VED-1
March 1, 1992

ACTION PLAN 1992

1.IMMUNOSCREENING OF EXPRESSION LIBRARY

2.Use aminoacid sequence information to synthesize primers for use in PCR.

3.USE PCR to isolate desired sequences from the tobacco root RNA and DNA.

a. RNA PCR(Perkin-Elmer kit,Forward and Reverse primers)

b. DNA PCR (Perkin-Elmer kit,Forward and Reverse Primers)

c. ANCHORED PCR:AMPLIFICATION WITH SINGLE-SIDED SPECIFICITY

1.Take tobacco root poly A RNA.Synthesise complementary strand of DNA using oligo dT as primer .Destroy RNA by alkali hydrolysis.Amplify Using

combinatins of forward primers and oligo dT25 .CLONE the PCR PRODUCT.SEQuence the insert. Enter the sequence into computer.use DNA STAR(findpro programme) to find PMT1.PRO,PMT2.PRO, PMT3.PRO PMT4. PRO sequences.

2.ANCHORED PCR of cDNA with OLIGO(dG)tails.Synthesise cDNA using REVERSE PCR PRIMERS or oligo dT.Add oligo dG to the newly synthesised c DNA strand.Amplify using REVERSE PCR PRIMERS and OLIGO dC.Clone the PCR product AND analyse as in 1.

d.LIGATION-MEDIATED ANCHORED PCR.Digest the genomic DNA with a Mbo1 OR its isoschizomer Sau3A.
If possible, Fill one base to prevent self-Ligation.

LIgate an anchor-primer on the ends of the DNA fragments.
Amplify with a specific primer as well as the anchor.
The choice of which strand the primer is derived from determines which side of the fragment is amplified .
This can be done for several enzymes that leave cohesive ends with a four-base 5'overhang. This could allow isolation of the promoter for the gene of interest.

4. GENERATION OF SINGLE-STRANDED LABELED PRIMERS AND THEIR USE IN NORTHERN AND SOUTHERN HYBRIDIZATION AND SCREENING OF LIBRARIES.

5. PMT POLYCLONALS(GPC FRACTION)

VED-4

IMMUNOSCREENING OF cDNA EXPRESSION LIBRARY.

1.EXPRESSION LIBRARY OF TOBACCO ROOT mRNA IS AT HAND.
2. A POLYCLONAL ANTIBODY AGAINST A SYNTHETIC PEPTIDE N-29 MER HAS BEEN RAISED
3. THIS POLYCLONAL ANTIBODY LIGHTS UP 60 KD REGION IN WESTERN BLOTS OF TOBACCO PROTEIN.
4.THE EXPRESSION LIBRARY(1) WILL BE SCREENED USING POLYCLONAL ANTIBODY(2).
5. IF A POSITIVE CLONE IS FOUND,THE PROTEIN OF THAT CLONE MAY BE PURIFIED USING ANTI betaGAL ANTIBODIES.

SEQUENCE INFORMATION

1. N-terminus of intact 60 kD protein, electroblot/sequenced. (code:PMT1.PRO)

```
PMT   L S S N F L F G T A S S Y Y Q Y E G A F L S D G V G L S N --- COOH
Deg   6 6 6 2 2 6 2 4 4 4 6 6 2 2 2 2 2 4 4 2 6 6 2 4 4 4 6 6 2
```

Alignments:

```
PNMT(PET) S(16)          S S   Y Q
PEMT(PhosphatidylEMT)              F Y G D F F L S
```

2. 6.2 kD CNBr Fragment of 60 kD protein, electroblot/sequenced (F-SG-T MOTIF, region I),(a met would precede all of the CNBr fragments). (code:PMT2.PRO)

```
PMT   N E P X F V A I S G Y R D X T --- COOH
Deg   2 2 4   2 4 4 3 6 4 2 6 2   4
```

Alignments:

```
erm C/G
RNA MT      I F E I G S G K G H F T
PNMT      (65)F A T G E S G R T
PNMT      (75)L I D I G S G P T Y N L
```

3. 3 kD CNBr Fragment of 60 kD after removal of N-terminus (DAL - Region II). (code:PMT3.PRO)

```
PMT   A D I E H Y S K L I D A L X I K G I Q F --- COOH
        G L   L                           A P
Deg             2 6 2 6 3 2 4 6   3 2 4 3
```

Alignments:

```
PNMT       (125)Y S                 K G
PNMT             (174)P A D A L
AdoHcyHMT          K     D A I
```

4. 15 kD CNBr Fragment of 60 kD protein. (code:PMT4.PRO)

```
PMT   F I T E N G F A G R S G R P(?)---   COOH
Deg   2 3 4 2 2 4 2 4 4 6 6 4 6
```

5. Possible sequence order of fragments obtained for 60 kD protein.

From this speculative "motif" examination, a deduced composite can be drawn for putative PMT as follows (cf. PNMT, PNAS 83,5455 (1986):

N-terminus(~3 kD) ----- 6.2 kD fragment ----- other 3 kD fragment ----- 15 kD fragment ---- COOH

Ref. Ingrosso etal., JBC 264,20131-20139 (1989); Baetge etal., PNAS 83,5454-5458. Also see. Lauster etal., FEBS Lett. 220, 167-176.

PCR AND PRIMERS

by VED MALIK

1.Importance of first PCR cycle AND 3' end of primers
2.Important to know where you are
3.Examination of PMT. SEquences.
4. N-terminal codons included in primers
5.C-terminal codons should appear in amplified product.
6.C-terminal codons can constitute the primers,and N-terminal codons can appear in the PCR product.

PMT1.PRO

VED-7

-- PAGE 1
PROBE#: 1
Melting Temp: 52
Number of possibilities: 128
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: taytaycartaygarggngc

PROBE#: 2
Melting Temp: 50
Number of possibilities: 128
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: aytaycartaygarggngc

PROBE#: 3
Melting Temp: 52
Number of possibilities: 256
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: taycartaygarggngcntt

PROBE#: 4
Melting Temp: 50
Number of possibilities: 256
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: taycartaygarggngcnt

PROBE#: 5
Melting Temp: 50
Number of possibilities: 256
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: aycartaygarggngcntt

PROBE#: 6
Melting Temp: 58
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: taytaycartaygarggngcntt

PROBE#: 7
Melting Temp: 56
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: taytaycartaygarggngcnt

PROBE#: 8
Melting Temp: 56
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: aytaycartaygarggngcntt

PROBE#: 9
Melting Temp: 54
Number of possibilities: 512
PMT1.PRO 12 Ser Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: ntaytaycartaygarggngc

PMT1.PRO

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-- PAGE 2
Oligonucleotide sequence: taytaycartaygarggngcn

PROBE#: 11
Melting Temp: 54
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: aytaycartaygarggngcnt

PROBE#: 12
Melting Temp: 54
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: ytaycartaygarggngcntt

PROBE#: 13
Melting Temp: 54
Number of possibilities: 512
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: taycartaygarggngcntty

PROBE#: 14
Melting Temp: 52
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: aytaycartaygarggngcn

PROBE#: 15
Melting Temp: 52
Number of possibilities: 512
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: ytaycartaygarggngcnt

PROBE#: 16
Melting Temp: 52
Number of possibilities: 512
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: aycartaygarggngcntty

PROBE#: 17
Melting Temp: 52
Number of possibilities: 512
PMT1.PRO 15 Gln Tyr Glu Gly Ala Phe Leu 21
Oligonucleotide sequence: cartaygarggngcnttyyt

PROBE#: 18
Melting Temp: 50
Number of possibilities: 512
PMT1.PRO 12 Ser Tyr Tyr Gln Tyr Glu Gly 18
Oligonucleotide sequence: wsntaytaycartaygargg

PROBE#: 19
Melting Temp: 50
Number of possibilities: 512
PMT1.PRO 12 Ser Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: ntaytaycartaygarggng

PMT 1. PRO

VED-9

-- PAGE 3
Oligonucleotide sequence: ytaycartaygarggngcn

PROBE#: 21
Melting Temp: 50
Number of possibilities: 512
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: ycartaygarggngcntty

PROBE#: 22
Melting Temp: 50
Number of possibilities: 512
PMT1.PRO 15 Gln Tyr Glu Gly Ala Phe Leu 21
Oligonucleotide sequence: cartaygarggngcnttyy

PROBE#: 23
Melting Temp: 60
Number of possibilities: 1024
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20 *
Oligonucleotide sequence: taytaycartaygarggngcntty

PROBE#: 24
Melting Temp: 58
Number of possibilities: 1024
PMT1.PRO 12 Ser Tyr Tyr Gln Tyr Glu Gly Ala 19
Oligonucleotide sequence: sntaytaycartaygarggngc

PROBE#: 25
Melting Temp: 58
Number of possibilities: 1024
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: aytaycartaygarggngcntty

PROBE#: 26
Melting Temp: 58
Number of possibilities: 1024
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe Leu 21
Oligonucleotide sequence: taycartaygarggngcnttyyt

PROBE#: 27
Melting Temp: 56
Number of possibilities: 1024
PMT1.PRO 13 Tyr Tyr Gln Tyr Glu Gly Ala Phe 20
Oligonucleotide sequence: ytaycartaygarggngcntty

PROBE#: 28
Melting Temp: 56
Number of possibilities: 1024
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe Leu 21
Oligonucleotide sequence: taycartaygarggngcnttyy

PROBE#: 29
Melting Temp: 56
Number of possibilities: 1024
PMT1.PRO 14 Tyr Gln Tyr Glu Gly Ala Phe Leu 21
Oligonucleotide sequence: aycartaygarggngcnttyyt

VED-10

PMT2.PRO

-- PAGE 1
PROBE#: 1
Melting Temp: 40
Number of possibilities: 768
PMT2.PRO 6 Val Ala Ile Ser Gly 10
Oligonucleotide sequence: gtngcnathwsngg

PROBE#: 2
Melting Temp: 38
Number of possibilities: 768
PMT2.PRO 7 Ala Ile Ser Gly Tyr 11
Oligonucleotide sequence: gcnathwsnggnta

PROBE#: 3
Melting Temp: 38
Number of possibilities: 1024
PMT2.PRO 9 Ser Gly Tyr Arg Asp 13
Oligonucleotide sequence: wsnggntaymgnga

PROBE#: 4
Melting Temp: 38
Number of possibilities: 1024
PMT2.PRO 9 Ser Gly Tyr Arg Asp 13
Oligonucleotide sequence: snggntaymgngay

PMT3.PRO

VED-11

-- PAGE 1
PROBE#: 1
Melting Temp: 46
Number of possibilities: 192
PMT3.PRO 1 Ala Asp Ile Glu His Tyr 6
Oligonucleotide sequence: gcngayathgarcaytay

PROBE#: 2
Melting Temp: 48
Number of possibilities: 384
PMT3.PRO 1 Ala Asp Ile Glu His Tyr Ser 7
Oligonucleotide sequence: gcngayathgarcaytayw

PROBE#: 3
Melting Temp: 46
Number of possibilities: 384
PMT3.PRO 1 Ala Asp Ile Glu His Tyr 6
Oligonucleotide sequence: ngcngayathgarcayta

PROBE#: 4
Melting Temp: 52
Number of possibilities: 768
PMT3.PRO 1 Ala Asp Ile Glu His Tyr Ser 7
Oligonucleotide sequence: gcngayathgarcaytayws

PROBE#: 5
Melting Temp: 48
Number of possibilities: 768
PMT3.PRO 1 Ala Asp Ile Glu His Tyr 6
Oligonucleotide sequence: ngcngayathgarcaytay

PROBE#: 6
Melting Temp: 48
Number of possibilities: 768
PMT3.PRO 1 Ala Asp Ile Glu His Tyr Ser 7
Oligonucleotide sequence: cngayathgarcaytayws

PROBE#: 7
Melting Temp: 48
Number of possibilities: 768
PMT3.PRO 2 Asp Ile Glu His Tyr Ser Lys 8
Oligonucleotide sequence: gayathgarcaytaywsnaa

PROBE#: 8
Melting Temp: 46
Number of possibilities: 768
PMT3.PRO 2 Asp Ile Glu His Tyr Ser Lys 8
Oligonucleotide sequence: gayathgarcaytaywsna

PMT 4. PRO

VED – 12

-- PAGE 1
PROBE#: 1
Melting Temp: 52
Number of possibilities: 384
PMT4.PRO 2 Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: athacngaraayggnttygc

PROBE#: 2
Melting Temp: 50
Number of possibilities: 384
PMT4.PRO 2 Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: thacngaraayggnttygc

PROBE#: 3
Melting Temp: 56
Number of possibilities: 512
PMT4.PRO 3 Thr Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: acngaraayggnttygcngg

PROBE#: 4
Melting Temp: 54
Number of possibilities: 512
PMT4.PRO 3 Thr Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: cngaraayggnttygcngg

PROBE#: 5
Melting Temp: 52
Number of possibilities: 512
PMT4.PRO 3 Thr Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: acngaraayggnttygcng

PROBE#: 6
Melting Temp: 50
Number of possibilities: 512
PMT4.PRO 3 Thr Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: cngaraayggnttygcng

PROBE#: 7
Melting Temp: 50
Number of possibilities: 512
PMT4.PRO 3 Thr Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: ngaraayggnttygcngg

PROBE#: 8
Melting Temp: 50
Number of possibilities: 512
PMT4.PRO 4 Glu Asn Gly Phe Ala Gly 9
Oligonucleotide sequence: garaayggnttygcnggn

PROBE#: 9
Melting Temp: 58
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: ttyathacngaraayggnttygc

PROBE#: 10
Melting Temp: 56
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8

PMT 4. PRO VED-13

-- PAGE 2
Oligonucleotide sequence: tyathacngaraayggnttygc

PROBE#: 11
Melting Temp: 54
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: ttyathacngaraayggnttyg

PROBE#: 12
Melting Temp: 54
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: yathacngaraayggnttygc

PROBE#: 13
Melting Temp: 52
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: tyathacngaraayggnttyg

PROBE#: 14
Melting Temp: 50
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe 7
Oligonucleotide sequence: ttyathacngaraayggntty

PROBE#: 15
Melting Temp: 50
Number of possibilities: 768
PMT4.PRO 1 Phe Ile Thr Glu Asn Gly Phe Ala 8
Oligonucleotide sequence: yathacngaraayggnttyg

PROBE#: 16
Melting Temp: 56
Number of possibilities: 1024
PMT4.PRO 4 Glu Asn Gly Phe Ala Gly Arg 10
Oligonucleotide sequence: garaayggnttygcnggnmg

PROBE#: 17
Melting Temp: 52
Number of possibilities: 1024
PMT4.PRO 4 Glu Asn Gly Phe Ala Gly Arg 10
Oligonucleotide sequence: garaayggnttygcnggnm

PROBE#: 18
Melting Temp: 52
Number of possibilities: 1024
PMT4.PRO 4 Glu Asn Gly Phe Ala Gly Arg 10
Oligonucleotide sequence: araayggnttygcnggnmg

PROBE#: 19
Melting Temp: 50
Number of possibilities: 1024
PMT4.PRO 4 Glu Asn Gly Phe Ala Gly Arg 10
Oligonucleotide sequence: raayggnttygcnggnmg

REVERSE PRIMERS=c mRNA EcoR1?

PMT4.PRO (15 kd CNBr fragment)

1 2 3 4 5 6 7 8 9
Met Phe Ile Thr glu Asn Gly Phe Ala
10 11 12 13 14
GLY Arg Ser Gly Arg

1, 5'-C CIG CRA AIC CRT TYT CIG TRA TRA ACA T -3',10(P-15).

1, 5'-ATC GAATTC CIG CRA AIC CRT TYT CIG TRA TRA ACA T -3,'10(P-16).
5'-ATC GAATT CCR TTY TCN GTD ATR AA CAT-3'(P-21,REVERSE OF P-17)

5'-AAN CCR TTY TCN GTD ATR AAC AT-3'(P-22, REVERSE OF P-18).

FORWARD PRIMERS=mRNA BamH1?
PMT4.PRO (15 kd CNBr fragment)

.

R:AG,Y:CT,H:ATC,D:ATG,B:GTC,N:AGCT

1 2 3 4 5 6 7 8 9
Met Phe Ile Thr glu Asn Gly Phe Ala
10 11 12 13 14
GLY Arg Ser Gly Arg

1 AAC GGATCC ATG TTY ATH ACN GAR AAY GG 7(P-17)
MELTING TEMPERATURE: 5O
Number of possibilities: 96

1 ATG TTY ATH ACN GAR AAY GGN TT 8(P-18)
Melting Temperature: 56
Number of Possibilities: 384

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FORWARD PRIMERS=mRNA,BamH1?

PMT1.PRO(N-29 mer OF 60 kd PROTEIN)

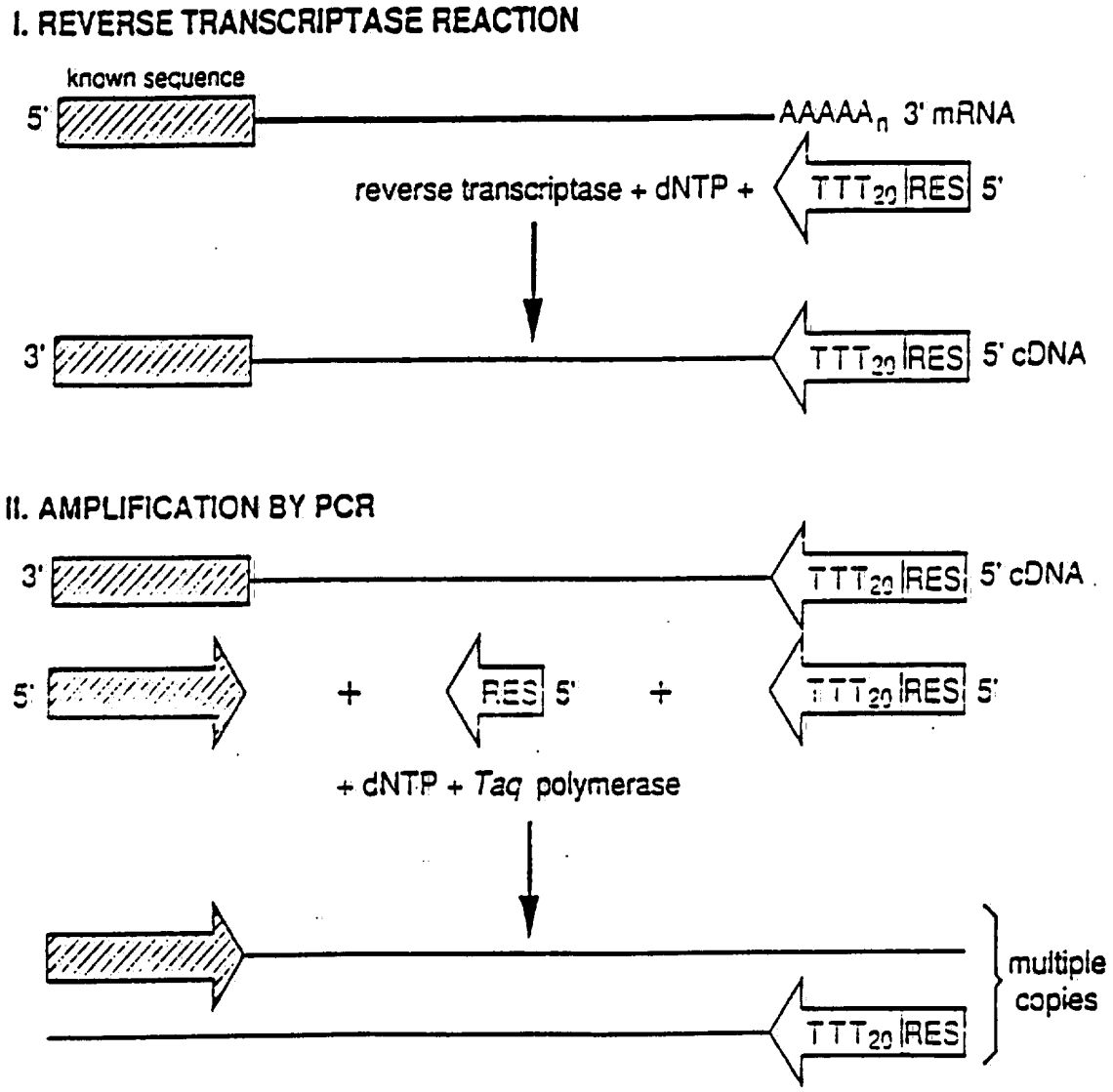
L S S N F L F G T A S S Y Y Q Y E G A F L S D G V G L S N

13 Tyr Tyr Gln Tyr Glu Gly Ala 19
ATC GGA TCC TAY TAY CAR TAY GAR GGI GC (P-19)
Melting temp: 52
Number of possibilities: 32

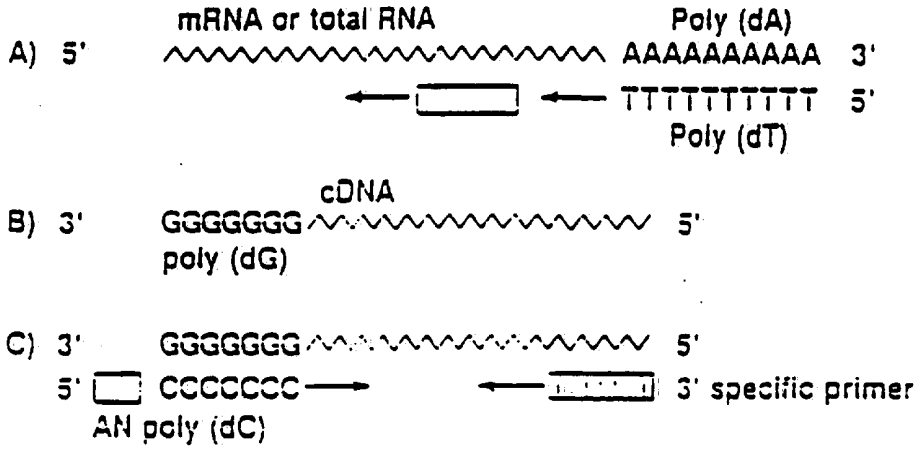
13 TAY TAY CAR TAY GAR GGI GCI TT 20.(P-20)
Melting temperature: 58
Number of possibilities: 32

ONE-SIDED PCR

Cloning of the 3' End of a cDNA with 5' Sequence Information

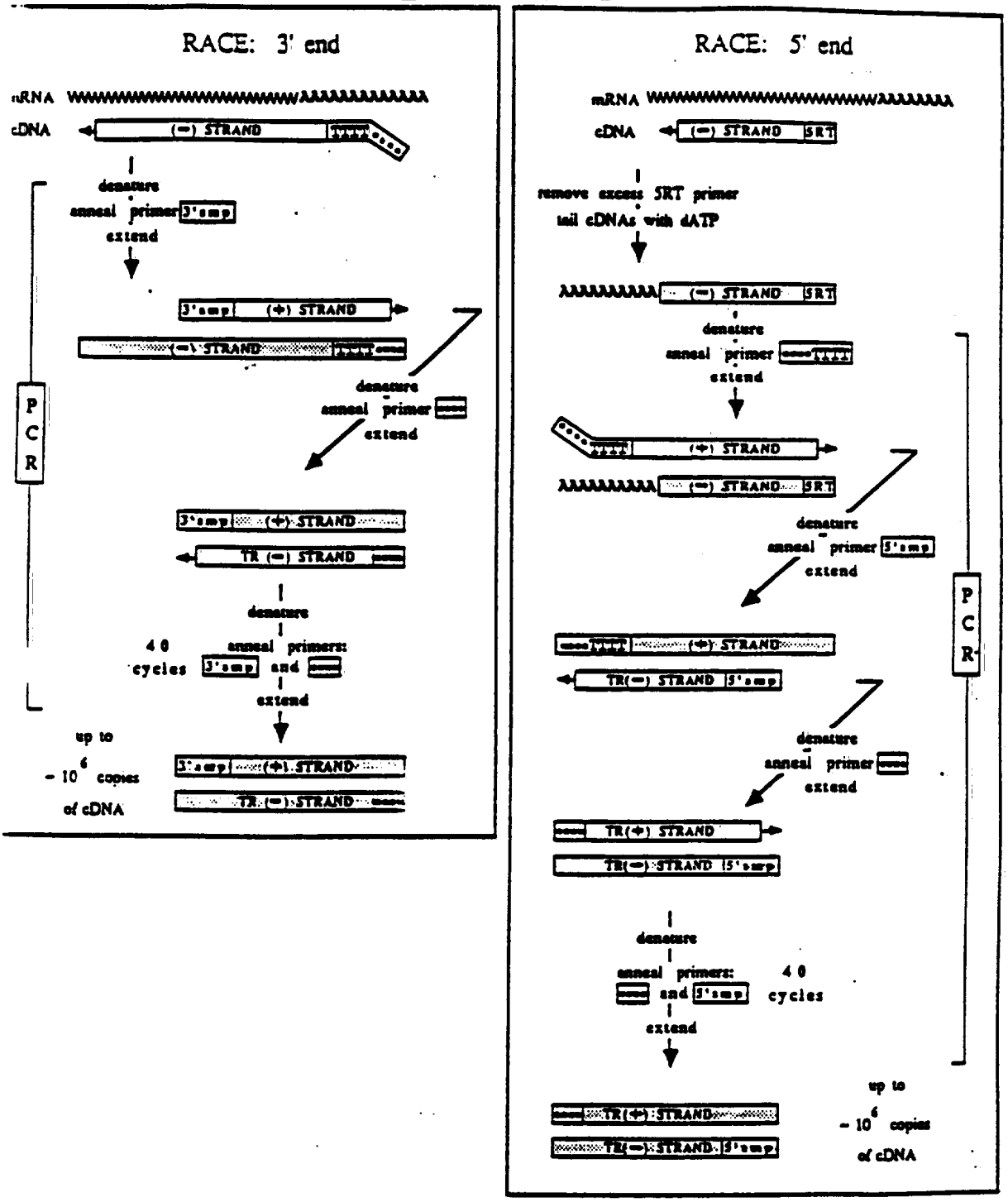


Anchor PCR



Loh, E.

M. Frohman, PNAS (1988) 85:8998-9002



BOVINE PNMT

```
          -40       -30       -20       -10       -1   1
           CGTGATGTCCTCAAAGTGGGCACAGGCGCTGAGCAGCAGC ATG AGC GGG ACA GAC CGG AGT
                                                     MET Ser Gly Thr Asp Arg Ser
         10                                                  20
CAG GCG GCG GGC GCA GTG CCC GAC TCA GAC CCG GGC CTG GCG GCG GTC TCC TCG GCC TAC
Gln Ala Ala Gly Ala Val Pro Asp Ser Asp Pro Gly Leu Ala Ala Val Ser Ser Ala Tyr
         30                                                  40
CAG CGC TTT GAG CCC CGC GCC TAC CTC CGC AAC AAC TAC GCG CCC CCG AGG GGG GAC CTG
Gln Arg Phe Glu Pro Arg Ala Tyr Leu Arg Asn Asn Tyr Ala Pro Pro Arg Gly Asp Leu
         50                                                  60
AGC TGC CCC GAC GGC GTC GGG CCT TGG AAG CTG CGC TGC TTG GCT CAG ACC TTC GCC ACC
Ser Cys Pro Asp Gly Val Gly Pro Trp Lys Leu Arg Cys Leu Ala Gln Thr Phe Ala Thr
         70                                                  80
GGT GAG GTG TCT GGC CGC ACC CTC ATT GAC ATC GGT TCA GGA CCC ACT ATA TAC CAG CTG
Gly Glu Val Ser Gly Arg Thr Leu Ile Asp Ile Gly Ser Gly Pro Thr Ile Tyr Gln Leu
         90                                                  100
CTC AGC GCC TGT GCC CAC TTT GAG GAC ATC ACC ATG ACA GAT TTC CTG GAG GTG AAC CGC
Leu Ser Ala Cys Ala His Phe Glu Asp Ile Thr MET Thr Asp Phe Leu Glu Val Asn Arg
         110                                                 120
CAG GAG CTG AGG CTC TGG CTG CGA GAA GAG CCT GGG GCT TTC GAC TGG AGC GTG TAC AGC
Gln Glu Leu Arg Leu Trp Leu Arg Glu Glu Pro Gly Ala Phe Asp Trp Ser Val Tyr Ser
         130                                                 140
CAG CAT GTC TGC CTC ATC GAG GGC AAG GGG GAA TCC TGG CAG GAG AAG GAG TGC CAG CTG
Gln His Val Cys Leu Ile Glu Gly Lys Gly Glu Ser Trp Gln Glu Lys Glu Cys Gln Leu
         150                                                 160
CGA GCC AGG GTG AAG AGG ATC CTG CCC ATC GAT GTG CAC CGG CCC CAG CCC CTG GGT GCT
Arg Ala Arg Val Lys Arg Ile Leu Pro Ile Asp Val His Arg Pro Gln Pro Leu Gly Ala
         170                                                 180
GGA GGC CTG GCA CCC CTG CCT GCC GAC GCC CTG GTC TCT GCC TTC TGC CTG GAG GCT GTG
Gly Gly Leu Ala Pro Leu Pro Ala Asp Ala Leu Val Ser Ala Phe Cys Leu Glu Ala Val
         190                                                 200
AGT CCA GAC CTG GCC AGC TTC CAG CGG GCC CTG GAC CAC ATC ACC ACA CTG CTG AGG CCT
Ser Pro Asp Leu Ala Ser Phe Gln Arg Ala Leu Asp His Ile Thr Thr Leu Leu Arg Pro
         210                                                 220
GGG GGG CAC CTC CTC CTC ATC GGA GCC CTG GAG GAG TCA TGG TAC CTG GCT GGG GAG GCC
Gly Gly His Leu Leu Leu Ile Gly Ala Leu Glu Glu Ser Trp Tyr Leu Ala Gly Glu Ala
         230                                                 240
AGG CTG GCG GTG GTG CCC GTG CGC GAG GAG GAG GTG AGG GAG GCC CTG GTG CGG AGC GCT
Arg Leu Ala Val Val Pro Val Arg Glu Glu Glu Val Arg Glu Ala Leu Val Arg Ser Ala
         250                                                 260
ATG AGG TGC GGG ATC TGC GCA CTA CAC CTA CAC CAT GCC TGC CCA CCT TCA GAC AGG TGT
MET Arg Cys Gly Ile Cys Ala Leu His Leu His His Ala Cys Pro Pro Ser Asp Arg Cys
         270                                                 280                 284
AGA CGA TGT CAA GGG CAT CTT CTT CAC CTG GCC CAG AAG AAG GTG GGG GTG TGA GGCCCC
Arg Arg Cys Gln Gly His Leu Leu His Leu Ala Gln Lys Lys Val Gly Val Ter
```

20231417S7

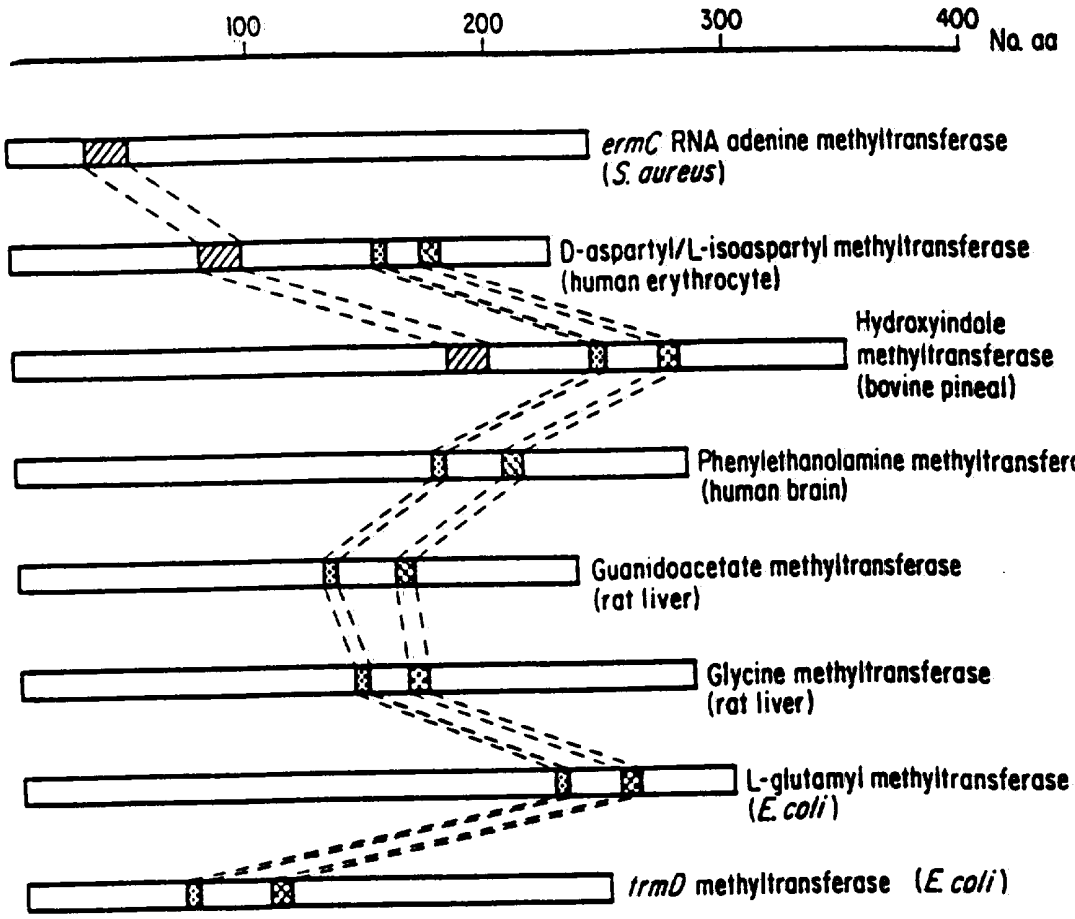


FIG. 6. **Comparison of the location of regions of sequence similarity of the human D-Asp/L-isoAsp and other methyltransferases along their polypeptide chains.** The homology regions, connected by *vertical lines*, are: Region I (*diagonal shading*), Region II (*dotted shading*), and Region III (*diamond shading*). Sequences are detailed in Tables IX (Region I) and X (Regions II and III). Although not listed, Region I is located at a similar position in the other bacterial RNA adenine methyltransferases.

ACTION PLAN 1992

THESE ARE ONLY SUGGESTED PLANS. PRIORITY DECREASES WITH THE INCREASING ASSIGNED NUMBER

1.Obtain aminoacid sequence (~~c-terminal~~) of PMT.

2.Use aminoacid sequence information to synthesize primers for use in PCR.

3.USE PCR to isolate desired sequences from the tobacco root RNA and DNA.

a. RNA PCR(Perkin-Elmer kit,Forward and Reverse primers)

b. DNA pcr(Perkin-Elmer kit,Forward and Reverse Primers)

c. ANCHORED PCR:AMPLIFICATION WITH SINGLE-SIDED SPECIFICITY

1.Take tobacco root poly A RNA.Synthesise complementary strand of

DNA using oligo dT as primer .Destroy RNA by alkali hydrolysis.Amplify Using combinatins of forward primers and oligo dT25 .CLONE the PCR PRODUCT.SEQuence the insert. Enter the sequence into computer.use DNA STAR(findpro programme) to find PMT1.PRO,PMT2.PRO, PMT3.PRO PMT4. PRO sequences.

2.ANCHORED PCR of cDNA with OLIGO(dG)tails.Synthesise cDNA using REVERSE PCR PRIMERS or oligo dT.Add oligo dG to the newly synthesised c DNA strand.Amplify using REVERSE PCR PRIMERS and OLIGO dC.Clone the PCR product AND analyse as in 1.

d.LIGATION-MEDIATED ANCHORED PCR.

Digest the genomic DNA with a Mbo1 OR its isoschizomer Sau3A.

If possible, Fill one base to prevent self-Ligation.
LIgate an anchor-primer on the ends of the DNA fragments.
Amplify with a specific primer as well as the anchor.
The choice of which strand the primer is derived from determines which side of the fragment is amplified .
This can be done for several enzymes that leave cohesive endswith a four-base 5'overhang. This could allow isolation of the promoter for the gene of interest.

4. GENERATION OF SINGLE-STRANDED LABELED PRIMERS AND THEIR USE IN NORTHERN AND SOUTHERN HYBRIDIZATION

5.IMMUNOSCREENING OF EXPRESSION LIBRARY>

SEQUENCE INFORMATION

1. N-terminus of intact 60 kD protein, electroblot/sequenced. (code:PMT1.PRO)

367

```
PMT   L S S N F L F G T A S S Y Y Q Y E G A F L S D G V G L S M --- COOH
Deg   6 6 6 2 2 6 2 4 4 4 6 6 2 2 2 2 2 4 4 2 6 6 2 4 4 4 6 6 2
```

Alignments:

```
PNMT(PET) S(16)          S S   Y Q
PEMT(PhosphatidylEMT)              F Y G D F F L S
```

2. 6.2 kD CNBr Fragment of 60 kD protein, electroblot/sequenced (F-SG-T MOTIF, Region I), (a met would precede all of the CNBr fragments). (code:PMT2.PRO)

```
PMT   M S P X F V A I S G Y R D X T --- COOH
Deg   2 2 4   2 4 4 3 6 4 2 6 2   4
```

Alignments:

```
C/G
RNA MT      I F E I G S G K G H F T
PNMT      (65)F A T G E S G R T
PNMT      (75)L I D I G S G P T Y N L
```

3. 3 kD CNBr Fragment of 60 kD after removal of N-terminus (DAL - Region II). (code:PMT3.PRO)

```
PMT   A D I E H Y S K L I D A L X I K G I Q F --- COOH
        G L   L                           A P
Deg             2 6 2 6 3 2 4 6   3 2 4 3
```

Alignments:

```
PNMT        (125)Y S              K G
PNMT               (174)P A D A L
AdoHcyHMT            K     D A I
```

4. 15 kD CNBr Fragment of 60 kD protein. (code:PMT4.PRO)

```
PMT   F I T E N G F A G R S G R P(?)---  COOH
Deg   2 3 4 2 2 4 2 4 4 6 6 4 6
```

5. Possible sequence order of fragments obtained for 60 kD protein.

From this speculative "motif" examination, a deduced composite can be drawn for putative PMT as follows (cf. PNMT, PNAS 83,5455 (1986):

N-terminus(~3 kD) ----- 6.2 kD fragment ----- other 3 kD fragment ----- 15 kD fragment ---- COOH

Ref. Ingrosso etal., JBC 264,20131-20139 (1989); Baetge etal., PNAS 83,5454-

IN ROOTING STAGE

172 TOTAL

121	VST
7A	46A
1A	49A
13A	19A
47A	9A
50A	17.3A
10.6A	3A & 10A

IN ROOTING STAGE

172 TOTAL

121	VST
7A	46A
1A	49A
13A	19A
47A	9A
50A	17.3A
10.6A	3A & 10A

GENERATION OF TRANSGENIC PLANTS
CURRENT STATUS

IN GREENHOUSE

77 TOTAL

121	13A
VST	46A
17.2A	1A
49A	12A
*47A	*19A
*3A & 10A	

After 10-14 days culture, separate developing calli and transfer to fresh medium

↓

As soon as morphologically acceptable shoots appear, transfer shoots to rooting medium containing 500 mg/L carbenicillin and 100 mg/L kanamycin

↓

After rooting, trangenic plantlets can be transferred to greenhouse or maintained as meristem cultures indefinitely until greenhouse space is available

LEAF DISC TRANSFORMATION

Grow transformed bacterial clones overnight in suspension culture

↓

Excise leaf material from axenic Burley 21 plantlets and cut lamina into 5-10 mm sections

↓

Dip sections into cell culture, blot on sterile gauze and transfer to shoot induction medium without antibiotics

↓

After 2 days incubation (28^{o}C, 16 hr photoperiod, 5000 lux), rinse sections in 500 mg/L carbenicillin, blot and transfer to shooting medium with 500 mg/L carbenicillin and 300 mg/L kanamycin

Electroporate cells at 100 μF capacitance, 250 μsec discharge interval, 480 v, .3mm gap for final field strength of 16000 v/cm

↓

Dilute cells 10-fold with nonselective culture medium and incubate at rt for 30-60 minutes

↓

Plate 10 μl and 70 μl on Luria + 100 μg/ml kanamycin

↓

After incubating for 2-3 days at 28°C, select 3 isolates from each construct and generate pure cultures for leaf transformation

AGROBACTERIUM ELECTROPORATION

Grow overnight suspension culture of Agrobacterium tumefaciens str. LBA4404

↓

Wash cells 2x in 1mM Hepes + 10% glycerol with a final wash in 10% glycerol alone (reduce volume to 500 µl)

↓

Mix 2 µl of DNA with 25 µl cells

↓

Transfer 8 µl of mix to Hoefer bacterial electrode (5.6mm)

GENERATION OF TRANSGENIC PLANTS VIA PLANT TISSUE CULTURE

GENERATION OF TRANSGENIC PLANTS VIA PLANT TISSUE CULTURE

Ask And Ye
Shall Receive

BGM

ASK And Ye
SHALL Receive

BSM

GENERATION OF TRANSGENIC PLANTS VIA PLANT TISSUE CULTURE