas –Exocrine acini	-	-	-	-
-Endocrine islets	-	-	-	-
Intestine –Glandular epithelium	-	-	-	-
-Smooth muscle	-	-	-	-
Thymus –Lymphocytes	-	-	-	-
Skin –Keratinocytes	-	-	-	-
-Sweat glands	-	-	-	-
-Hair follicles	-	-	-	-
Skeletal muscle	-	-	-	-
Heart	-	-	-	-
Spleen –Lymphocytes	-	-	-	-
Ovary –Follicular epithelium	-	-	-	-
-Stroma	-	-	-	-
Uterus –Myometrium	-	-	-	-
-Cervix uteri	-	-	-	-
Testis –Tubular epithelium	nt	nt	nt	-
Connective tissue	-	-	-	-

nt, not tested

Example 10: **Construction of cell lines producing chimeric and reshaped anti-FAP monoclonal antibodies**

The objective of this experiment was to demonstrate stable cell lines according to the invention expressing L_AH_C, L_AH_A, L_BH_B, L_BH_D, and cF19 in CHO DG44 cells. Stable cell lines transfected with humanized or chimeric F19 antibodies were produced and their identity was confirmed by PCR amplification of heavy and light variable regions using genomic DNA derived from each transfectant as template.

CHO DG44 cells maintained under serum-free conditions in SFM-II medium. Lipofectin and SFM-II serum-free medium were obtained from Gibco/BRL. Geneticin and all restriction enzymes were obtained from Boehringer Mannheim. Pfu polymerase was obtained from Stratagene.

DNA for transfections was purified from E. coli cells using QiaFilter Maxi Cartridges (Qiagen) as directed by the manufacturer. All DNA preparations were examined by restriction enzyme digestion. Sequences of L_AH_C variable regions in their respective vectors were confirmed using an ABI PRISM 310 Sequencer (Perkin-Elmer).

Further information regarding the vectors and DNA sequences employed is available in the prior examples.

Transfection of CHO DG44 cells

Cells in logarithmic growth were plated into 6 well plates containing 1 ml fresh SFM-II medium. Plasmids encoding heavy and light chains of humanized or chimeric F19 versions were cotransfected into CHO DG44 cells using liposomal transfection. Liposomes were prepared using 6 µl Lipofectin reagent and 0.5 µg of each vector (one for the desired heavy chain and one for the light) as described for LipofectAMINE transfections except that SFM-II medium was used to dilute all reagents. Twenty-four hours later, cells were diluted 1:10 into SFM-II medium containing 300 µg/ml Geneticin.

After the initial phase of cell killing was over (10-14 days), the concentration of Geneticin was reduced to 200 mg/ml and methotrexate was added to a final concentration of 5 nM. Methotrexate concentrations were increased after 10-14 days to a final concentration of 20 nM.

PCR Amplification of transfectant DNA

10^7 CHO DG44 cells were centrifuged in an Eppendorf microcentrifuge briefly at full speed, washed once with PBS, and pelleted once again. Genomic DNA was prepared by ethanol precipitation after SDS lysis and Proteinase K treatment of the cell pellets.

A mixture containing one of the following primer pairs, dNTPs, buffer, and Pfu polymerase was used to amplify either the heavy or light chain variable region using genomic DNA as template. The resulting PCR products were digested with the appropriate restriction enzyme and analyzed by agarose gel electrophoresis to confirm their identity.

Light chain primer set:

5'- GAG ACA TTG TGA CCC AAT CTC C - 3' PKN 1690

5'- GAC AGT CAT AAA CTG CCA CAT CTT C - 3' PKN.1930.R

Heavy chain primer set:

5'- TTG ACA CGC GTC TCG GGA AGC TT - 3' PG 5863

5'- GGC GCA GAG GAT CCA CTC ACC T - 3' PG 6332.R

The undigested heavy chain PCR product has a predicted size of 469 bp while the light chain PCR product has a predicted size of 286 bp. Verification of identity was determined by restriction enzyme digest with BstEII (heavy chain) or NlaIV (light chain).

CHO cell lines were transfected with L_AH_C, L_AH_A, L_BH_B, L_BH_D, as well as cF19. Geneticin-resistant cells were obtained and these cells were further selected for resistance to methotrexate. PCR amplification, followed by restriction enzyme cleavage of the light and heavy chain DNA produced the expected bands and confirmed the identity of L_AH_C, L_BH_B, L_AH_A and L_BH_D transfectants..

The cells described were maintained under serum-free conditions at all times and were not treated with animal-derived products such as trypsin.

Producer cell lines transfected with expressing monoclonal L_AH_C, L_AH_A, L_BH_B, L_BH_D and cF19 antibodies were produced. Their identities were confirmed using PCR amplification and restriction enzyme cleavage of the resulting PCR products of both their heavy and light chain variable regions.

Example 11: Expression of antibody proteins in Chinese hamster ovary DG44 cells and their purification

The objective of this experiment was to express and purify L_AH_C, L_AH_A, L_BH_B, and L_BH_D mAbs to enable their characterization. Other goals included the establishment of a quantitative ELISA to permit measurement of antibody concentrations in both crude media samples as well as purified Ig samples and determination of relative expression levels of various humanized F19 constructs using this assay.

Serum-free CHO DG44 cells and USP-grade methotrexate were obtained from the Biotechnical Production Unit of the Dr. Karl Thomae GmbH, Biberach, Germany; both products are also commercially available. Cells were maintained under serum-free con-

ditions at all times. SFM-II serum-free medium was obtained from Gibco/BRL. Protein A agarose was from Pierce Chemical (Indianapolis, IN, USA). Human IgG1 standards (Cat. No. I 3889), p-Nitrophenyl phosphate tablets (N 2640), bovine serum albumin (BSA) (A 7906), and goat anti-human kappa chain specific alkaline phosphatase-conjugated antibody (A 3813) were obtained from Sigma Chemical (St. Louis, MO, USA). Goat anti-human gamma-chain specific alkaline phosphatase-conjugated antibody was obtained from Jackson Immunoresearch Laboratories (through Stratech Scientific). Tris-buffered saline (TBS) consisted of 150 mM NaCl, 50 mM Tris, pH 7.5.

Cell culture conditions for antibody expression

Cells were cultured and maintained in T-175 flasks in SFM-II serum-free medium without agitation. The medium contained 200 µg/ml Geneticin and 20 nM methotrexate without antibiotics. Cells were passaged by dilution, were not adherent, and grew in small clusters. When the cells reached stationary phase, the medium was collected and centrifuged to remove cells and frozen at -20°C until needed.

Purification of L_AH_C

All purification steps were carried out at 4°C. A C10/10 column (Pharmacia Fine Chemicals) was packed with Protein A agarose (3 ml bed volume). The column was washed with TBS and preeluted once with 0.1 M Na citrate, pH 3.0 to insure that no loosely bound material remained on the column. The column was then immediately reequilibrated with TBS and stored at 4°C. Spent culture supernatants were thawed and centrifuged at 10,000 x g for 30 minutes prior to Protein A chromatography to remove debris and diluted with an equal volume of TBS. This material was loaded onto the Protein A column at 0.5 ml/min using a P-1 peristaltic pump (Pharmacia) and washed with TBS until the absorbance at 280 nm was undetectable. Elution of the antibody was initiated with 0.1 M Na citrate pH 3.0 at approximately 0.2 ml/min. The elution was monitored at 280 nm and one ml fractions of the eluted material were collected into tubes containing sufficient Tris base pH 9 to neutralize the citrate buffer. Protein-con-

taining fractions were pooled and concentrated using an Amicon filtration apparatus with a YM-30 filter and dialyzed against PBS. The column was immediately regenerated with TBS. Protein dye-binding assays were performed with the BioRad (Hercules, California) protein determination kit, according to the manufacturer's instructions, using bovine serum albumin as a standard.

Human IgG (gamma immunoglobulin) ELISA

ELISA plates were coated overnight with 100 µl goat anti-human gamma-chain specific alkaline phosphatase-conjugated antibody at 0.4 mg/ml in coating buffer at 4°C. Coating antibody was removed and plates were blocked with 2% BSA in PBS for 2 hours. All subsequent steps were performed at 37°C. Blocking buffer was replaced with antibody samples or human IgG1 standard diluted in dilution buffer, serially diluted in a 200 ml volume, and incubated for one hour. Negative controls included dilution buffer and/or culture medium of nontransfected cells. Wells were washed and 100 µl goat anti-human kappa chain specific alkaline phosphatase-conjugated antibody diluted 1:5000 was added and incubated for one hour. Wells were washed and 100 µl reaction buffer was added and incubated for 30 minutes. The reaction was stopped by addition of 1 M NaOH and absorbance read at 405 nm in an ELISA plate reader. Results were analyzed by four-parameter iterative curve fitting.

Amino acid analysis was performed according to methods available in the state of the art.

Monoclonal antibody L_AH_C was produced and purified to homogeneity using Protein A affinity chromatography. ELISA assays using human IgG1 as standard indicated L_AH_C recoveries exceeding 70%. The purity of the material was estimated to be >90% by SDS-polyacrylamide gel electrophoresis. Representative expression data and typical purification yields are shown in Table 21.

Table 21: Expression data and purification yields FAP antibody proteins in CHO cells

Antibody	Expression levels in crude media samples (ELISA)	Purified antibody yields	Yield improvement [purified antibody]
L_AH_C	7 - 10 mg /l	~ 5 - 7 mg/l	500 - 700
L_AH_A	5 - 7 mg/l	~ 3 - 4 mg/l	300 - 400
L_BH_B	0.5 – 1 mg/l	~ 0.2 – 0.5 mg/l	20 - 50
L_BH_D	0.8 - 1.5 mg/l	~ 0.3 – 0.8 mg/l	30 - 60
Chimeric F19	~ 0.02 mg/l	< 0.01 mg/l	1

Representative expression data for each of the anti-FAP antibodies produced in this study are shown. Recoveries after Protein A agarose affinity chromatography were based on protein dye-binding measurements of the purified Ig using BSA as a standard.

Example 12: **Binding of monoclonal antibody L_AH_C to isolated recombinant human FAP**

The objective of this study was to characterize binding of L_AH_C to isolated recombinant human FAP.

CD8-FAP ELISA

ELISA plates were coated overnight with 100 µl of mouse anti-rat antibody (Sigma Chemical R0761) at 1:2000 in coating buffer at 4°C. Coating antibody was removed and plates were blocked with 2% BSA in PBS for one hour. All subsequent steps were performed at room temperature. Blocking buffer was replaced with 100 ml of 1 µg/ml rat anti-CD8 antibody (Pharmingen 01041D) and incubated for one hour. Plates were washed and 100 µL CD8-FAP culture supernatant (see Example 14) (1:2 in PBS) was added and allowed to bind for one hour. Plates were washed and antibody samples were added (two-fold serial dilutions) in a 100 µl volume and incubated for one hour. Negative controls included human IgG and/or culture medium of nontransfected cells. Wells were washed and 100 µl of horseradish peroxidase (HRP) conjugated mouse anti-human IgG1 antibody (Zymed 05-3320) diluted 1:500 in dilution buffer were added

and incubated for one hour. Wells were washed and 100 µl HRP substrate, (azino-bis (3-ethylbenzthiazoline 6-sulfonic) acid, Sigma Chemical A9941), were added and incubated for 60 minutes. The reaction was stopped by addition of 1 M NaOH and absorbance read at 405/490 nm in an ELISA plate reader. Results were analyzed by four-parameter curve iterative curve fitting.

Alternatively, plates were coated directly with cF19. FAP (recombinant human FAP, see example 13) was allowed to bind to these plates as above and biotinylated L_AH_C (~1 µg/ml) was then added. Antibody binding was detected with HRP-streptavidin conjugate as above.

Solubilization of membrane-bound human FAP

FAP-expressing 293FAP I/2 cells or control 293 cells were washed with PBS and lysed with 1% Triton X-114 in Tris-buffered saline. Nuclei and debris were removed by centrifugation at 10,000 x g. The supernatant was phase-partitioned (Estreicher A, Wohlend A, Belin D, Scheuning WD Vasalli JD. Characterization of the cellular binding site for the urokinase-type plasminogen activator. (1989) *J Biol Chem* **264**:1180-1189) to enrich membrane proteins. The detergent phase was collected and diluted in buffer containing 1% Empigen BB (Calbiochem) to prevent reaggregation of the Triton X-114. This material was subjected to Concanavalin A agarose chromatography (Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Ozer HL, Schwab, M, Albino AP, Old LJ. Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. (1993) *Cancer Res* **53**:3327-3335).

Biotinylation of L_AH_C

L_AH_C (1-2 mg) was dialyzed against 50 mM bicarbonate buffer and biotinylated with a ten-fold molar excess of sulfosuccinimidyl-6-biotinamido hexanoate (NHS-LC

biotin, Pierce Chemical, Rockford, Illinois, USA) for 2 hours at room temperature. Unreacted product was removed by repeated microdialysis in a microconcentrator.

Transient transfections

COS-7 cells (American Type Tissue Culture Collection, reference number CRL 1651) were cotransfected by electroporation with the heavy and light chain vectors encoding L_AH_C.

Anti-CD8 monoclonal antibody was immobilized onto microtiter plates. CD8-FAP from medium of insect cells infected with CD8-FAP baculovirus was allowed to bind to these plates. Spent medium from COS-7 cell cultures transiently transfected with two separate vectors encoding L_AH_C was serially diluted and added to the wells containing the immobilized CD8-FAP. L_AH_C bound to isolated immobilized CD8-FAP protein (Figure 35). Culture supernatants from mock-transfected COS-7 cells failed to demonstrate binding.

Recombinant membrane-bound FAP from detergent extracts of 293FAP I/2 cells or control extracts was serially diluted and immobilized via chimeric F19 monoclonal antibody bound to microtiter plates. Biotinylated L_AH_C bound recombinant human FAP immobilized with cF19 (Figure 36) in a concentration-dependent manner.

L_AH_C recognized isolated immobilized recombinant human FAP carrying the epitope for murine F19. L_AH_C bound to both CD8-FAP produced in insect cells, as well as FAP protein produced in 293FAP I/2 cells.

Culture supernatants from COS7 cells transfected with either heavy and light chain vectors encoding L_AH_C or without DNA (Control) were collected three days posttransfection. CD8-FAP was immobilized via an anti-CD8 antibody as described in the text. Serial dilutions of the COS7 supernatants were allowed to bind to the immobilized CD8-FAP and subsequently detected with an HRP-conjugated anti-human IgG1 antibody.

Detergent extracts of FAP-expressing 293FAP I/2 cells or control 293 cells were serially diluted and added to cF19-coated microtiter plates. Biotinylated L_AH_C was added and binding of biotinylated L_AH_C was detected with HRP-conjugated streptavidin.

Example 13: **Characterization of HT-1080 fibrosarcoma cells and 293 human embryonic kidney cells transfected with cDNA for human FAP**

Fibroblast activation protein (FAP) is a cell-surface, membrane-bound protein which carries the F19 epitope and is expressed on tumor stromal fibroblasts. Cell lines expressing recombinant FAP protein and matched controls lacking FAP were generated for the characterization of anti-FAP monoclonal antibodies.

Cells used were HT-1080 cells (reference number CCL 121) and 293 human embryonic kidney cells (reference number CRL 1573) were obtained from the American Type Culture Collection (Maryland, USA). Transfectam was obtained from Promega (Madison, WI). Geneticin and all restriction enzymes were obtained from Boehringer Mannheim. DNA for transfections was purified from E. coli cells using QiaFilter Maxi Cartridges (Qiagen) as directed by the manufacturer. All DNA preparations were examined by restriction enzyme digestion. Vector sequences were confirmed using an ABI PRISM 310 Sequencer.

Further information regarding the vectors and DNA sequences employed has been described in Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, Old LJ, Rettig WJ. Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. (1992) *Proc Natl Acad Sci USA* **89**:10832-10836. The FAP cDNA sequence has been deposited in Genbank (accession number HS09287).

Cell culture and immunoassays

HT-1080 cells were transfected with 1 mg DNA using Transfectam according to the manufacturer's instructions. Human embryonic kidney 293 cells were transfected by calcium phosphate transfection (Brann MR; Buckley NJ; Jones SVP; Bonner TI. Expression of cloned muscarinic receptor in A9 L cells. (1987) *Mol Pharmacol* **32**: 450-455) with 10 mg DNA. Twenty-four hours later, cells were diluted 1:10 into fresh medium containing 200 mg/ml Geneticin. Colonies were picked and examined by immunofluorescence for FAP expression as described in Rettig WJ; Garin-Chesa P; Beresford HR; Oettgen HF; Melamed MR; Old LJ. Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells.(1988) *Proc Natl Acad Sci USA* **85**: 3110-3114.

Immunoprecipitations with cF19 were performed with metabolically labelled cells as described in Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Ozer HL, Schwab, M, Albino AP, Old LJ. Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. (1993) *Cancer Res* **53**:3327-3335.

HT-1080 and 293 cells were tested for FAP antigen expression in immunofluorescence assays with anti-FAP antibodies and were found to be antigen-negative. Transfection of these cells with FAP.38 vector resulted in the generation of Geneticin-resistant colonies. Isolated colonies were picked and analyzed by immunofluorescence for FAP expression. Two cell clones were identified, designated HT-1080FAP clone 33 and 293FAP I/2, which express cell surface-bound FAP protein, as recognized by cF19 antibody. Staining of nonpermeabilized HT-1080FAP clone 33 cells and 293FAP I/2 with cF19 antibody confirmed the cell surface localization of the FAP protein.

Immunoprecipitation of radiolabelled FAP protein with cF19 from extracts of ^{35}S-methionine labelled HT-1080FAP clone 33 cells or 293FAP I/2 cells resulted in the ap-

pearance of a 93 kilodalton band after autoradiography. This band is not detectable in immunoprecipitates of parental HT-1080 or 293 cell extracts.

Two stably transfected cell lines, HT-1080FAP clone 33 and 293FAP I/2, express FAP on the cell surface as determined in immunological assays with anti-FAP mAbs. Neither parental HT-1080 cells nor parental 293 cells express detectable levels of FAP.

Example 14: **Generation and characterization of CD8-FAP fusion protein**

A soluble form of human FAP (fibroblast activation protein) in the form of a CD8-FAP fusion protein was produced in insect cells for the characterization of L_AH_C containing the binding site for anti-FAP mAbs. Murine CD8 was chosen to permit secretion of the protein and to provide an additional epitope tag.

The cDNA encoding the extracellular domain of CD8, consisting of the first 189 amino acids of murine CD8α (Genbank M12825), was linked to that of the extracellular domain of FAP (amino acids 27 to 760), essentially as described by Lane, et al. (Lane P, Brocker T, Hubele S, Padovan E, Lazavecchia A, McConnell. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. (1993) *J Exp Med* **177**:1209-1213) using standard PCR protocols. The authenticity of all clones was verified by DNA sequencing. The resulting DNA was inserted into the pVL1393 vector (Invitrogen) and transfection of Sf9 cells (Invitrogen) with this vector and amplification of the resulting recombinant baculovirus were performed as described (Baculovirus Expression Vectors. A Laboratory Manual. O'Reilly DR, Miller LK, Luckow VA, (Eds.), Oxford University Press: New York, 1994). The spent medium of High Five™ cells (Invitrogen) infected with recombinant CD8-FAP baculovirus for four days was collected and cleared by ultracentrifugation.

The CD8-FAP ELISA (enzyme-linked immunosorbent assay) has been described above (Example 12).

Insect cell cultures infected with CD8-FAP virus secreted a fusion protein into the medium which carries the F19 epitope and is recognized by an anti-FAP antibody (Figure 1). Neither the cell culture medium alone nor medium from insect cells infected with CD8-CD40L fusion protein bound anti-FAP antibody.

Soluble CD8-FAP protein carrying the F19 epitope was secreted into the medium of infected insect cell cultures. Culture supernatant from cells infected with a control construct did not contain antigen bearing the F19 epitope.

A soluble form of FAP, CD8-FAP, was produced in insect cells and CD8-FAP was shown to carry the epitope recognized by cF19.

Supernatants from insect cells infected with recombinant baculovirus encoding either CD8-FAP or CD8-CD40L fusion protein were collected four days postinfection. Cell culture medium without cells was used as an additional control (medium). Serial dilutions of these materials were added to anti-CD8 antibody-coated microtiter plates and allowed to bind. cF19 (1 mg/ml) was subsequently added and allowed to bind. Bound cF19 was detected with horseradish peroxidase-conjugated anti-human antibody.

Claims

1. An antibody protein having the complementary determining regions of the monoclonal antibody F19 (ATCC Accession No. HB 8269), said antibody protein specifically binding to fibroblast activation protein, characterized in that it has framework modifications resulting in the improved producibility in host cells as compared to a chimeric antibody having the variable regions of F19 and foreign constant regions.

2. An antibody protein characterised in that it has a variable light chain region and a variable heavy chain region according to claim 1, each joined to a human constant region.

3. The antibody protein of claim 2, wherein said human constant region of the light chain is a human kappa constant region.

4. The antibody protein of claim 2, wherein said human constant region of the heavy chain is a human gamma-1 constant region.

5. An antibody protein according to any one of claims 1 to 4, characterised in that its expression level in crude media samples as determined by ELISA and/or purified antibody yield exceeds the expression level and/or purification yield of the chimeric antibody without framework modifications by at least a factor of 10.

6. An antibody protein according to any one of claims 1 to 4, characterised in that its expression levels in crude media samples as determined by ELISA and/or purified antibody yield exceeds the expression level and/or purification yield of the chimeric antibody without framework modifications by at least a factor of 20.

7. An antibody protein according to any one of claims 1 to 4, characterised in that its expression level in crude media samples as determined by ELISA and/or purified antibody yield exceeds the expression level and/or purification yield of the chimeric antibody without framework modifications by at least a factor of 100.

8. An antibody protein according to any one of claims 1 to 7, characterised in that it displays improved producibility in eukaryotic cells.

9. The antibody protein according to claim 8 wherein said eukaryotic cell is a Chinese hamster ovary cell (CHO cell).

10. An antibody protein according to any one of claims 1 to 9, wherein the amino acid in Kabat position 87 of the light chain region is not asparagine.

11. The antibody protein of claim 10, wherein the amino acid in Kabat position 87 of the light chain region is selected from aromatic or aliphatic amino acids.

12. The antibody protein of claim 11, wherein said aromatic amino acid in Kabat position 87 of the light chain region is a tyrosine or phenylalanine.

13. The antibody protein according to any one of claims 1 to 12, wherein the amino acid in Kabat position 36 of the light chain region is selected from aromatic amino acids.

14. An antibody protein according to any one of claims 1 to 13 that contains the variable region of the light chain as set forth in SEQ ID NO: 2.

15. An antibody protein of claim 14 characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1.

16. An antibody protein according to any one of claims 1 to 13 that contains the variable region of the light chain as set forth in SEQ ID NO: 6.

17. An antibody protein of claim 16 characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 5.

18. An antibody protein according to any one of claims 1 to 17 containing a variable region of the heavy chain as set forth in any one of SEQ ID NOs: 8, 10, 12, 14.

19. An antibody protein according to claim 18 characterised in that the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in SEQ ID NOs: 7, 9, 11, 13.

20. An antibody protein according to any one of claims 1 to 14 containing the variable region of the light chain as set forth in SEQ ID NO: 2 and the variable region of the heavy chain as set forth in SEQ ID NOs: 12.

21. The antibody protein of claim 20 characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1 and the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 11.

22. An antibody protein according to claim 20 or claim 21 containing the constant region of the light chain as set forth in SEQ ID NO: 20 and the constant region of the heavy chain as set forth in SEQ ID NO: 22.

23. An antibody protein according to any one of claims 1 to 13, 18 or 19 containing the variable region of the light chain as set forth in SEQ ID NO: 2 and the variable region of the heavy chain as set forth in SEQ ID NOs: 8.

24. The antibody protein of claim 23 characterised in that the variable region of the light chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 1 and the variable region of the heavy chain is encoded by a nucleotide sequence as set forth in SEQ ID NO: 7.

25. A nucleotide sequence encoding an antibody protein according to any one of claims 1 to 24.

26. A recombinant DNA vector that contains a nucleotide sequence of claim 25.

27. The recombinant DNA vector of claim 26, said vector being an expression vector.

28. A host cell carrying a vector according to claims 26 or 27.

29. The host cell of claim 28, wherein said host cell is a eukaryotic cell.

30. The host cell of claim 29, wherein said eukaryotic host cell is a mammalian cell.

31. The host cell of claim 30, wherein said host cell is a CHO or a COS cell.

32. A method of producing antibody proteins according to any one of claims 1 to 24, said method comprising the steps of:
    (a) cultivating a host cell according to any one of claims 28 to 31 under conditions where said antibody protein is expressed by said host cell, and
    (b) isolating said antibody protein.

33. The method of claim 32, wherein said host cell is a mammalian cell, preferably a CHO or COS cell.

34. The method of claim 32 or 33, wherein said host cell is cotransfected with two plasmids carrying the expression units for light and heavy chains respectively.

35. An antibody protein according to any one of claims 1 to 24, wherein said antibody protein is conjugated to a therapeutic agent.

36. The antibody protein of claim 35, wherein said therapeutic agent is a therapeutic agent selected from the group consisting of radioisotopes, toxins, toxoids, inflammatory agents and chemotherapeutic agents.

37. The antibody protein of claim 36, wherein said radioisotope is a ß-emitting radioisotope.

38. The antibody protein of claim 37, wherein said radioisotope is selected from the group consisting of ^{186}Rhenium, ^{188}Rhenium, ^{131}Iodine and ^{90}Yttrium.

39. An antibody protein according to any one of claims 1 to 24, characterised in that it is labelled.

40. The antibody protein of claim 39, wherein said label is a detectable marker.

41. The antibody protein of claim 40, wherein the detectable marker is a detectable marker selected from the group consisting of enzymes, dyes, radioisotopes, digoxygenin, and biotin.

42. An antibody protein according to any one of claims 1 to 24 conjugated to an imageable agent.

43. The antibody protein of claim 42, wherein the imageable agent is a radioisotope.

44. The antibody protein of claim 43, wherein said radioisotope is a γ-emitting radioisotopes.

45. The antibody protein of claim 44, wherein said radioisotope is ^{125}I.

46. A pharmaceutical composition containing an antibody protein according to any one of claims 1 to 24 and a pharmaceutically acceptable carrier.

47. A pharmaceutical composition containing an antibody protein according to any one of claims 35 to 38 and a pharmaceutically acceptable carrier.

48. A pharmaceutical composition containing an antibody protein according to any one of claims 42 to 45 and a pharmaceutically acceptable carrier.

49. The pharmaceutical composition of claims 46 to 48, for use in the treatment or imaging of tumors, wherein said tumors are associated with activated stromal fibroblasts, preferably wherein said tumors are tumors selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain.

50. Use of an antibody protein according to anyone of claims 1 to 24 for the treatment of cancer.

51. Use of an antibody protein according to anyone of claims 35 to 38 for the treatment of cancer.

52. Use of an antibody protein according to anyone of claims 42 to 45 for imaging activated stromal fibroblasts.

53. Use of an antibody protein according to anyone of claims 39 to 41 for detecting the presence of activated stromal fibroblasts in a sample.

54. A method of treating tumors, wherein the tumor is associated with activated stromal fibroblasts capable of specifically forming a complex with antibody proteins according to any one of claims 1 to 24 or 35 to 38, which comprises contacting the tumor with an amount of said antibody proteins effective to treat the tumor.

55. The method of claim 54, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain.

56. The method of claim 54, wherein the contacting is effected in vitro.

57. The method of claim 54, wherein the contacting is effected in vivo.

58. A method of detecting the presence of activated stromal fibroblasts in wound healing, inflammation or a tumor, characterised in that

(a) a sample, possibly containing activated stromal fibroblasts, is contacted with an antibody protein according to any one of claims 1 to 24 or 39 to 41 under conditions suitable for the formation of a complex between said antibody and antigen,

(b) detecting the presence of said complex, thereby detecting the presence of activated stromal fibroblasts in wound healing, inflammation or a tumor.

59. The method of claim 58, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain.

60. The method of claim 58 or 59, wherein the antibody protein is a protein according to any one of claims 39 to 41.

61. A method of imaging the presence of activated stromal fibroblasts in a healing wound, inflamed skin or a tumor, in a human patient, characterised in that
    (a) an antibody protein according to any one of claims 1 to 24 conjugated to an imageable agent is administered to a human patient under conditions suitable for the formation of an antibody-antigen complex,
    (b) imaging any complex formed in this manner.

62. The method of claim 61, wherein the tumor is a tumor having cancer cells selected from the cancer group consisting of colorectal cancers, non-small cell lung cancers, breast cancers, head and neck cancer, ovarian cancers, lung cancers, bladder cancers, pancreatic cancers and metastatic cancers of the brain.

63. A method of detecting tumor-stroma, characterised in that
    (a) a suitable sample is contacted with an antibody protein according to any one of claims 1 to 24, under conditions suitable for the formation of an antibody-antigen complex,
    (b) detecting the presence of any complex so formed,
    (c) relating the presence of said complex to the presence of tumor-stroma.

64. The method of claim 62, wherein said antibody is labelled with a detectable marker.

65. A method of imaging tumor-stroma in a human patient, which comprises
    (a) administering to the patient an antibody protein according to any one of claims 42 to 45, under conditions suitable for the formation of an antibody-antigen complex,
    (b) imaging any complex so formed, and thereby imaging the presence of tumor-stroma in a human patient.

66. An antibody protein containing an amino acid sequence as set forth in SEQ ID NO: 2.

67. An antibody protein according to claim 66 further containing an amino acid sequence as set forth in SEQ ID NO: 12.

68. An antibody protein according to claim 66 or 67 further containing an amino acid sequence as set forth in SEQ ID NO: 20 and an amino acid sequence as set forth in SEQ ID NO: 22.

69. A DNA molecule coding for an antibody protein according to any one of claims 66 to 68.

70. A host cell carrying a DNA molecule according to claim 69.

71. A method of producing an antibody protein of any one of claims 66 to 68, said method comprising the steps of

(a) cultivating the host cell of claim 70 under conditions where said antibody protein is expressed by said host cell, and

(b) isolating said protein.

72. An antibody protein according to any one of claims 66 to 68 which is conjugated to a radioisotope, preferably ^{131}I, ^{125}I, ^{186}Re, ^{188}Re, or ^{90}Y.

73. A pharmaceutical composition comprising an antibody protein according to any one of claims 66 to 68, or 72, and a pharmaceutically acceptable carrier.