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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: REDDY1.3 EXT

In re Patent of:	)	Conf. No.: 4565
Vermuri REDDY et al	)	
Patent No.: 5,639,639	)	
Issued: June 17, 1997	)	Washington, D.C.
For: RECOMBINANT HETERODIMERIC HUMAN FERTILITY HORMONES, AND METHODS, CELLS, VECTORS AND DNA FOR THE PRODUCTION THEREOF	)	December 7, 2004

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OFFICE OF PETITIONS

APPLICATION FOR EXTENSION OF PATENT TERM

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
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Arlington, VA 22202

Sir:

In accordance with 35 U.S.C. §156, patentee, Genzyme Corporation, through the undersigned attorney, hereby applies for extension of the term of the above-identified patent. Following is the information required by 37 C.F.R. §1.740.

(a)(1) The approved product is lutropin alfa also known as recombinant human luteinizing hormone (r-hLH). Lutropin alfa is a water soluble glycoprotein consisting of two non-covalently linked subunits - designated α and β - consisting of 92 and 121 amino acid residues, respectively,

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with carbohydrate moieties linked to Asn-52 and Asn-78 (on the α subunit) and Asn-30 (on the β subunit). The full amino acid sequence of the α and β subunits of lutropin alfa is as follows:

α subunit:

Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro
1 5 10 15

Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys
20 25 30

Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu
35 40 45

Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser
50 55 60

Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr
65 70 75 80

Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
115 120

β subunit:

Ser Arg Glu Pro Leu Arg Pro Trp Cys His Pro Ile Asn Ala Ile Leu
1 5 10 15

Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr
20 25 30

Ile Cys Ala Gly Tyr Cys Pro Thr Met Met Arg Val Leu Gln Ala Val
35 40 45

Leu Pro Pro Leu Pro Gln Val Val Cys Thr Tyr Arg Asp Val Arg Phe
50 55 60

Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val Asp Pro Val Val
65 70 75 80

Ser Phe Pro Val Ala Leu Ser Cys Arg Cys Gly Pro Cys Arg Arg Ser
85 90 95

Thr Ser Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys Asp His
100 105 110

Pro Gln Leu Ser Gly Leu Leu Phe Leu
115 120

Asn: N-glycosylation site

(a)(2) The product was approved under Section 505(b) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. §355(b)).

(a)(3) The product received permission for commercial marketing or use under Section 505(b) of the Federal Food, Drug and Cosmetic Act on October 8, 2004.

(a)(4) As the present product is a human biological product and not a drug product (as those terms are used in the Federal Food, Drug and Cosmetic Act and the Public Health Service Act), 37 C.F.R. §1.740(a)(4) is not applicable.

(a)(5) The present application is being submitted within the sixty-day period permitted for submission pursuant to 37 C.F.R. §1.720(f). It is required that the application be filed within sixty days following the date of the FDA approval letter of October 8, 2004. The sixty-day period expires on December 7, 2004, which is, thus, the last date on which the application can be filed.

(a)(6) The patent for which an extension is being sought is U.S. patent 5,639,639, of which the inventors are Vermuri B. Reddy, Nancy Hsiung, Anton K. Beck, and Edward

George Bernstine. The date of issue was June 17, 1997. Because of the filing of a terminal disclaimer, the date of expiration is June 20, 2006.

(a)(7) A copy of patent 5,639,639 is attached hereto as Exhibit A, including the entire specification (including claims and drawings).

(a)(8) Attached hereto as Exhibit B is a copy of the Terminal Disclaimer filed during the prosecution of the applications that led to the issuance of the '639 patent. Please note that the Terminal Disclaimer was filed in Application No. 07/515,481. The '639 application issued from Application No. 08/268,734, which was a file-wrapper continuation of said Application No. 07/515,481. Thus, the Terminal Disclaimer of Exhibit B is effective with respect to the '639 patent. Also attached hereto as Exhibits C and D are copies of receipts of maintenance fee payments establishing that the first and second maintenance fees were timely paid with respect to this patent.

(a)(9) The patent claims a method of manufacturing the approved product. Each claim of the patent which claims a method of manufacturing the approved product is identified as claims 1, 3, 6 and 8. Attached hereto as Exhibit E are portions of the original NDA submitted during the FDA approval process. Exhibit E includes pages numbered 036-045. These

documents are a summary of the chemistry and the manufacturing process of the approved product. This manufacturing process of the approved product falls within the scope of claim 1 of the '639 patent. Also part of Exhibit E as page 096 is Table BAA-1 showing batch analysis results. Exhibit E also includes pages numbered 242-264, which relate to the development genetics that led to the approved product of the '639 product.

The following showing demonstrates the manner in which claim 1 reads on the method of manufacturing the approved product.

Patent Claim	Approved Product
A process for producing biologically active heterodimeric human fertility hormone selected from the group consisting of human chorionic gonadotropin, human luteinizing hormone and human follicle stimulating hormone, comprising:	Page 036 of Exhibit E states that the active substance that is produced is recombinant human luteinizing hormone, which is one of the products listed in the Markush group in the preamble of claim 1. That it is biologically active is evident from the last line of the table on page 096 of Exhibit E, which shows a specific bioactivity in the various batches running between 25,819 to 31,229 IU/mg. Thus, as the hLH can be measured in international units, biologically active hLH is produced by the method.

transforming a eukaryotic cell	See page 248 of Exhibit E under the heading "Preparation of the production cell line", where it states, "The employed CHO cell line was co-transfected with two expression plasmids, encoding the α- and β-subunits." Thus, it is clear that CHO cells were transformed. Page 041 of Exhibit E, under section 3.4.1.4.2, defines CHO cells as Chinese hamster ovary cells. The Chinese hamster is a mammal, and it is well known that all mammalian cells are eukaryotic.
with at least one vector constructed so as to insert into said cell DNA coding for each of the two subunits of the hormone,	As seen in the above-quoted portion from page 248 of Exhibit E, the CHO cell line was co-transfected with two expression plasmids encoding the α- and β-subunits. The claimed "at least one vector" encompasses two vectors. That the vectors were constructed so as to insert the DNA of the subunits into the cell is evident from the fact that the cell lines ultimately produce both subunits of hLH so as to produce a biologically active product, as can be seen from page 096 of Exhibit E, discussed above.

the translation of the DNA of each said subunit being controlled by a separate promoter; and	That each subunit is controlled by a separate promoter is evident from the third paragraph on page 242 of Exhibit E. The α-genomic fragment was inserted into the expression vector downstream of and juxtaposed to the mouse metallothionein (MMT) gene promoter. Later, it says that the second vector was of similar design but contained the β-hLH cDNA in place of the α-subunit gene and the selectable ODC gene in place of the DHFR gene. Thus, it is apparent from the fact that a similar design was used, that the second vector also contains the MMT gene promoter. This is also evidenced by the restriction enzyme map of pHαDHFR at page 257 of Exhibit E, clearly showing that an MMT-1 promoter is present in this plasmid, and the restriction enzyme map of pHLHβODC on page 260 of Exhibit E, which shows that the β-subunit is also controlled by an MMT promoter. Thus, each subunit is being controlled by a separate promoter.
culturing said cell under conditions appropriate to permit expression of the hormone therefrom,	Reference is made to section 3.4.1.4.3.1 on page 042 of Exhibit E, teaching the conditions of cell culture and the expression of r-hLH therefrom (see the last line of Figure 2 on page 045 of Exhibit E.

wherein said eukaryotic cell is one which permits appropriate post-translational modification of the subunits so produced such that the formed protein is biologically active.	That post-translational modification occurs is evident from the glycosylation pattern and cysteine bridges in the subunits, discussed on pages 036-038 of Exhibit E. Note the statement in the fourth paragraph from the end on page 038, which states, "Correct pairing of the cysteines in the recombinant molecules can be assumed as the *in vivo* bioactivity and immunoactivty of the r-hLH molecule are maintained." Thus, it is apparent that there is post-translational modification and that the formed protein is biologically active.

(a)(10)(i) The Investigational New Drug (IND) application was submitted on December 8, 1993, and received on December 10, 1993. The FDA letter acknowledging receipt was mailed on December 15, 1993, stating that studies may begin 30 days after the date of receipt, i.e., January 9, 1994. The IND application was assigned IND number 44,108. The New Drug Application was initially submitted on April 30, 2001, was received on May 1, 2001, and was assigned NDA number 21-322. The NDA was approved on October 8, 2004.

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(11) The following is a brief description of significant activities undertaken by the marketing applicant during the applicable review period:

IND submitted - protocols for Phase II/III Study 6253 and Study 6905	December 8, 1993
FDA Action Letter and Acknowledgement of Receipt	December 15, 1993
Study 6253 Complete	April 1995
Study 6905 Complete	July 1997
IND Amendment - Protocol for Phase III Study 21008	July 1, 1999
IND Amendment - Protocol for Extension Study 21415	January 31, 2000
Study 21008 Complete	September 2000
Pre-NDA meeting - Study Results Presented	December 12, 2000
NDA Submitted	April 30, 2001
NDA Received	May 1, 2001
Study 21415 Complete	August 2001
Not Approvable Letter	March 1, 2002
NDA Amendment - Report for Study 21415 Submitted	April 28, 2003
Reproductive Health Drugs Advisory Committee	September 30, 2003
NDA Amendment - Phase IV Protocol	October 5, 2004
Approval Letter	October 8, 2004

(a)(12) Applicant is of the opinion that patent 5,639,639 is eligible for patent term extension. Applicant claims a length of extension of 1780 days, which will extend the patent through May 5, 2011. The length of extension was determined as follows using the following dates and time periods as set forth in 37 C.F.R. §1.775:

(c)(1)	01/09/1994 through 05/01/2001	=	2669
(c)(2)	05/01/2001 through 10/08/2004	=	1256
(c)	(c)(1)+(c)(2)	=	3925
(d)(1)(i)	01/09/1994 through 06/17/1997	=	1255
(d)(1)(ii)	not known	=	0
(d)(1)(iii)	((c)(1)-(d)(1)(i))÷2	=	707
(d)(1)	(c)-(d)(1)(i)-(d)(1)(ii)-(d)(1)(iii)	=	1963
(d)(2)	06/20/2006 + (d)(1)	=	11/04/2011
(d)(3)	10/08/2004 + 14 years	=	10/08/2018
(d)(4)	earliest of (d)(2) and (d)(3)	=	11/04/2011
(d)(5)(i)	06/20/2006 + 5 years	=	06/20/2011
(d)(5)(ii)	earliest of (d)(4) and (d)(5)(i)	=	06/20/2011
(d)	(d)(5)(ii)	=	06/20/2011

(a)(13) Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services or the Secretary of Agriculture any information that is material to the determination of entitlement to the extension sought.

(a)(14) Attached hereto is PTO Form 2038, Credit Card Payment Form, authorizing payment in the amount of $1,120.00 in accordance with 37 C.F.R. §1.20(j)(1) for receiving and acting upon the application for extension.

(a)(15) The name, address and telephone number of the person to whom inquiries and correspondence relating to the present application are to be directed is as follows:

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Respectfully submitted,

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Patent 5,639,639

APPLICATION FOR EXTENSION OF PATENT TERM

EXHIBIT A

US005639639A

United States Patent [19]

Reddy et al.

[11] Patent Number: 5,639,639

[45] Date of Patent: *Jun. 17, 1997

[54] **RECOMBINANT HETERODIMERIC HUMAN FERTILITY HORMONES, AND METHODS, CELLS, VECTORS AND DNA FOR THE PRODUCTION THEREOF**

[75] Inventors: **Vermuri B. Reddy**, Westboro; **Nancy Hsiung**, Wellesley, both of Mass.; **Anton K. Beck**, Pfeffiagen, Switzerland; **Edward George Bemstine**, Boston, Mass.

[73] Assignee: **Genzyme Corporation**, Boston, Mass.

[*] Notice: The portion of the term of this patent subsequent to Jun. 20, 2006, has been disclaimed.

[21] Appl. No.: **268,734**

[22] Filed: **Jun. 30, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 515,481, Apr. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 323,772, Mar. 15, 1989, abandoned, and Ser. No. 696,647, Jan. 30, 1985, Pat. No. 4,923,805, which is a continuation-in-part of Ser. No. 548,228, Nov. 2, 1983, Pat. No. 4,840,896, said Ser. No. 323,772, is a continuation of Ser. No. 548,228.

[51] **Int. Cl.**[6] **C12N 15/16**

[52] **U.S. Cl.** **435/69.4**; 435/320.1; 435/325; 435/354; 435/363; 435/365.1

[58] **Field of Search** 530/399; 435/69.4, 435/69.8, 172.1, 172.3, 240.2, 252.3, 320.1; 536/23.51; 935/9, 34, 44

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Primary Examiner—Stephen G. Walsh
Assistant Examiner—Lorraine M. Spector
Attorney, Agent, or Firm—Browdy and Neimark

[57] **ABSTRACT**

Biologically active heterodimeric human fertility hormones composed of two different subunits, each subunit being synthesized in the same cell transformed by at least one cell expression vector having heterologous DNA encoding each subunit with each subunit being controlled by a separate promoter. Preferred human fertility hormones include hCG, hLH and hFSH.

26 Claims, 13 Drawing Sheets

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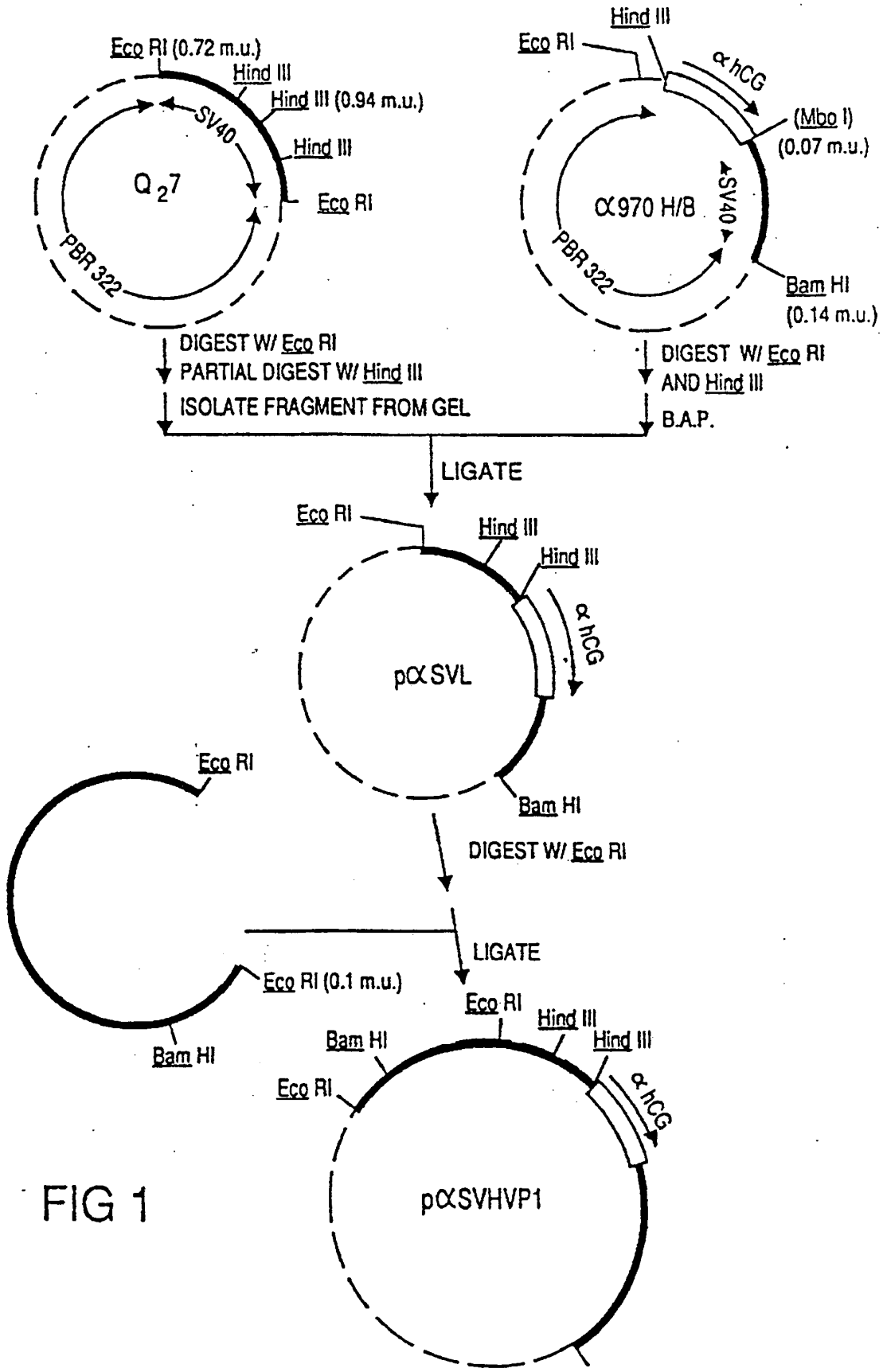
Eco RI (0.72 m.u.)
Hind III
Hind III (0.94 m.u.)
Hind III
Eco RI
SV40
Q 27
PBR 322
Hind III
Eco RI
α hCG
(Mbo I)
(0.07 m.u.)
α970 H/8
SV40
PBR 322
Bam HI
(0.14 m.u.)
DIGEST W/ Eco RI
PARTIAL DIGEST W/ Hind III
ISOLATE FRAGMENT FROM GEL
DIGEST W/ Eco RI
AND Hind III
B.A.P.
LIGATE
Eco RI
Hind III
Hind III
α hCG
pα SVL
Bam HI
Eco RI
DIGEST W/ Eco RI
LIGATE
Eco RI (0.1 m.u.)
Bam HI
Eco RI
Hind III
Hind III
Bam HI
Eco RI
α hCG
pαSVHVP1

FIG 1

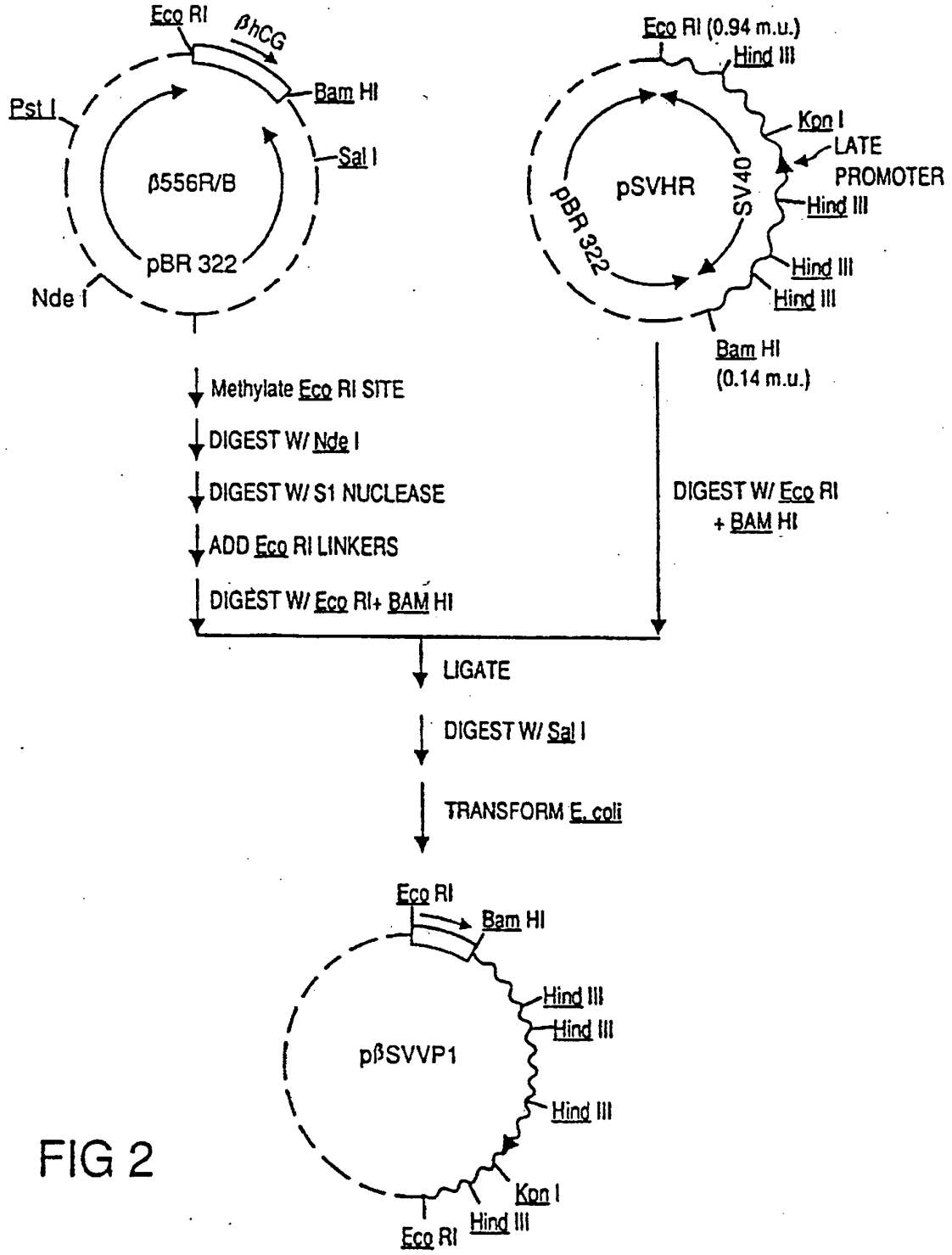
Eco RI
βhCG
Bam HI
Pst I
Sal I
β556R/B
pBR 322
Nde I
Eco RI (0.94 m.u.)
Hind III
Kpn I
LATE
PROMOTER
Hind III
pSVHR
SV40
pBR 322
Hind III
Hind III
Bam HI
(0.14 m.u.)
Methylate Eco RI SITE
DIGEST W/ Nde I
DIGEST W/ S1 NUCLEASE
ADD Eco RI LINKERS
DIGEST W/ Eco RI+ BAM HI
DIGEST W/ Eco RI
+ BAM HI
LIGATE
DIGEST W/ Sal I
TRANSFORM E. coli
Eco RI
Bam HI
Hind III
Hind III
pβSVVP1
Hind III
Kpn I
Hind III
Eco RI

FIG 2

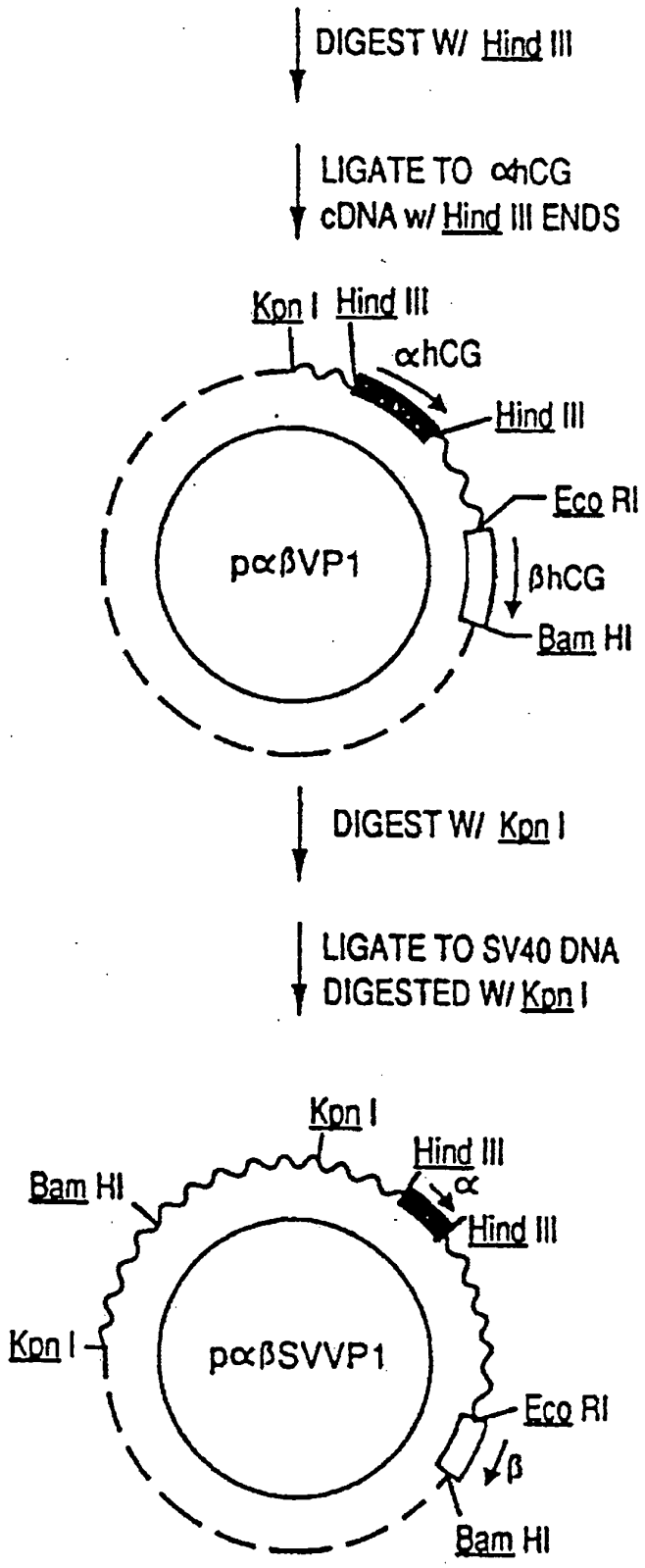
DIGEST W/ Hind III
LIGATE TO αhCG
cDNA w/ Hind III ENDS
Kpn I
Hind III
αhCG
Hind III
Eco RI
pαβVP1
βhCG
Bam HI
DIGEST W/ Kpn I
LIGATE TO SV40 DNA
DIGESTED W/ Kpn I
Kpn I
Hind III
α
Hind III
Bam HI
Kpn I
pαβSVVP1
Eco RI
β
Bam HI

FIG 3

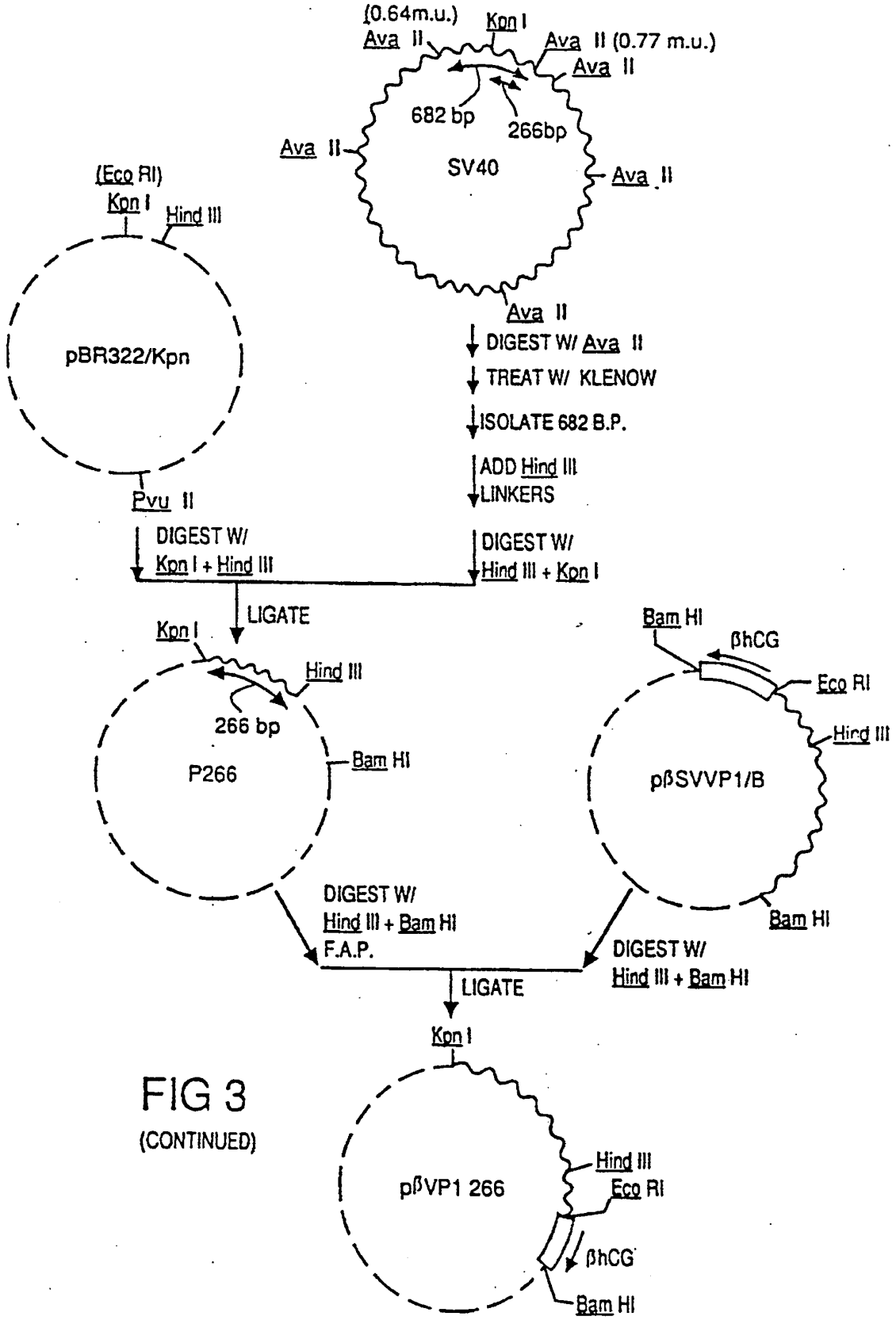

FIG 3
(CONTINUED)

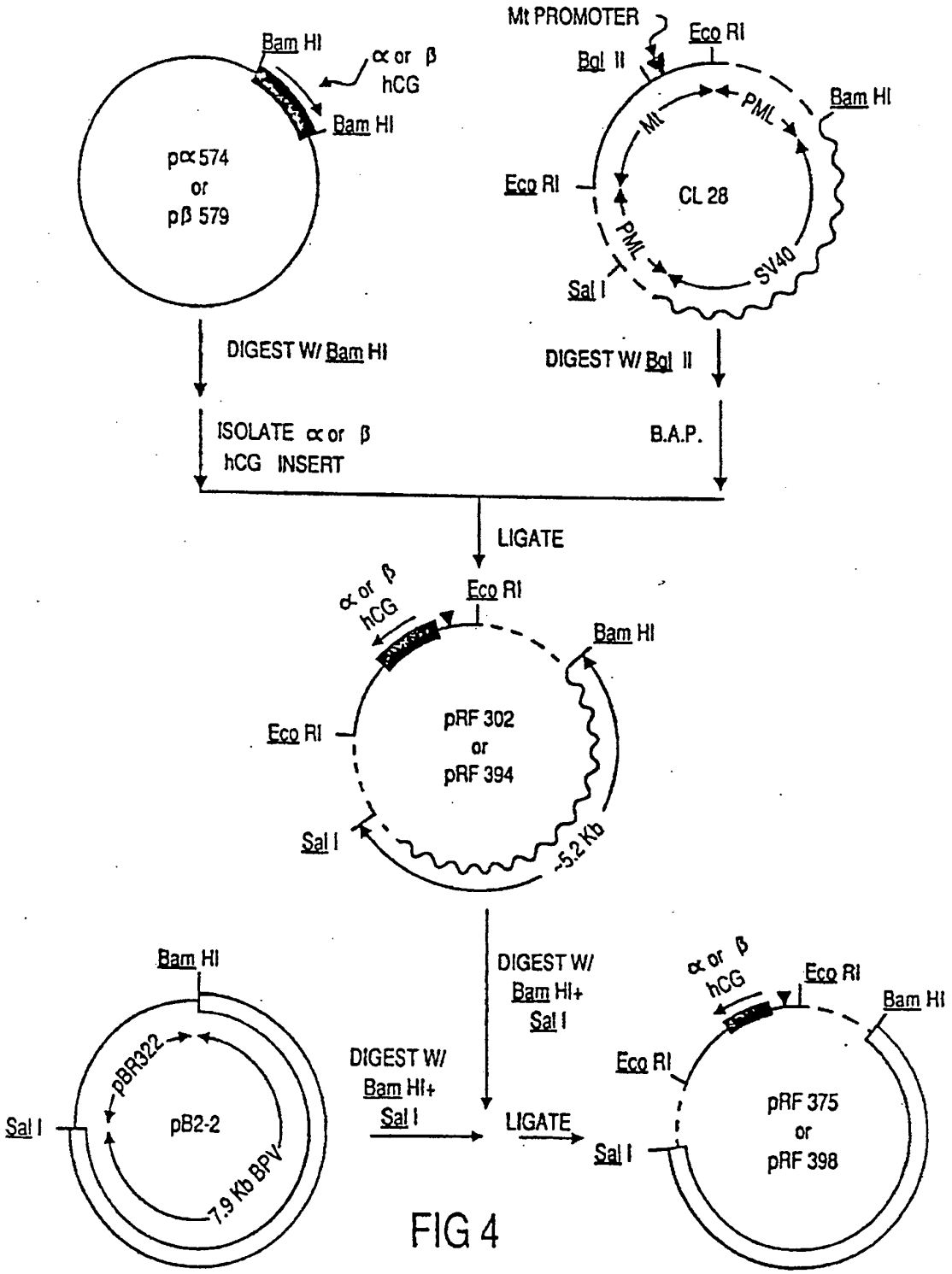
Bam HI
α or β
hCG
Bam HI
pα574
or
pβ 579
Mt PROMOTER
Eco RI
Bgl II
Bam HI
Mt
PML
Eco RI
CL 28
PML
SV40
Sal I
DIGEST W/ Bam HI
DIGEST W/ Bgl II
ISOLATE α or β
hCG INSERT
B.A.P.
LIGATE
α or β
hCG
Eco RI
Bam HI
Eco RI
pRF 302
or
pRF 394
Sal I
~5.2 Kb
Bam HI
DIGEST W/
Bam HI+
Sal I
α or β
hCG
Eco RI
Bam HI
pBR322
Sal I
pB2-2
7.9 Kb BPV
DIGEST W/
Bam HI+
Sal I
LIGATE
Eco RI
pRF 375
or
pRF 398
Sal I

FIG 4

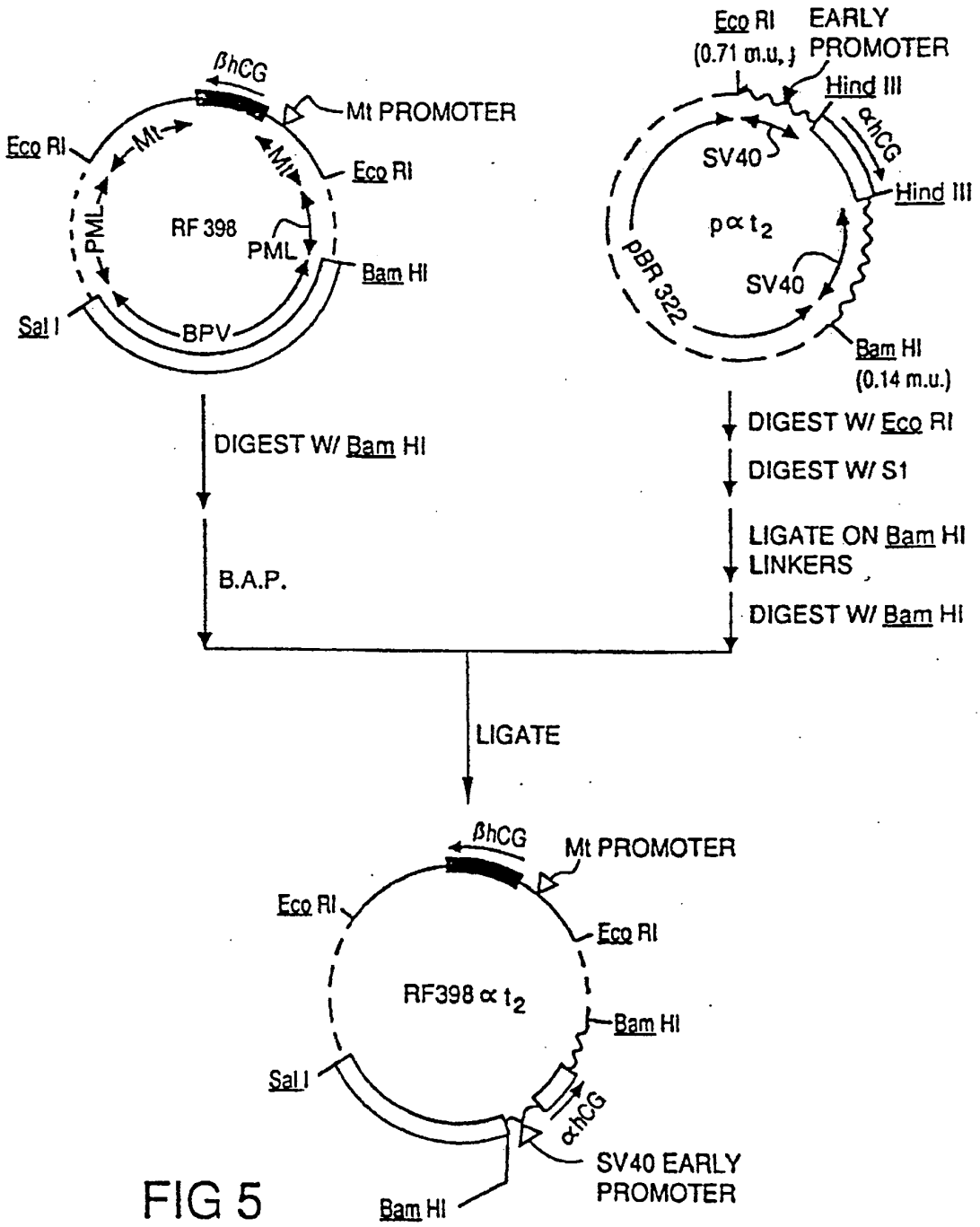
βhCG
Mt PROMOTER
Eco RI
Mt
Mt
Eco RI
PML
RF 398
PML
Bam HI
Sal I
BPV
Eco RI
(0.71 m.u.)
EARLY
PROMOTER
Hind III
αhCG
SV40
Hind III
pαt2
pBR 322
SV40
Bam HI
(0.14 m.u.)
DIGEST W/ Bam HI
B.A.P.
DIGEST W/ Eco RI
DIGEST W/ S1
LIGATE ON Bam HI
LINKERS
DIGEST W/ Bam HI
LIGATE
βhCG
Mt PROMOTER
Eco RI
Eco RI
RF398 αt2
Bam HI
Sal I
αhCG
SV40 EARLY
PROMOTER
Bam HI

FIG 5

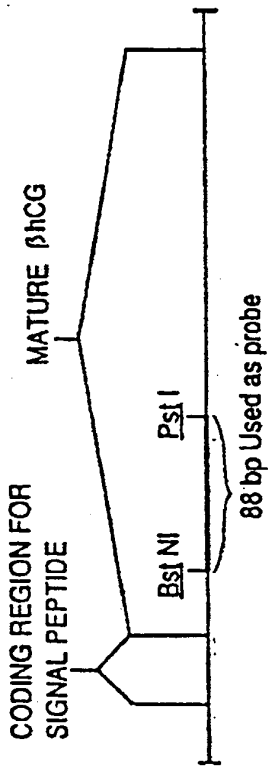
CODING REGION FOR SIGNAL PEPTIDE
MATURE βhCG
Bst NI
Pst I
88 bp Used as probe

FIG 6

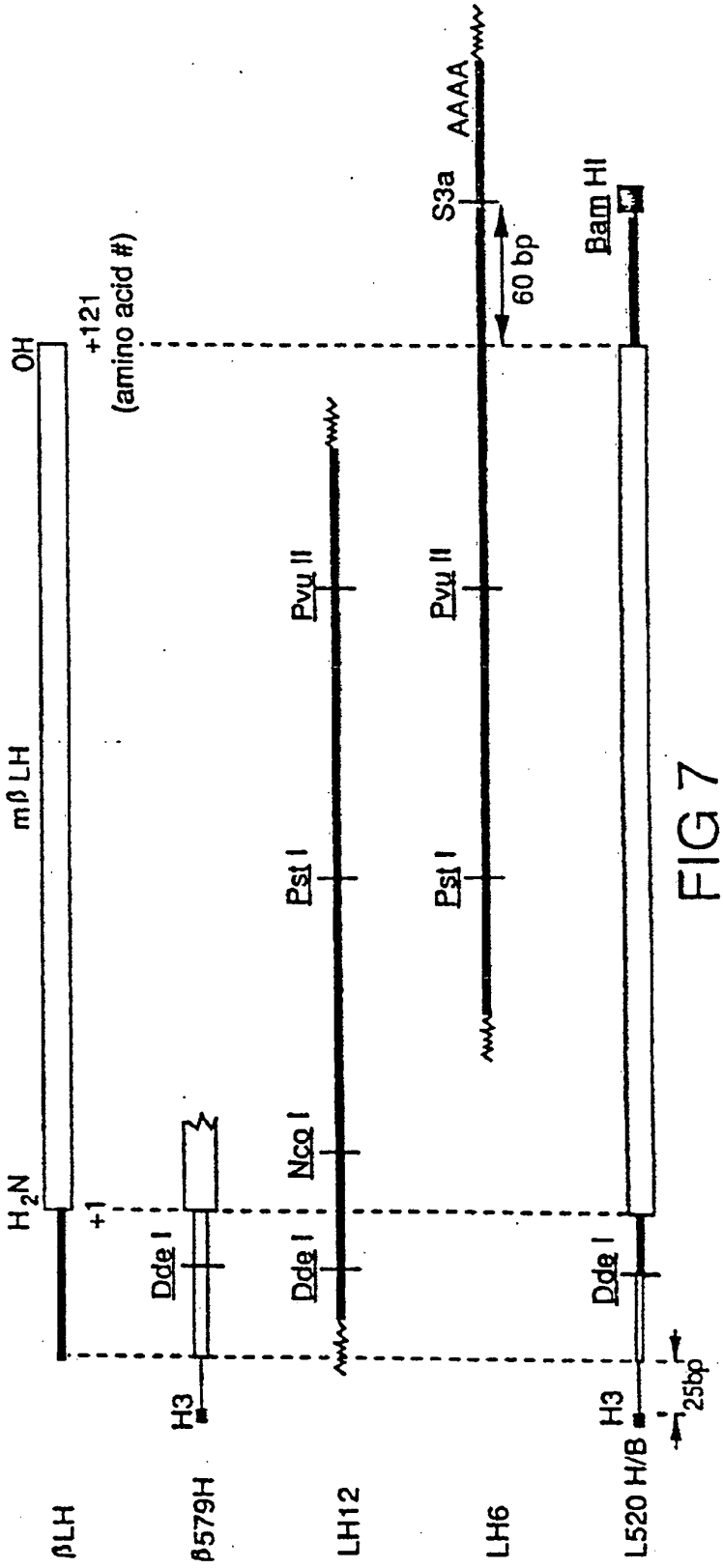
βLH
β579H
LH12
LH6
L520 H/B
H3
Dde I
Nco I
Pst I
Pvu II
H2N
+1
mβ LH
OH
+121
(amino acid #)
S3a
AAAA
60 bp
Bam HI
25bp

FIG 7

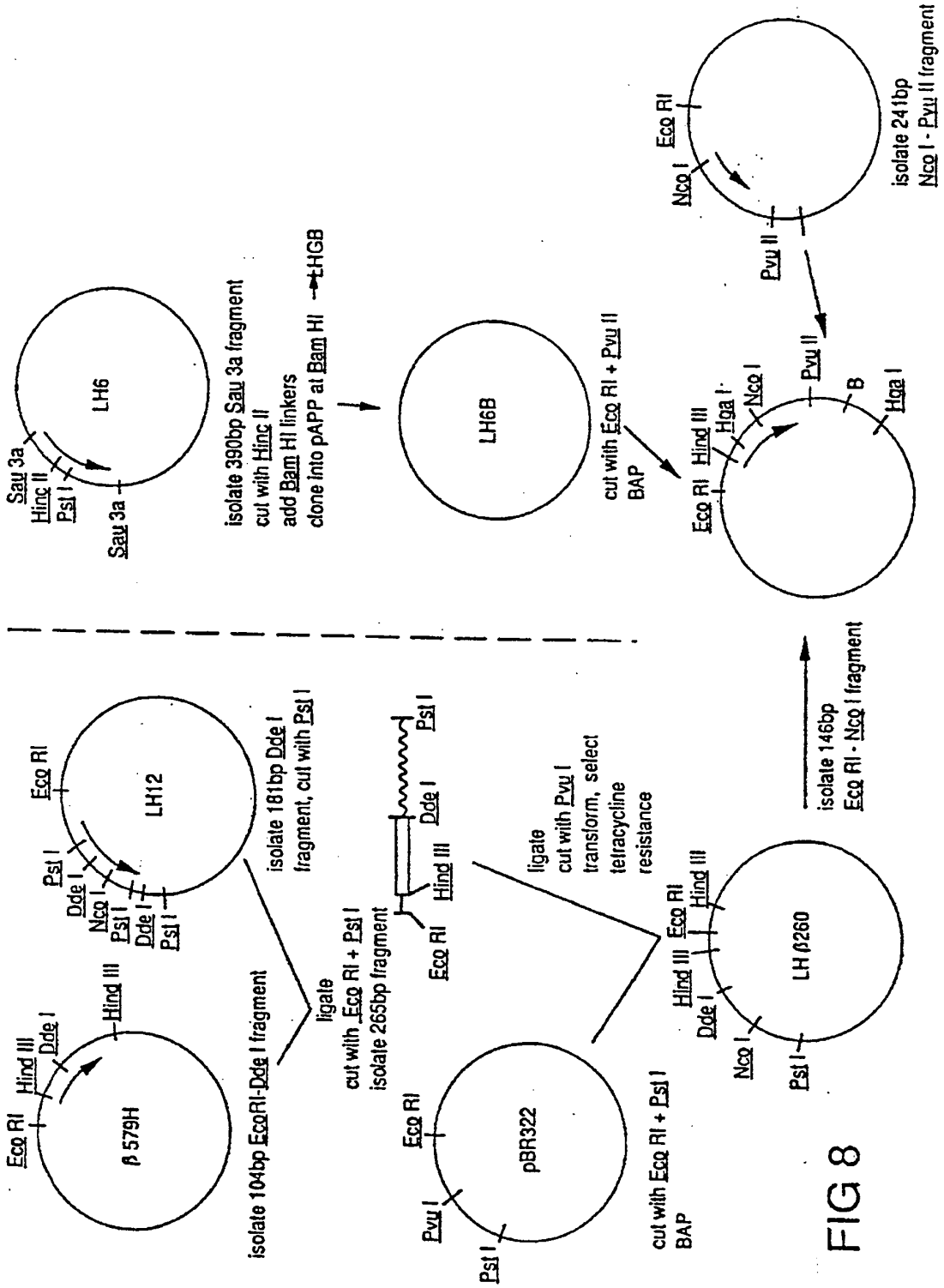
LH6
Sau 3a
Hinc II
Pst I
Sau 3a
isolate 390bp Sau 3a fragment
cut with Hinc II
add Bam HI linkers
clone into pAPP at Bam HI
tHGB
LH6B
cut with Eco RI + Pvu II
BAP
Eco RI
Hind III
Hga I
Nco I
Pvu II
B
Hga I
Pvu II
Nco I
Eco RI
isolate 241bp
Nco I - Pvu II fragment
isolate 146bp
Eco RI - Nco I fragment
LH12
Eco RI
Pst I
Dde I
Nco I
Pst I
Dde I
Pst I
isolate 181bp Dde I
fragment, cut with Pst I
ß 579H
Eco RI
Hind III
Dde I
Hind III
isolate 104bp EcoRI-Dde I fragment
ligate
cut with Eco RI + Pst I
isolate 265bp fragment
Eco RI
Hind III
Dde I
Pst I
pBR322
Eco RI
Pvu I
Pst I
ligate
cut with Pvu I
transform, select
tetracycline
resistance
cut with Eco RI + Pst I
BAP
LH Δ260
Eco RI
Hind III
Hind III
Dde I
Nco I
Pst I

FIG 8

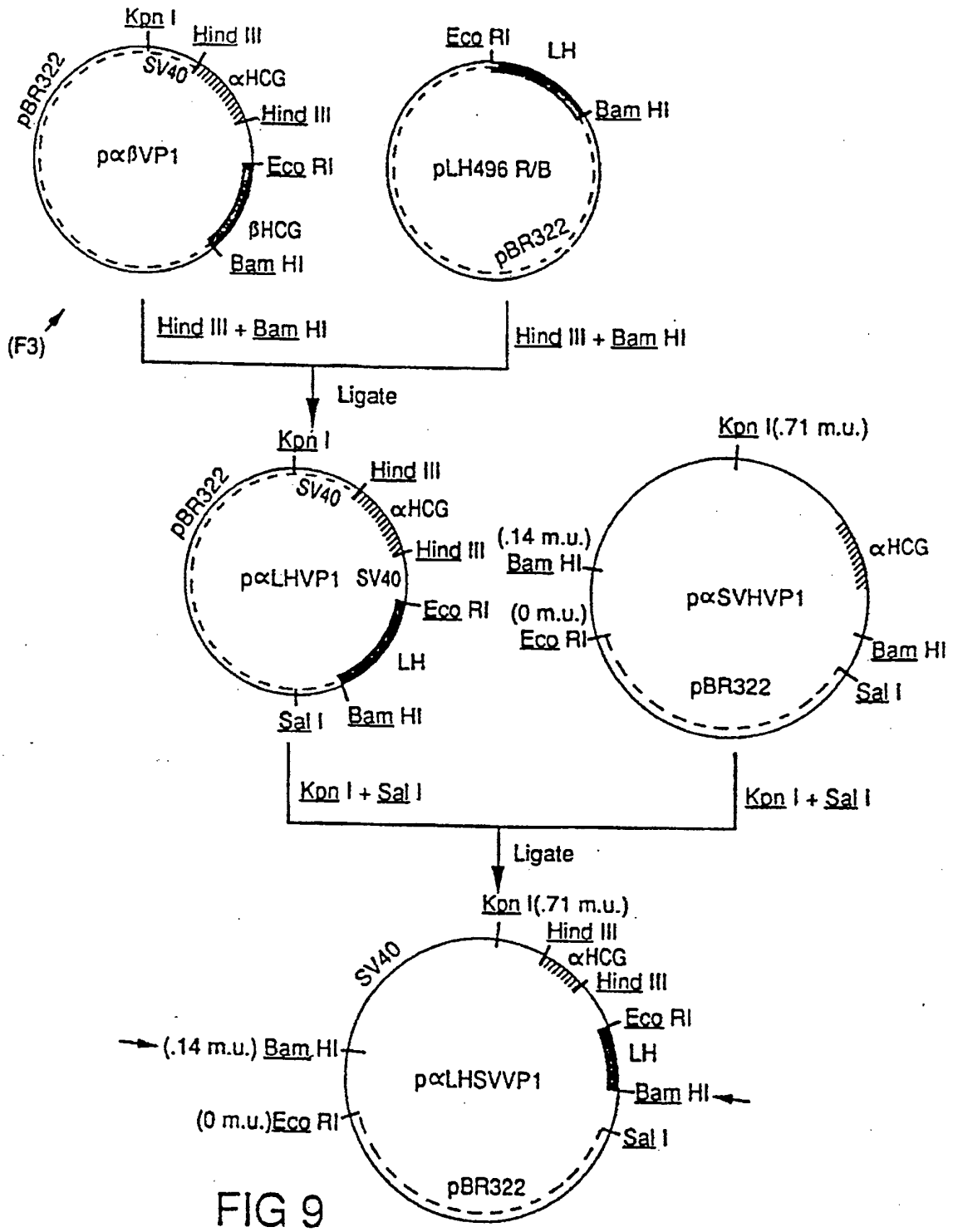
Kpn I
Hind III
SV40
αHCG
Hind III
pBR322
pαβVP1
Eco RI
βHCG
Bam HI
Eco RI
LH
Bam HI
pLH496 R/B
pBR322
(F3)
Hind III + Bam HI
Hind III + Bam HI
Ligate
Kpn I
Hind III
SV40
αHCG
pBR322
Hind III
pαLHVP1
SV40
Eco RI
LH
Sal I
Bam HI
Kpn I(.71 m.u.)
(.14 m.u.)
Bam HI
αHCG
pαSVHVP1
(0 m.u.)
Eco RI
Bam HI
pBR322
Sal I
Kpn I + Sal I
Kpn I + Sal I
Ligate
Kpn I(.71 m.u.)
Hind III
αHCG
SV40
Hind III
Eco RI
(.14 m.u.) Bam HI
LH
pαLHSVVP1
Bam HI
(0 m.u.)Eco RI
Sal I
pBR322

FIG 9

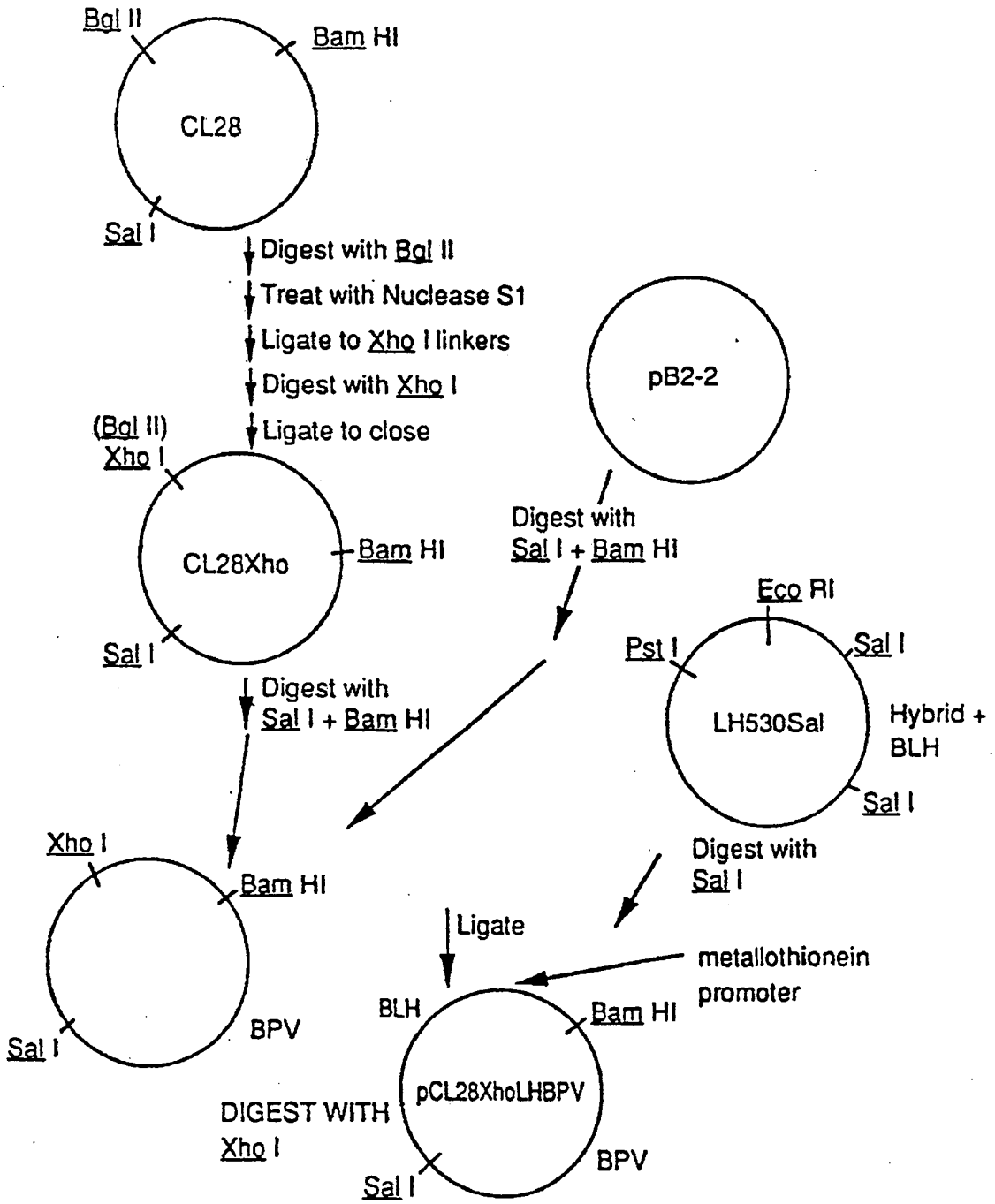
Bgl II
Bam HI
CL28
Sal I
Digest with Bgl II
Treat with Nuclease S1
Ligate to Xho I linkers
Digest with Xho I
Ligate to close
(Bgl II)
Xho I
CL28Xho
Bam HI
Sal I
pB2-2
Digest with
Sal I + Bam HI
Eco RI
Pst I
Sal I
LH530Sal
Hybrid +
BLH
Sal I
Digest with
Sal I + Bam HI
Xho I
Bam HI
BPV
Sal I
Digest with
Sal I
Ligate
metallothionein
promoter
BLH
Bam HI
pCL28XhoLHBPV
DIGEST WITH
Xho I
BPV
Sal I

FIG 10

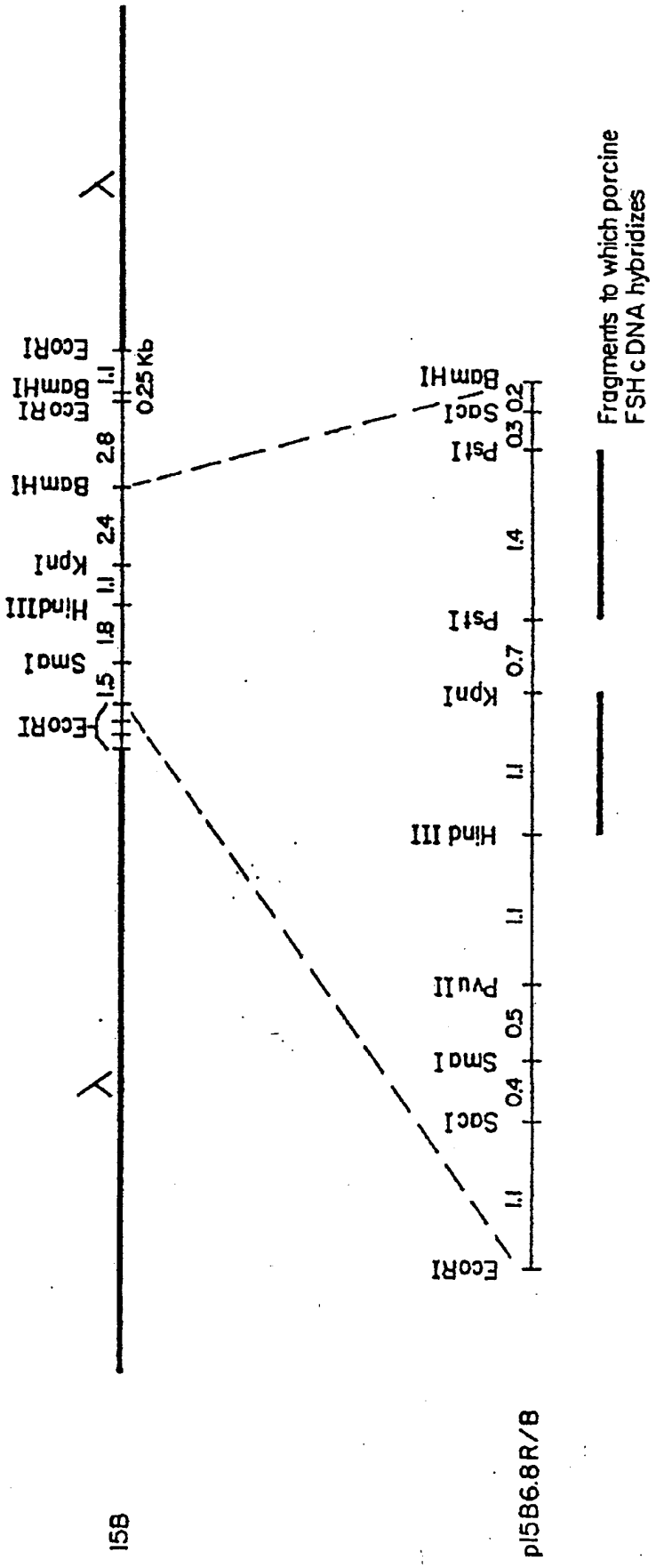
15B
EcoRI
1.1
BamHI
EcoRI
0.25 Kb
2.8
BamHI
2.4
KpnI
1.1
HindIII
1.8
SmaI
1.5
EcoRI
p15B6.8R/B
EcoRI
1.1
SacI
0.4
SmaI
0.5
PvuII
1.1
Hind III
1.1
KpnI
0.7
PstI
1.4
PstI
0.3 0.2
SacI
BamHI
Fragments to which porcine FSH c DNA hybridizes

FIG 11

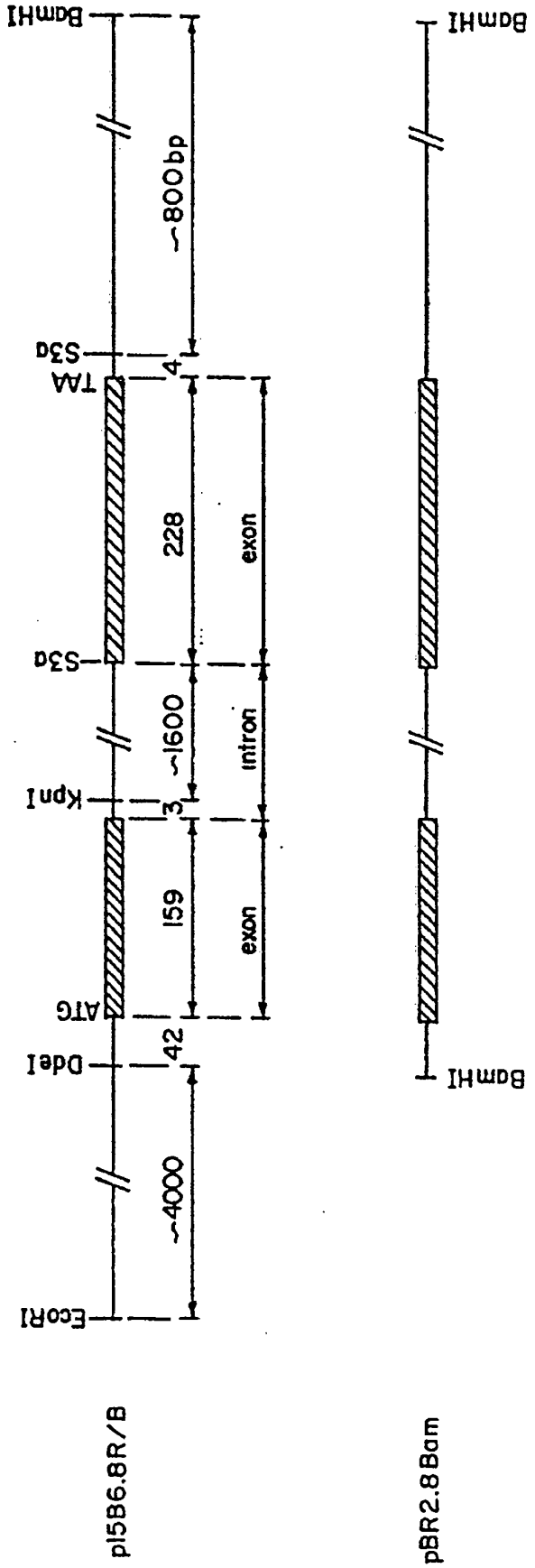
EcoRI
~4000
DdeI
42
ATG
159
exon
KpnI
3
~1600
intron
S3a
228
exon
TAA
4
S3a
~800bp
BamHI
p15B6.8R/B
BamHI
BamHI
pBR2.8Bam

FIG 12

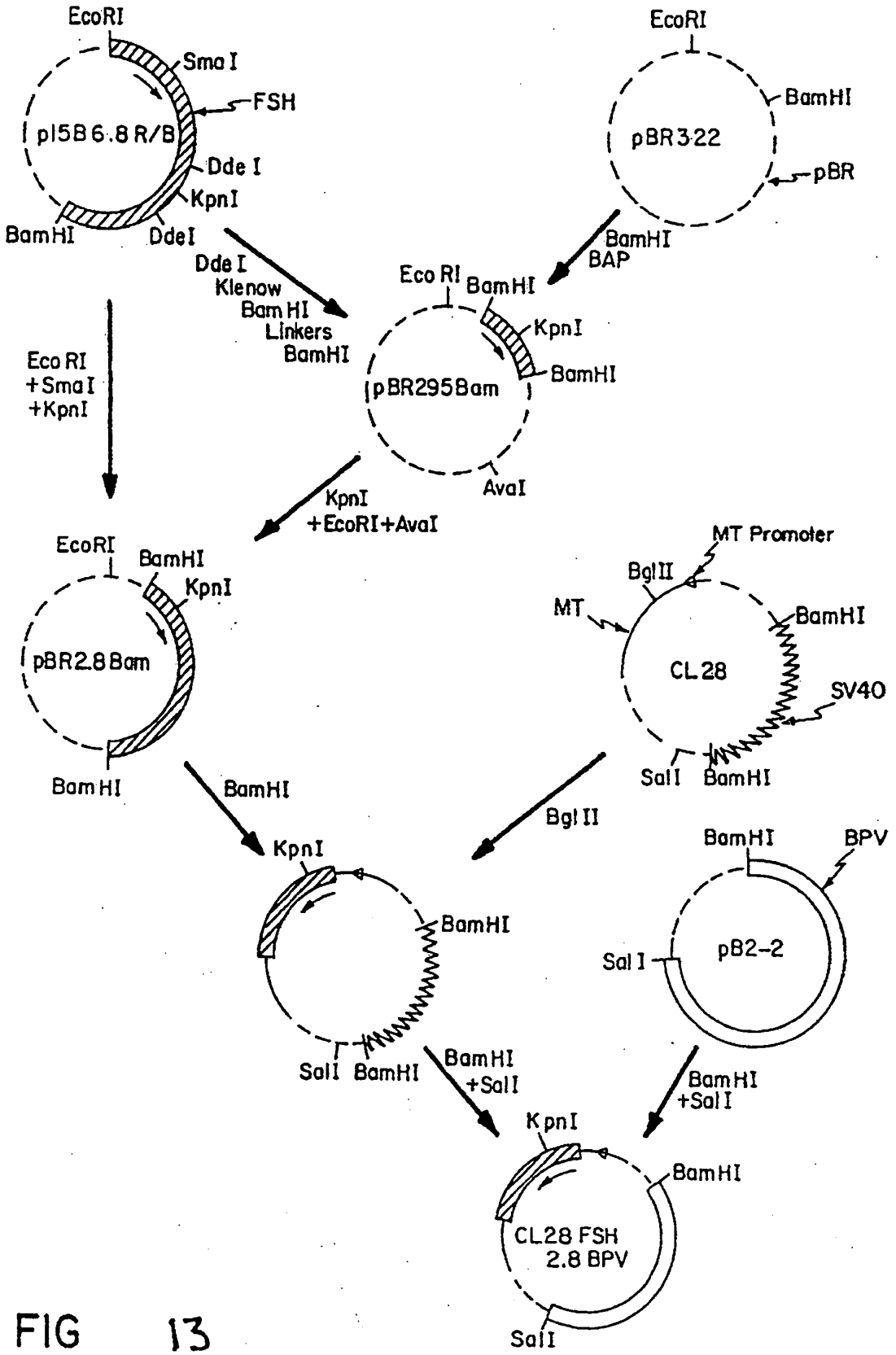
EcoRI
Sma I
FSH
pI5B6.8 R/B
Dde I
KpnI
BamHI
DdeI
Dde I
Klenow
Bam HI
Linkers
BamHI
EcoRI
BamHI
pBR322
pBR
BamHI
BAP
Eco RI
BamHI
KpnI
BamHI
pBR295Bam
AvaI
Eco RI
+Sma I
+KpnI
KpnI
+EcoRI+AvaI
EcoRI
BamHI
KpnI
pBR2.8Bam
BamHI
MT Promoter
BglII
MT
BamHI
CL28
SV40
SalI
BamHI
BamHI
BglII
KpnI
BamHI
SalI
BamHI
BamHI
BPV
SalI
pB2-2
BamHI
+SalI
BamHI
+SalI
KpnI
BamHI
CL28 FSH
2.8 BPV
SalI

FIG 13

RECOMBINANT HETERODIMERIC HUMAN FERTILITY HORMONES, AND METHODS, CELLS, VECTORS AND DNA FOR THE PRODUCTION THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation of application Ser. No. 07/515,481, filed Apr. 27, 1990, now abandoned, which application is a continuation-in-part of both Ser. No. 07/323,772, filed Mar. 15, 1989, now abandoned, and Ser. No. 06/696,647, filed Jan. 30, 1985, now U.S. Pat. No. 4,923,805, the entire contents of both of which are hereby incorporated herein by reference. Ser. No. 07/323,772 is a continuation of Ser. No. 06/548,228, filed Nov. 2, 1983 now U.S. Pat. No. 4,840,896. Ser. No. 06/696,647 is a continuation-in-part of said Ser. No. 06/548,228.

BACKGROUND OF THE INVENTION

The present invention relates to the use of recombinant DNA techniques to produce heterodimeric human fertility hormones.

Various polypeptide chains have been expressed, via recombinant DNA technology, in host cells such as bacteria, yeast, and cultured mammalian cells. Fiddes, J. C. and Goodman, H. M. *Nature,* vol. 281, pp. 351–356 (1979) and Fiddes, J. C. and Goodman, H. M., *Nature,* vol. 286, pp. 684–687 (1980) describe the cloning of, respectively, the alpha and beta subunits of human choriogonadotropin (hCG).

Sugimoto U.S. Pat. Nos. 4,383,034, 4,383,035 and 4,383,036 describe processes for producing FSH, LH and hCG, respectively, in which human lymphoblastoid cells are implanted into a laboratory animal, harvested from the animal, and cultured in vitro; accumulated hormone is then harvested from the culture. This technique is not capable of producing substantially pure hormone free of any other human fertility hormone.

Cohen et al, U.S. Pat. No. 4,468,464 mentions the production of fertility hormones by recombinant DNA techniques. However, Cohen et al only uses a prokaryotic system which cannot produce biologically active human fertility hormone.

Pierce et al, *Ann. Rev. Biochem.,* 50, 465–95 (1981) states that the alpha and beta subunits of LH are known to associate in vitro. The subunits referred to in this paper are obtained by dissociating naturally occurring dimeric hormone and allowing the units to reassociate. Such a disclosure does not permit the prediction that when synthesized by non-specialized cells transformed with recombinant DNA, the subunits would be properly glycosylated and folded for association so as to produce a biologically active hormone.

While many human proteins have been produced by recombinant DNA techniques the production of biologically active heterodimeric hormones by such techniques has not heretofore been accomplished. Heterodimeric fertility hormones are produced in the human body by highly specialized, differentiated cells which have evolved over a long period of time to carry out the specialized function of producing each particular hormone. The mechanism by which post-translational heterodimeric assembly occurs intracellularly in these differentiated cells is not known, but it is known that proper assembly is necessary for biological activity. Undifferentiated cells do not, as far as is known, normally produce hormones. Thus, whether or not a biologically active heterodimeric hormone could be produced in undifferentiated cells transformed with DNA encoding the alpha and beta subunits was totally unpredictable.

SUMMARY OF THE INVENTION

The present invention stems from the unpredictable discovery that biologically active heterodimeric human fertility hormones can be produced in eukaryotic cells transformed by vectors containing the alpha and beta subunits of the hormone controlled by separate promoters. While alpha and beta subunits produced in separate cultures will not reassociate to form biologically active hormones, it has unexpectedly been discovered that when both subunits are produced in the same cell, a hormone is expressed which is biologically active.

Thus, the present invention includes the substantially pure heterodimeric human fertility hormones which can now be made totally free of other fertility hormones as well as any other human proteins. It also includes the process for production of such hormones, eukaryotic cells which have been transformed to so produce the hormones and vectors containing the DNA of both the alpha and beta subunits.

The present invention also includes the DNA encoding the beta subunits of hLH and hFSH, including cDNA coding for the beta subunit of human FSH, expression vectors containing such DNA and cells transfected therewith. The invention also includes DNA derivatives according to the genetic code which on expression code for the beta subunit of human FSH according to the present invention. The polypeptide structure of the beta subunit of hFSH has never before been accurately set forth.

Thus, in general, the present invention features, in one aspect, a substantially pure heterodimeric human fertility hormone composed of two different subunits and the process for the production thereof by means of which the two subunits are synthesized by single cell line, each cell having been transformed by an expression vector containing heterologous DNA encoding both subunits under control of separate promoters or two expression vectors each containing heterologous DNA encoding the separate subunits. The cell line is composed of eukaryotic cells which permit appropriate post-translational modification of the subunits such that the formed protein is biologically active. Because of the recombinant DNA technique which is used, the hormones produced are substantially pure and free of any other fertility hormones or any other human proteins. The preferred fertility hormones which are produced in accordance with the present invention are hCG, luteinizing hormone (LH) and follicle stimulating hormone (FSH).

In another aspect, the present invention features a cell transformed by at least one expression vector, which cell is capable of producing a biologically active heterodimeric protein that is encoded at least in part by the vector. In preferred embodiments: a second expression vector encodes a second portion of the protein or at least two subunits of the protein are encoded by a single expression vector; the vectors are autonomously replicating, preferably a replicating virus or a plasmid; the cell is a mammalian cell, such as a monkey or mouse cell; transcription of the different subunits is under the control of the SV40 late promoter; transcription of the alpha subunit of the protein is under the control of the SV40 early promoter and transcription of the beta subunit is under control of the mouse metallothionein promoter, or transcription of both subunits is under the control of the mouse metallothionein promoter; and the expression vector which includes the mouse metallothionein

promoter also includes at least the 69% transforming region of the bovine papilloma virus (BPV) genome.

In another aspect, the invention features an autonomously replicating expression vector including two genes encoding two different heterologous proteins, the genes being under the control of two different promoters, most preferably a metallothionein promoter and a BPV promoter; the use of different promoters advantageously minimizes the possibility of deleterious recombinations.

In a further aspect, the invention features the DNA for the beta subunits of hLH and hFSH.

As used herein, "subunit" refers to a portion of a protein, which portion, or homologue or analogue thereof, is encoded in nature by a distinct mRNA. Thus, for example, a heavy chain and a light chain of an IgG immunoglobulin are each considered a subunit. Insulin, on the other hand, is composed of two chains which are not considered subunits, because both are, in nature, encoded by a single mRNA, and cleavage into two chains naturally occurs only after translation.

The term "expression vector" refers to a cloning vector which includes heterologous (to the vector) DNA under the control of control sequences which permit expression in a host cell. Such vectors include replicating viruses, plasmids, and phages. Preferred vectors are those containing at least the 69% transforming region, and most preferably all, of the bovine papilloma virus genome.

The invention permits the production of a biologically active heterodimeric human fertility hormone from a single culture of transformed cells which hormone undergoes, in the culture, post-translational modification, e.g. glycosylation and proteolytic processing, for biological activity and stability.

In preferred embodiments, each expression vector is autonomously replicating, i.e., not integrated into the chromosome of the host cell. The use of autonomously replicating expression vectors prevents undesirable influence of the desired coding regions by control sequences in the host chromosome.

Other advantages and features of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

We turn now to the preferred embodiments of the invention, first briefly describing the drawings thereof.

FIG. 1 is a diagrammatic illustration of the construction of the plasmid p alpha SVHVPl, which contains the alpha hCG cDNA clone, portions of SV40 viral DNA, and sequences of the plasmid pBR322.

FIG. 2 is a diagrammatic illustration of the construction of plasmid p beta SVVPl, which incorporates the beta hCG cDNA clone, regions of SV40 DNA and a portion of pBR322 including the region conferring resistance to ampicillin on host *E. coli*.

FIG. 3 is a diagrammatic illustration of the construction of the plasmid p alpha beta SVVPl in which the alpha and beta hCG cDNA clones are inserted into SV40 DNA.

FIG. 4 is a diagrammatic illustration of the construction of the plasmids pRF375 and pRF398.

FIG. 5 is a diagrammatic illustration of the construction of the plasmid RF398 alpha t_2.

FIG. 6 is a diagram illustrating the location of an 88 bp probe within the beta hCG cDNA clone.

FIG. 7 illustrates the beta LH restriction map, and the pieces used in the construction shown in FIG. 8.

FIG. 8 is a diagrammatic illustration of the construction of a plasmid, LH520H/B, containing the complete mature beta LH cDNA clone.

FIG. 9 is a diagrammatic illustration of the construction of the viral vector p alpha LHSVVPl.

FIG. 10 is a diagrammatic illustration of the construction of the BPV-containing plasmid pCL28XhoLHBPV, encoding the beta subunit of LH.

FIG. 11 is a partial restriction map of the lambda clone 15B and the beta FSH-containing 6.8 kb EcoRI-BamHI fragment that is inserted into pBR322.

FIG. 12 is a partial restriction map of the beta FSH coding region and the BamHI fragment that is inserted into a BPV based expression vector.

FIG. 13 is a diagrammatic illustration of the construction of the BPV-containing plasmid CL28FSH2.8BPV, encoding the beta subunit of FSH.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

The cloning vectors of the invention have the general structure recited in the Summary of the Invention, above. Preferred vectors have the structures shown in the Figures, and are described in more detail below.

Construction of Cloning Vectors

Isolation of cDNA Clones Encoding the Alpha and Beta Subunits of hCG

All of the techniques used herein are described in detail in Maniatis et al, (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory), hereby incorporated by reference.

RNA is extracted from placental tissue by the following method. Homogenization of the tissue is carried out in a 1:1 mixture of phenol:100 mM Na-acetate (pH 5.5) containing 1 mM EDTA, that has been warmed to 60° C. for 20 min. After cooling on ice for 10 min., the phases are separated by centrifugation. The hot phenol extraction is repeated twice more followed by two extractions with chloroform.

RNA is precipitated from the final aqueous phase by the addition of 2.5 volumes of ethanol.

In order to enrich for poly A^+ mRNA, placental RNA is passed over oligo (dT)-cellulose in 0.5M NaCl buffered with 10 mM Tris-HCl, pH 7.5, and washed with the same solution. Poly A^+ mRNA is eluted with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.05% SDS and precipitated twice with ethanol. Typical initial yields are 1.5–2.0 mg of total RNA per g of tissue, of which about 2% is poly A^+ mRNA.

Placental cDNA libraries are constructed by reverse transcription of placental mRNA, second strand synthesis using *E. coli* DNA polymerase I (large fragment), treatment with S1 nuclease, and homopolymer tailing (dC) with terminal deoxynucleotidyl transferase; all such procedures are by conventional techniques.

In a typical preparation, 20–30% conversion of mRNA to single strand (ss) cDNA; 70% resistance to digestion with nuclease S1 after second strand synthesis; and dC "tails" of ten to twenty-five bases in length, are obtained. These cDNA molecules are then annealed to DNA fragments of the plasmid pBR322, which has been digested with PstI, and to which dG "tails" have been added. These recombinant

plasmids are then used to transform *E. coli* cells to generate a cDNA library (transformed cells are selected on the basis of tetracycline resistance).

In order to identify the human alpha hCG clone, a 219 bp fragment of a mouse alpha thyroid stimulating hormone (TSH) clone is used as a hybridization probe. This probe has 77% sequence homology with the human clone. It is radioactively labeled by nick translation and hybridized to the cDNA library under conditions that take into account the extent of homology. Strongly hybridizing clones are analysed by restriction mapping and clones containing the complete coding sequence of alpha hCG are verified by DNA sequencing.

Construction of Plasmid p alpha SVHVPl

Referring to FIG. 1, in order to construct the plasmid alpha 970 H/B, a cDNA clone containing the alpha hCG fragment is digested with NcoI. The NcoI site, just 5' to the ATG codon signalling initiation of translation, is filled in and ligated to a synthetic HindIII linker. Similarly, the natural HindIII site in the 3' untranslated region of the clone is cut, filled in with *E. coli* DNA polymerase Klenow, and then ligated to a synthetic BamHI linker. This fragment is cloned into the plasmid pBR322 between its HindIII and BamHI sites to generate the plasmid alpha 574 H/B. This plasmid is digested with BamHI, treated with alkaline phosphatase, and ligated to the 396 bp Sau3A fragment of SV40 DNA (from 0.07 to 0.14 map units) which has been isolated from a polyacrylamide gel. The ligation mix is used to transform *E. coli* to ampicillin resistance and the desired plasmid, alpha 970 H/B, is identified among the transformants.

The plasmid Q_27 is constructed by cutting SV40 at its HsaII site, making flush ends by digestion with nuclease S1, ligating on EcoRI linkers, digesting with EcoRI, and cloning the resulting 1436 bp fragment into the EcoRI site of pBR322.

Referring to FIG. 1, Q_27 is digested completely with EcoRI and partially with HindIII; the fragment from 0.72 to 0.94 map units is isolated and cloned into alpha 970 H/B, which has been digested with ScoRI and HindIII and treated with alkaline phosphatase. The ligation mix is used to transform *E. coli*, and the desired plasmid, p alpha SVL, is identified among the transformants by restriction mapping.

p alpha SVL is digested with EcoRI and the fragment of SV40, with EcoRI ends, extending from 0 to 0.72 map units, and containing the SV40 origin of replication and the intact early region, is ligated to it to generate the plasmid p alpha SVHVPl, which is isolated from *E. coli* transformants.

Construction of Plasmid p beta SVVPl

A 579 bp cDNA clone coding for beta hCG was obtained from John C. Fiddes at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (Fiddes et al, *Nature*, vol. 286, pp. 684–687 (1980)). This fragment is ligated at each end to synthetic BamHI linkers. After digestion by HgaI restriction enzyme, the ends are filled in with Klenow DNA polymerase and synthetic EcoRI linkers are ligated on so that an EcoRI site is about 10 bp 5' to the ATG codon of the signal peptide coding sequence. A BamHI site is about 60 bp 3' to the nonsense codon marking the end of the coding sequence. Referring to FIG. 2, this 556 bp EcoRI-BamHI fragment is isolated and cloned into pBR322, between the EcoRI and BamHI sites, to give the plasmid p beta 556 R/B.

In order to construct the plasmid pSVHR (FIG. 2), SV40 DNA is partially digested with HindIII to yield linear molecules, digested with nuclease S1 to make flush ends, ligated to synthetic EcoRI linkers and digested with EcoRI and BamHI. The fragment from 0.94 to 0.14 map units, containing the SV40 origin of replication and early region, is cloned into pBR322 as an EcoRI-BamHI piece.

Referring still to FIG. 2, the EcoRI site of the plasmid p beta 556 R/B is methylated in a reaction catalyzed by EcoRI methylase, following which the plasmid is cut with NdeI. EcoRI linkers are ligated to the S1 treated NdeI flush ends and activated by digestion with EcoRI, which is followed by digestion with BamHI.

The SV40 fragment of pSVHR from the EcoRI site to the BamHI site is isolated and ligated in a reaction mix containing the digestion fragments of p beta 556 R/B. Following ligation, the mix is digested with SalI to eliminate plasmids which have re-inserted the EcoRI (NdeI) to BamHI piece of pBR322. *E. coli* is transformed with the digested ligation mix and p beta SVVPl is identified and isolated.

Construction of the Plasmid p Alpha Beta SVVPl

Referring to FIG. 3, pBR322/Kpn is derived from pBR322 by inserting a KpnI linker into its unique EcoRI site, after this site is deleted by digestion with EcoRI, followed by digestion with S1 nuclease.

Referring still to FIG. 3, SV40 DNA is digested with AvaII. The staggered ends of the resulting fragments are filled in by Klenow DNA polymerase to form flush ends, and the mixture is then fractionated on a polyacrylamide gel. The 682 base pair fragment (0.64 to 0.77 map units) containing the origin of replication and to the unique KpnI site is isolated from the gel, ligated to synthetic HindIII linkers, and digested with HindIII and KsnI.

The resulting fragments are ligated to pBR322/Kpn. p266, which contains the 266 base pair KpnI HindIII fragment, including the SV40 late promoter region, is isolated. p266 is cut with HindIII and BamHI, and treated with bacterial alkaline phosphatase.

Still referring to FIG. 3, p beta SVVPI/B is constructed as follows: p beta SVVPI (FIG. 2) is cut with EcoRI, followed by ligation to eliminate pBR322 sequences. Subsequently, this DNA is cut with BamHI and cloned into the BamHI site of pBR322.

The resulting plasmid, p beta SVVPI/B, is then digested with HindIII and BamHI and the 1003 base pair HindIII-BamHI fragment is ligated into p266 to yield the plasmid p beta VPl 266, in which the beta hCG cDNA is positioned downstream from the SV40 late promoter in such a way that its RNA transcript would be spliced as if it were the viral VP1 transcript.

The alpha hCG cDNA is inserted into p beta VPl 266 as a HindIII fragment, which has been cut at its HindIII site and treated with bacterial alkaline phosphatase. *E. coli* transformants derived from this ligation are screened by restriction mapping, and plasmids are isolated that have the desired structure, in which the alpha hCG cDNA has replaced VP2 in the correct orientation, followed downstream by the beta hCG cDNA, which has replaced VP1.

One such isolated plasmid, p alpha beta VP1, is used to complete the construction of p alpha beta SVVPl. The plasmid is cut with KpnI, and the full SV40 genome, cut with KpnI, is inserted by ligation into this site. Following transformation of *E. coli*, a plasmid with the required structure, p alpha beta SVVPl, is isolated. This plasmid contains DNA encoding both the alpha and beta subunits of hCG, and thus is capable of directing the expression, in host

mammalian cells, of both subunits, whereby biologically functional, glycosylated heterodimeric hCG is produced (glycosylation occurs post-translationally).

Construction of Plasmids pRF 375 and pRF 398

Referring to FIG. 4, the plasmid CL28 (identical to plasmid JYMMT(E); Haner et al., *J. Mol. Applied Gen.*, 1, 273–288 (1983)), containing the murine metallothionein promoter, SV40 DNA, and pBR322 sequences, is cut with the restriction endonuclease Bgl II. At this site are inserted cDNA clones of either alpha hCG or beta hCG, containing untranslated regions of about 10 and 30 bp at their 5' and of about 220 and 60 bp at their 3' ends. These clones have been genetically engineered by the addition of synthetic BamHI linkers at their termini.

The resulting plasmids pRF 302 (alpha) or pRF 394 (beta) are digested with restriction enzymes BamHI and SalI to release the SV40 DNA sequences.

Plasmid pB2-2, which contains the entire BPV genome, and some pBR322 sequences, is digested with BamHI and SalI to yield the BPV genome with BamHI/SalI ends; this fragment is ligated into pRF 302 (alpha) and pRF 394 (beta) containing the metallothionein-hCG sequences.

Following transformation of *E. coli*, plasmids pRF 375 and pRF 398 are identified and isolated. They encode alpha hCG or beta hCG, respectively, under the control of the mouse metallothionein promoter.

Construction of the Plasmid RF 398 alpha t_2

Referring to FIG. 5, the plasmid p alpha t_2 is derived by cloning the alpha hCG 574 HindIII fragment into plasmid pVBt2 (V. B. Reddy et al., *PNAS*, 79, 2064–2067, 1982). p alpha t_2, which contains the alpha hCG cDNA under the control of the SV40 early promoter, is digested with EcoRI. The 5' overhangs are removed by S1 nuclease digestion prior to the addition of synthetic BamHI linkers by blunt end ligation.

Plasmid RF 398 (FIG. 4) is digested with BamHI and treated with bacterial alkaline phosphatase. The 1735 base pair BamHI fragment of p alpha t_2 is inserted in to RF 398. The resulting plasmid RF 398 alpha t_2 is isolated from *E. coli* transformants. This plasmid thus has the beta hCG cDNA in a transcriptional unit under control of the mouse metallothionein promoter and the alpha hCG cDNA in a transcriptional unit controlled by the SV40 early promoter.

Expression of Luteinizing Hormone (LH) cDNA Clones

Construction of a Human Pituitary cDNA Library

RNA is prepared from human pituitaries by homogenizing 5 to 10 grams of the frozen glands in 20 ml of a solution containing 4M guanidine thiocyanate, 1M 2-mercaptoethanol, 0.05M Na-acetate (pH 5.0), and 0.001M EDTA. One g CsCl is added per ml of homogenate and the suspension is centrifuged at 2,000 rpm for 15 min. The supernatant is layered carefully over a 15 ml cushion of CsCl solution (containing 1.25 ml of 1M Na-acetate (pH 5), 62.5 microliters of 0.4M EDTA and 39.8 g of CsCl in a final volume of 35 ml) and centrifuged at 45,000 rpm in the Ti 70 rotor of a Beckman ultracentrifuge for 18–24 h at 20° C. The RNA visible as a pellicle in the gradient is removed with a syringe, diluted, and precipitated by the addition of two volumes of ethanol. Following three cycles of dissolution and reprecipitation, the RNA pellet is dissolved in H_2O and brought to 0.01M Tris-HCl (pH 7.5) and 0.5M NaCl by the addition of concentrated stock solutions. The preparation is then enriched for poly A^+ mRNA by two passes over oligo dT-cellulose, as described above in the case of placental RNA.

A human pituitary cDNA library is constructed from the poly A^+ mRNA as described above for placental poly A^+ mRNA except that both the large fragment *E. coli* DNA polymerase I and the avian myeloblastosis virus reverse transcriptase are used sequentially for second strand cDNA synthesis. Reverse transcriptase is used first. The reaction is stopped by phenol extraction. The aqueous phase of the centrifuged extract is applied to a 5 ml column of BioGel A-5m. Fractions containing high molecular weight material are pooled, concentrated, precipitated with two volumes of ethanol, dried, and dissolved in 100 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 20 mM 2-mercaptoethanol, 1 mM of each of the four deoxyribonucleoside triphosphates, for reverse transcription. Reverse transcriptase is added to about 20 units per microgram of cDNA. Double stranded cDNA is then treated with nuclease S1, tailed, and cloned as described above.

Isolation of Beta LH cDNA Clones

Colonies grown on nutrient agar plates containing 25 micrograms per ml of tetracycline are transferred to nitrocellulose filters. Colonies are lysed in situ by treatment with 0.5M NaOH and neutralized with 0.5M Tris-HCl (pH 7.4) containing 1.5M NaCl. Liberated DNA is fixed to the filter by baking at 80° C. in a vacuum oven for 2 h. The filters are screened by hybridization to a ^{32}P labeled 88 base pair fragment of the beta hCG clone corresponding to amino acids 16 to 45 of the mature hCG beta chain, which has 29 of 30 amino acids in common with this region of the beta LH polypeptide (FIG. 6). Hybridization is carried out overnight at 32° C. in 50% formamide, 0.75M NaCl, 0.075M Na-citrate (pH 7.0), 2.5% dextran sulfate, 0.1% polyvinylpyrollidone, 0.1 mg per ml bovine serum albumin, and at least 10^5 cpm per filter of ^{32}P-labeled 88 bp beta hCG fragment. Filters are washed several times in 0.15M NaCl, 0.015M Na-citrate at 37° C. before autoradiography. One of the positive isolated clones LH12 (FIG. 7), is used further. LH12 is 365 bp long and includes sequences coding for 15 amino acids of the pre-beta signal sequence plus 105 amino acids of the mature beta LH polypeptide. Its nucleotide sequence is determined. Since the complete mature beta LH is not coded by LH12, further screening of the human pituitary cDNA library is carried out using a 240 bp NcoI-PvuII fragment of LH12 (FIG. 7) as a ^{32}P labeled hybridization probe. The clone LH6 (FIG. 7) is isolated from this screening. LH6 contains the complete 3' end of beta LH, including the region corresponding to the untranslated portion of the mRNA through 27 A residues of the poly A "tail" of the mRNA. No clones are found that extended further than LH12 in the 5' direction. DNA sequencing of the complete, combined mature beta LH coding regions reveals two differences in the amino acid sequence of beta LH from the published protein sequence data: position 42 is a methionine and position 55 is a valine. Also, the mature beta LH contains 121 amino acids, based on the cDNA sequence.

A clone containing an intact signal peptide coding sequence and the complete mature beta LH sequence is constructed as shown in FIG. 8, using the restriction fragments illustrated in FIG. 7. A 104 bp EcoRI-DdeI fragment is isolated from the plasmid beta 579 H and ligated to an isolated 181 bp DdeI fragment, subsequently digested with PstI, from the LH12 plasmid. Following ligation overnight

at 15° C., the ligation mix is digested with EcoRI and PstI and fractionated on a 7% polyacrylamide gel from which the desired 256 bp fragment is isolated. This fragment fuses the beta hCG signal sequence to that of the pre-beta LH in such a way as to provide a coding sequence for a 20 amino acid signal peptide.

The 256 bp EcoRI-PstI fragment is cloned into pBR322 digested with EcoRI and PstI so as to give the plasmid LH beta 260. The 146 bp EcoRI-NcoI fragment indicated in FIG. 8 is isolated from a polyacrylamide gel and used later in the construction as described below.

The LH6 plasmid (FIG. 8) is digested with Sau3a and the 390 bp fragment is isolated by polyacrylamide gel electrophoresis. This fragment is then digested with HincII, ligated to BamHI linkers, digested with BamHI, and cloned into the plasmid pAPP at the BamHI site. pAPP is derived from pBR322 by digestion with AvaI, filling in the 5' overhang with the dNTP's and the large fragment DNA polymerase I of *E. coli*, digestion with PvuII, and ligation to close the plasmid so as to eliminate the PvuII site. The plasmid LH6B, isolated from the ligation of the 340 bp BamHI fragment into the BamHI site of pAPP, is digested with EcoRI and PvuII, and treated with bacterial alkaline phosphatase. The fragments are ligated to a mixture of the 145 bp EcoRI-NcoI fragment of LH beta 260, described above, and the isolated 241 bp NcoI-PvuII fragment from the plasmid LH12 shown in FIG. 8. The ligation mix is used to transform *E. coli* to ampicillin resistance. The plasmid LH 520 H/B is found among the transformants. LH 520 H/B contains a complete beta LH coding sequence including a hybrid signal peptide sequence.

Construction of p Alpha LHSVVP1

In order to express this pre-beta LH clone is an SV40-based vector, as had been done for the pre-alpha and pre-beta hCG clones described previously, it is desirable to place an EcoRI site very close to the ATG of the pre-beta coding sequence. This is accomplished by digesting LH520 H/B with HgaI, filling in the 5' overhang, ligating on synthetic EcoRI linkers, digesting with EcoRI and BamHI, and cloning the isolated 496 bp EcoRI-BamHI fragment into pBR322 digested with EcoRI and BamHI and treated with bacterial alkaline phosphatase. The plasmid pLH496 R/B is isolated from *E. coli* transformed with this ligation mix and is used as the source of the 496 bp fragment to be expressed.

The plasmid p alpha beta VPl, whose construction and use in expression both subunits of hCG is described earlier (FIG. 3), is digested with EcoRI and BamHI and ligated in a reaction mix containing the plasmid pLH496 R/B which had been digested with both of these enzymes (FIG. 9). The plasmid p alpha LHVPl is identified among the *E. coli* transformants. As shown in FIG. 9. the intact SV40 viral early region is taken from p alpha 8VHVPl (FIG. 1) and inserted by ligation as a KsnI-SalI fragment into p alpha LHVPl which had been digested with KsnI and SalI to give the plasmid p alpha LHSVVPl. By cutting this plasmid with BamHI and religating, the virus alpha LHSVVPl is formed. This virus contains cloned cDNA's for the common (to LH and hCG, as well as FSH and TSH) alpha subunit and the specific beta LH subunit under control of the SV40 late promoter. The cloned cDNA's are positioned in such a way that the common alpha insert replaced the viral VP1 protein coding sequence and the beta LH insert replaced the viral VP2 coding sequence.

Insertion of the Beta LH cDNA (With Beta hCG 5' End of Signal Peptide) into a BPV-Based Expression System

LH 520 H/B (FIG. 8) is digested with HindIII and BamHI, treated with the *E. coli* DNA polymerase (Klenow), ligated to synthetic SalI linkers, digested with SalI, and cloned into the SalI site of pBR322. The resulting plasmid, LH 530 Sal, is used as a source of the LH cDNA clone for insertion into the mouse metallothionein gene of the plasmid CL28 as described in FIG. 10.

CL28 is cut with BslII, treated with nuclease S1, and ligated to XhoI linkers. Following digestion with XhoI, ligation and digestion with BslII, *E. coli* is transformed with the reaction mix to give the plasmid CL28Xho. This plasmid is digested with BamHI and SalI and ligated to a BamHI plus 8alI digest of the plasmid pB2-2 (FIG. 4) to give the plasmid CL28XhoBPV. The latter LH insert is then ligated into the XhoI site of CL28XhoBPV as a SalI fragment, since the 5' overhang of SalI digests is complementary to that of XhoI digests. Following digestion with XhoI to eliminate background, *E. coli* is transformed and the desired plasmid pCL28XhoLHBVP containing the (hybrid) pre-beta LH insert, in a BPV-based plasmid, under control of the mouse metallothionein promoter, is isolated.

Transfection and Infection of Host Monkey Cells

The incorporation of virus-containing vectors into eukaryotic cells for the production of a heteropolymeric protein is generally accomplished as follows. First, if the viral DNA and homopolymeric protein-encoding DNA are incorporated into a plasmid, which is maintained, in, say, *E. coli*. the plasmid sequences (e.g. the pBR322 sequences) are removed and the resulting DNA is ligated to form circular DNA including the viral region and the heteropolymeric protein-encoding sequence or sequences. This circular DNA generally does not contain all of the genetic information needed to produce a replicating virus, the other necessary sequences (e.g. those encoding coat protein) having been replaced by the heteropolymeric protein-encoding sequence or sequences. The circular DNA, minus the plasmid DNA, must be close enough in size to the naturally occurring viral DNA from which it is derived to permit the DNA to enter and replicate in appropriate host mammalian cells.

The circular DNA is used to transfect host cells in order to produce virus stock for later infections. Since some of the DNA necessary to produce virus is missing, the transfection must occur in conjunction with helper virus DNA encoding enough of the missing function to produce replicating virus.

Transfected host cells are grown and incubated until lysed by replicating virus. The resulting replicating virus stock, including helper virus, is then used to infect host cells for production of the heteropolymeric protein. Virus stock is maintained, since it generally needs to be reused to infect fresh batches of host cells, as each culture of infected, protein-producing host cells generally is eventually lysed by the virus.

The specific recombinant DNA sequences described above are used to transfect, and then infect, host cells, as follows.

The pBR322 sequences are removed from the above-described SV40-containing plasmids to produce transfecting viral DNA. In the case of p alpha SVHVPl and p alpha beta SVVPl, this is accomplished by digestion with BamHI, followed by ligation under conditions favoring circularization of the fragments to give (among other products) alpha SVHVP1 and alpha beta SVVPl. For p beta 8VVPl. digestion with EcoRI followed by re-ligation brings the SV40 late promoter and VPl splice region into juxtaposition with the beta hCG cDNA insert at the same time that it eliminates pBR322 sequences and forms beta SVPl. At the same time. PtsA58 Bam (tsA58 SV40 viral DNA cloned into the

pBR322 BamHI site) is cut with BamHI and ligated to obtain self-ligated circles. Analogous methods are used for the LH vectors. Separate virus stocks are prepared as described below.

The DNA's, which are cut and ligated as described above, are ethanol precipitated and dissolved in sterile water. Approximately 1 μg of ptsA58 Bam DNA (helper virus) and 10 μg of recombinant DNA (encoding alpha and/or beta hCG or LH) are combined in a sterile test tube, mixed with 2 ml of TBS buffer (G. Kimura and R. Dulbecco 1972, Virology, 49, 79–81) and 1 ml of 2 mg/ml DEAE-dextran solution and added to a monolayer of confluent monkey CV-1 cells previously washed twice with 10 ml of TBS in a T-75 flask. The cells are left at 37° C. for 1–2 hrs with occasional shaking, washed with TBS twice, fed with 10 ml of DMEM containing 5% fetal calf serum, and left at 40° C. for 10–15 days. After complete cell lysis, the medium is transferred to a test tube, frozen and thawed five times, and centrifuged at 3000 rpm for five minutes. The resulting supernatants serve as virus stocks for infection of fresh SV-1 cells.

To accomplish an infection, CV-1 cells are grown to confluence in a T-150 flask. 1 ml of one of the virus stocks (made as described above) is added to the flask and the cells are incubated at 40° C. for 5 days.

For mixed infections, CV-1 cells are grown to confluence in a T-150 flask, alpha SVHVPl and beta SVVI viruses are mixed in a 1:1 ratio and 1 ml of the mixed virus is used to infect CV-1 cells at 40° C.

Transfection of Mouse Cells

To produce heterodimeric hCG using a mixed transfection, five μg of each BPV plasmid, i.e., pRF 375 (alpha hCG) and pRF 398 (beta hCG), are mixed and added to 0.5 ml of a 250 mM $CaCl_2$ solution containing 10 μg of salmon sperm DNA as carrier. This mixture is bubbled into 0.5 ml of 280 mM NaCl, 50 mM Hepes and 1.5 mM sodium phosphate. The calcium phosphate precipitate is allowed to form for 30–40 minutes at room temperature.

24 hours prior to transfection, 5×10^5 cells of mouse C127 cells (available from Dr. Dean Hamer, National Cancer Institute, NIH, Bethesda, Md.) are placed in a 100 mm dish or T-75 flask. Immediately before adding the exogenous DNA, the cells are fed with fresh medium (Dulbecco's Modified Medium, 10% fetal calf serum). One ml of calcium phosphate precipitate is added to each dish (10 ml), and the cells are incubated for 6–8 hours at 37° C.

The medium is aspirated and replaced with 5 ml of 20% glycerol in phosphate buffered saline, pH 7.0 (PBS) for 2 minutes at room temperature. The cells are washed with PBS, fed with 10 ml of medium, and incubated at 37° C. After 20–24 hours, the medium is changed and subsequent refeeding of the cells is carried out every 3–4 days. Individual clones are grown in T-25 cm flasks. After 7–21 days, cell clones can be transferred to larger flasks for analysis.

To produce heterodimeric hCG using a single transfection, plasmid RF 398 alpha t_2 is employed in the same manner as the above two plasmids were employed for a mixed infection.

To make heterodimeric LH, plasmids pRF 375 and pCL28XhoLHBPV are mixed, as described above in the case of hCG.

An interesting observation is that culturing cells containing beta hCG or beta LH-encoding vectors alone, in the absence of alpha-encoding cells, produces practically no beta subunit, while cells containing alpha and beta-encoding sequences produce not only heterodimer, but free beta subunit as well. This lends support to the notion that the production of both subunits in a single cell culture has the additional advantage of somehow permitting the presence of the alpha subunit to stabilize the beta subunit.

A further interesting observation was that mouse C127 cells transformed by an expression vector for the alpha subunit of hCG, without the additional vector for the beta subunit, produces an alpha subunit which will not associate with the complementary urinary beta subunit (combination<5%). The recombinant alpha subunit apparently has more glycosylations than the native subunit and is thus prevented from combining with beta subunit to produce a biologically active hormone. However, when both subunits are produced by the same cells, proper association and glycosylation occurs in vivo so that biologically active hormone substantially identical to the native hormone is expressed.

Isolation of the Human beta FSH Gene

A human genomic library in phage lambda (Lawn et al., *Cell*, 15, 1157–1174 (1978)) is screened using "guessed" long probes. The idea behind such probes, set forth in Jaye et al., *Nucleic Acids Research*, 11, 2325 (1983), is that if the amino acid sequence of a desired protein is at least partially known, a long probe can be constructed in which educated guesses are made as to the triplet encoding any amino acid which can be encoded by more than one, and not more than four, different triplets. Any correct guesses increase the amount of homology, and improve the specificity, of the results.

To isolate desired regions of DNA, two labeled 45-mer probes are used: TB36, homologous with amino acids 56–70 of human beta FSH; and TB21, homologous with amino acids 73–87. These probes have the following nucleotide compositions (corresponding amino acids are also given):

```
TB36:        Val—Tyr—Glu—Thr—Val—Lys—Val—
(AA56-70) 3' CAC ATG CTC TGG CAC TCT CAC

             Pro—Gly—Cys—Ala—His—His—Ala—Asp
             GGT CCG ACG CGG GTG GTG CGA CTG 5'

TB21:        Tyr—Thr—Tyr—Pro—Val—Ala—Thr—
(AA73-87) 3' ATG TGC ATG GGT CAC CGA TGT

             Glu—Cys—His—Cys—Gly—Lys—Cys—Asp
             CTC ACA GTG ACG CCG TTT ACG CTG 5'
```

The above probes are used to screen the human genomic library as follows. TB21 is labeled with ^{32}P and used to screen approximately 5×10^5 lambda plaques on duplicate filters by the in situ plaque hybridization technique of Benton and Davis, *Science*, 196, 180–182 (1977). The prehybridization solution is maintained at 55° C. for several hours and has the following composition: 0.75M NaCl; 0.15M Tris/HCl, pH 8.0; 10 mM EDTA; 5×Denhardt's Solution; 0.1% sodium pyrophosphate; 0.1% SDS; 100 microgram/ml *E. coli* t-RNA. The hybridization solution has the same composition except that it is maintained overnight at 45° C., and contains labeled probe in a concentration of about 0.5×10^6 cpm/ml. After hybridization, the filters are washed four times in 1×SSC (=0.15M NaCl, 0.015M Na_3-citrate) and exposed to x-ray film.

This screening procedure yields 50 plaques which hybridize to TB21 on both sets of filters. These 50 individual plaques are picked and combined into 10 culture pools

containing 5 plaques each. The 10 cultures are grown and DNA is isolated from 50 ml phage lysates. This DNA is then digested with EcoRI and fractionated on two identical 1% agarose gels. after which it is transferred to nitrocellulose paper according to the method of Southern, *J. Mol. Biol.*, 98, 503–517 (1975).

The DNAs on the two filters are hybridized to ^{32}P labeled TB21 and TB36, respectively. Individual plaques from the pool containing a restriction fragment which strongly hybridizes to both probes are then screened according to the above procedure, except that the DNAs are digested with EcoRI, BamHI, and EcoRI plus BamHI. In this way the 6.8 kb EcoRI-BamHI fragment containing human beta FSH is isolated.

A partial restriction map of clone 15B, containing the 6.8 kb EcoRI-BamHI fragment is shown in FIG. 2. In order to locate the position of the beta FSH sequences within the clone, the 6.8 kb EcoRI-BamHI fragment of clone 15B is subcloned into pBR322 to yield plasmid p15B6.8R/B (FIG. 2). p15B6.8R/B is then digested with various restriction enzymes and the products are fractionated by agarose gel electrophoresis using conventional methods. The DNA is blotted to nitrocellulose paper and hybridized to fragments of a porcine beta FSH cDNA clone labeled with ^{32}P by nick translation.

As shown in FIG. 2, the porcine beta FSH-probe hybridizes to only two fragments of the human DNA, namely a 1.1 kb HindIII-KpnI and a 1.4 kb PstI fragment. Partial DNA sequencing of these two fragments shows that this DNA indeed codes for human beta FSH and that the entire coding region for beta FSH is contained in these two fragments.

As shown by the restriction map of FIG. 3, the beta FSH coding sequence is interrupted by an intervening sequence of approximately 1.6 kb between amino acids 35 and 36 of mature beta FSH. The nucleotide sequence of the entire human beta FSH coding region and some of the flanking and intervening sequences are given below. The amino acid sequence deduced from the nucleotide sequence is given for the coding region.

```
                                                  30                                                  60
GCT TAC ATA ATG ATT ATC GTT CTT TGG TTT CTC AGT TTC TAG TGG GCT TCA TTG TTT GCT

                                                  90                                                 120
TCC CAG ACC AGG ATG AAG ACA CTC CAG TTT TTC TTC CTT TTC TGT TGC TGG AAA GCA ATC
                Met Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile

                                                 150                                                 180
TGC TGC AAT AGC TGT GAG CTG ACC AAC ATC ACC ATT GCA ATA GAG AAA GAA GAA TGT CGT
Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu Cys Arg

                                                 210                                                 240
TTC TGC ATA AGC ATC AAC ACC ACT TGG TGT GCT GGC TAC TGC TAC ACC AGG GTA GGT ACC
Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys Tyr Thr Arg

                                                 270                                                 300
//  ATG TTA GAG CAA GCA GTA TTC AAT TTC TGT CTC ATT TTG ACT AAG CTA AAT AGG AAC

                                                 330                                                 360
TTC CAC AAT ACC ATA ACC TAA CTC TCT TCT TAA ACT CCT CAG GAT CTG GTG TAT AAG GAC
                                                        Asp Leu Val Tyr Lys Asp

                                                 390                                                 420
CCA GCC AGG CCC AAA ATC CAG AAA ACA TGT ACC TTC AAG GAA CTG GTA TAT GAA ACA GTG
Pro Ala Arg Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val

                                                 450                                                 480
AGA GTG CCC GGC TGT GCT CAC CAT GCA GAT TCC TTG TAT ACA TAC CCA GTG GCC ACC CAG
Arg Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr Gln

                                                 510                                                 540
TGT CAC TGT GGC AAG TGT GAC AGC GAC AGC ACT GAT TGT ACT GTG CGA GGC CTG GGG CCC
Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys Thr Val Arg Gly Leu Gly Pro

                                                 570
AGC TAC TGC TCC TTT GGT GAA ATG AAA GAA TAA AAA TCA GTG GAC ATT TC
Ser Tyr Cys Ser Phe Gly Glu Met Lys Glu
```

Still referring to the above sequence, there is a box around the ATG initiation codon of the 18 amino acid signal peptide, which is cleaved post-translationally. The mature protein begins with the amino acid Asn encoded by the circled triplet AAT. The exon-intron boundaries are marked by arrows; they are flanked by the consensus sequence GT for the splice donor and AG for the splice acceptor site. There is a box around the stop codon TAA, the end of the coding region.

Below is a reproduction of the above sequence not broken into triplets, showing restriction sites; the ATG beginning and the TAA ending the coding region are boxed and the exon-intron junctions are marked by arrows.

```
        10         20         30         40         50         60
GCTTACATT TGATTATCGT TCTTTGGTTT CTCAGTTTCT AGTGGGCTTC ATTGTTTGCT
                                D
                                D
                                E
                                1

        70         80         90        100        110        120
TCCCAGACCA GGATGAAGAC ACTCCAGTTT TTCTTCCTTT TCTGTTGCTG GAAAGCAAT C
       B   F   M                  M
       S   O   B                  B
       T   K   O                  O
       1   1   2                  2

       130        140        150        160        170        180
TGCTGCAATA GCTGTGAGCT GACCAACATC ACCATTGCAA TAGAGAAAGA AGAATGTCGT
 B       A       A            H                      H
 B       L       L            P                      B
 V       U       U            H                      O
 1       1       1            1                      2

       190        200        210        220        230        240
TTCTGCATAA GCATCAACAC CACTTGGTGT GCTGGCTACT GCTACACCAG GGTAGGTACC
           S                                      B        KR
           F                                      S        PS
           A                                      T        NA
           1                                      1        11

       250        260        270        280        290        300
//  ATGTTAG AGCAAGCAGT ATTCAATTTC TGTCTCATTT TGACTAAGCT AAATAGGAAC
                                              D  A
                                              D  L
                                              E  U
                                              1  1

       310        320        330        340        350        360
TTCCACAATA CCATAACCTA ACTCTCTTCT TAAACTCCTC AGGATCTGGT GTATAAGGAC
                          M            SD    XS              A
                          B            AD    HA              V
                          O            UE    OU              A
                          2            11    2A              2

       370        380        390        400        410        420
CCAGCCAGGC CCAAAATCCA GAAAACATGT ACCTTCAAGG AACTGGTATA TGAAACAGTG
    B  S                  A   R
    S  A                  F   S
    T  U                  L   A
    1  1                  3   1

       430        440        450        460        470        480
AGAGTGCCCG GCTGTGCTCA CCATGCAGAT TCCTTGTATA CATACCCAGT GGCCACCCAG
      NH      H   H          H        S              B H
      CP      G   P          I        N              A A
      IA      I   H          N        A              L E
      12      1   1          1        1              1 3

       490        500        510        520        530        540
TGTCACTGTG GCAAGTGTGA CAGCGACAGC ACTGATTGTA CTGTGCGAGG CCTGGGGCCC
                                          R        MSH  B    AS
                                          S        NTA  S    PA
                                          A        LUE  T    AU
                                          A        113  1    11

       550        560        570        580        590
AGCTACTGCT CCTTTGGTGA AAT GAAAGAA TAAAGATCAG TGGACATTTC
A               H                    S
L               P                    A
U               H                    U
1               A                    A
```

Insertion of the Beta FSH DNA into a BPV-Based Expression Vector

Referring to FIG. 3, a synthetic BamHI linker is inserted at the DdeI site of p15B6.8R/B, which is located 42 nucleotides 5. of the ATG initiation codon. Referring to FIG. 4, p15B6.8R/B is digested with DdeI and treated with *E. coli* DNA polymerase (Klenow), after which it is ligated to synthetic BamHI linkers and digested with BamHI. The 295 bp fragment containing the first exon of FSH is isolated and cloned into the BamHI site of pBR322. The resulting plasmid pBR295Bam is digested with KpnI plus EcoRI plus AvaI and ligated to p15B6.8R/B which has been digested

with KpnI plus EcoRI plus SmaI. The ligation mix is then used to transform *E. coli*, and the plasmid pBR2.8Bam containing the human beta FSH DNA sequence as a BamHI fragment is identified from among the transformants by restriction mapping.

As shown in FIG. 4, expression plasmid CL28FSH2.8BPV is prepared according to the same method used to prepare pRF375 (FIG. 1), except that the 2.8 kb BamHI fragment of pBR2.8Bam is used in place of the alpha hCG cDNA clone. Plasmid CL28FSH2.8BPV can be used to transform mammalian host cells using conventional methods, and human beta FSH can be isolated and purified.

Transfection of Mouse Cells

To produce heterodimeric FSH using a mixed transfection, five μg of each BPV plasmid, i.e., pRF375 (alpha subunit) and CL28FSH2.8BPV (beta FSH). are mixed and added 0.5 ml of a 250 mM $CaCl_2$ solution containing 10 μg of salmon sperm DNA as carrier. This mixture is bubbled into 0.5 ml 280 mM NaCl, 50 mM Hepes and 1.5 mM sodium phosphate. The calcium phosphate precipitate is allowed to form for 30–40 minutes at room temperature.

24 hours prior to transfection, 5×10^5 cells of mouse C127 cells (available from Dr. Dean Hamer, National Cancer Institute, NIH, Bethesda, Md.) are placed in a 100 mm dish or T-75 flask. Immediately before adding the exogenous DNA, the cells are fed with fresh medium (Dulbecco's Modified Medium. 10% fetal calf serum). One ml of calcium phosphate precipitate.e is added to each dish (10 ml). and the cells are incubated for 6–8 hours at 37° C.

The medium is aspirated and replaced with 5 ml of 2 glycerol in phophate buffered saline, pH 7.0 (PBS) for 2 minutes at room temperature.

The cells are washed with PBS, fed with 10 ml of medium, and incubated at 37° C. After 20–24 hours, the medium is changed and subsequent refeeding of the cells is carried out every 3–4 days. Individual clones are grown in T-25 flasks. After 7–21 days, cell clones can be transferred to larger flasks for analysis.

Deposits

The following, described above, have been deposited in the American Type Culture Collection, Rockville, Md.:

alpha beta SVVPl, ATCC VR 2077;

alpha SVHVPl, ATCC VR 2075;

beta SVVPl, ATCC VR 2075;

pRF 375 in C127 cells, ATCC CRL 8401;

pRF 398 in C127 cells, ATCC CRL 8401;

pCL28XhoLHBPV *E. coli*, ATCC 39475;

pRF 398 alpha t_2 in C127 cells, ATCC CL 8400.

The following, described above, has been deposited in the Agricultural Research Culture Collection (NRRL), Peoria, Ill. 61604:

CL28FSH2.8BPV in *E. coli*, NRRL B-5923.

Use

The transformed cell lines of the invention are used to produce glycosylated biologically active heterodimeric human fertility hormones. hCG and LH made according to the invention, for example, have a number of well-known medical uses associated with human fertility. Furthermore, FSH can be used, alone or in conjunction with hCG or LH, to induce ovulation or superovulation for in vitro fertilization. In addition, heterodimeric FSH, or the beta subunit alone, can be used in diagnostic tests for fertility and pituitary functions.

Human fertility hormones produced by recombinant cells have the advantage, compared to such hormones obtained from natural sources, of being free from contamination by other human proteins, in particular other fertility hormones.

Other Embodiments

Other host cells, vectors, promoters, transforming sequences, and viruses can also be employed. The host cell employed generally is dependent on the vector being used. For example, when the vector is a replicating virus or a non-replicating viral DNA, the host cells are cells capable of being infected or transfected, respectively, by those vectors; e.g., SV40-containing vectors require monkey host cells, preferably CV-1 cells. Where the cloning vector is a plasmid having procaryotic control sequences, prokaryotic host cells, e.g., *E. coli*, are used. Where the cloning vector is a plasmid having eukaryotic control sequences, appropriate eukaryotic host cells, e.g., mouse C127 cells, are used. Besides autonomously replicating vectors, suitable vectors can be used to insert the DNA into the genome of the host cell, as is well known to those skilled in the art. As is also well known in the art, the vector may also contain an amplifiable marker, such as DHFR, particularly when used to transfect a $DHFR^-$ CHO cell line.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation.

We claim:

1. A process for producing biologically active heterodimeric human fertility hormone selected from the group consisting of human chorionic gonadotropin, human luteinizing hormone and human follicle stimulating hormone, comprising:

transforming a eukaryotic cell with at least one vector constructed so as to insert into said cell DNA coding for each of the two subunits of the hormone, the translation of the DNA of each said subunit being controlled by a separate promoter; and

culturing said cell under conditions appropriate to permit expression of the hormone therefrom,

wherein said eukaryotic cell is one which permits appropriate post-translational modification of the subunits so produced such that the formed protein is biologically active.

2. A process in accordance with claim 1, wherein said hormone is human chorionic gonadotropin.

3. A process in accordance with claim 1, wherein said hormone is human luteinizing hormone.

4. A process in accordance with claim 1, wherein said hormone is human follicle stimulating hormone.

5. A process in accordance with claim 1, wherein said transforming step comprises transforming the cell with a single vector containing DNA for both of said subunits under the control of separate promoters.

6. A process in accordance claim 1, wherein said transforming step comprises transforming the cell with two

vectors each containing DNA for one of said subunits and an appropriate promoter.

7. A process in accordance with claim 1, wherein said at least one vector is autonomously replicating.

8. A process in accordance with claim 1, wherein said at least one vector is a plasmid or a replicating virus.

9. A process in accordance with claim 1, wherein each of said promoters are different.

10. A eukaryotic cell transformed by at least one vector constructed so as to insert into said cell heterologous DNA coding for each of the two subunits of a heterodimeric human fertility hormone selected from the group consisting of human chorionic gonadotropin, human luteinizing hormone and human follicle stimulating hormone, the translation of the DNA of each said subunit being controlled by a separate promoter, said eukaryotic cell being of a species which permits appropriate post-translational modification of the subunits so produced such that the formed protein is biologically active.

11. A cell in accordance with claim 10, wherein said hormone is human chorionic gonadotropin.

12. A cell in accordance with claim 10, wherein said hormone is human luteinizing hormone.

13. A cell in accordance with claim 10, wherein said hormone is human follicle stimulating hormone.

14. A cell in accordance with claim 10, wherein the cell has been transformed with a single vector containing DNA for both of said subunits under the control of separate promoters.

15. A cell in accordance with claim 10, wherein the cell has been transformed with two vectors each containing DNA for one of said subunits and an appropriate promoter.

16. A cell in accordance with claim 10, wherein said at least one vector is autonomously replicating.

17. A cell in accordance with claim 10, wherein said at least one vector is a plasmid or a replicating virus.

18. A cell in accordance with claim 10, wherein each of said promoters are different.

19. An expression vector containing DNA encoding each of the two subunits of a heterodimeric human fertility hormone selected from the group consisting of human chorionic gonadotropin, human luteinizing hormone and human follicle stimulating hormone, with separate promoters for each said subunit.

20. An expression vector in accordance with claim 19, wherein said hormone is human chorionic gonadotropin.

21. An expression vector in accordance with claim 19, wherein said hormone is human luteinizing hormone.

22. An expression vector in accordance with claim 19, wherein said hormone is human follicle stimulating hormone.

23. An expression vector in accordance with claim 19, wherein each said promoter is different.

24. An expression vector in accordance with claim 23, wherein said two different promoters are derived from two different species.

25. An expression vector in accordance with claim 19, which is autonomously replicating.

26. An expression vector in accordance with claim 19 comprising a plasmid or a replicating virus.

* * * * *

Patent 5,639,639
APPLICATION FOR EXTENSION OF PATENT TERM

EXHIBIT B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: REDDY et al.

Serial No.: 07/515,481

Filed: April 27, 1990

For: RECOMBINANT HETERODIMERIC HUMAN FERTILITY HORMONES

Art Unit: 1812

Examiner: G. Wang

Washington, D.C.

Atty.'s Docket: REDDY=1.2

Date: March 17, 1994

THE COMMISSIONER OF PATENTS AND TRADEMARKS

Washington, D.C. 20231

Sir:

Transmitted herewith is an [ ] Amendment [XX] TERMINAL DISCLAIMER in the above-identified application.

[ ] Small entity status of this application under 37 CFR 1.9 and 1.27 has been established by a verified statement previously submitted.

[ ] A verified statement to establish small entity status under 37 CFR 1.9 and 1.27 is enclosed.

[XX] Terminal Disclaimer fee of $110.00.

The fee has been calculated as shown below:

	(Col. 1) CLAIMS REMAINING AFTER AMENDMENT		(Col. 2) HIGHEST NO. PREVIOUSLY PAID FOR	(Col. 3) PRESENT EXTRA	SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN A SMALL ENTITY RATE	OTHER THAN A SMALL ENTITY ADDITIONAL FEE
TOTAL	*	MINUS	** 20	=	x 11	$		x 22	$
INDEP.	*	MINUS	*** 3	=	x 37	$		x 74	$
FIRST PRESENTATION OF MULTIPLE DEP. CLAIM					+115	$		+ 230	$
					TOTAL ADDITIONAL FEE	$	OR	TOTAL	

* If the entry in Col. 1 is less than the entry in Col. 2, write "0" in Col. 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, write "20" in this space.

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, write "3" in this space.

The "Highest Number Previously Paid For" (total or independent) is the highest number found from the equivalent box in Col. 1 of a prior amendment of the number of claims originally filed.

[XX] Conditional Petition for Extension of Time

If any extension of time for a response is required applicant requests that this be considered a petition therefor.

[ ] It is hereby petitioned for an extension of time in accordance with 37 CFR 1.136(a). The appropriate fee required by 37 CFR 1.17 is calculated as shown below:

Small Entity
Response Filed Within
[ ] First - $ 55.00
[ ] Second - $180.00
[ ] Third - $420.00
[ ] Fourth - $660.00
Month After Time Period Set

Other Than Small Entity
Response Filed Within
[ ] First - $ 110.00
[ ] Second - $ 360.00
[ ] Third - $ 840.00
[ ] Fourth - $1320.00
Month After Time Period Set

[XX] Please charge my Deposit Account No. 02-4035 in the amount of $ 110.00. A duplicate copy of this sheet is attached.

[ ] A check in the amount of $_____ is attached (Check No.)

[XX] The Commissioner is hereby authorized and requested to charge any additional fees which may be required in connection with this application or credit any overpayment to Deposit Account No. 02-4035. This authorization and request is not limited to payment of all fees associated with this communication, including any Extension of Time fee, not covered by check or specific authorization, but is also intended to include all fees for the presentation of extra claims under 37 CFR Section 1.16 and all patent processing fees under 37 CFR Section 1.17 throughout the prosecution of the case. This blanket authorization does not include patent issue fees under 37 CFR Section 1.18.

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:atm

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit: 1812
REDDY et al.	)	Examiner: G. Wang
Serial No.: 07/515,481	)	Washington, D.C.
Filed: April 27, 1990	)	March 17, 1994
For: RECOMBINANT HETERODIMERIC HUMAN FERTILITY HORMONES	)	Attorney's Docket: REDDY=1.2

TERMINAL DISCLAIMER

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Genzyme Corporation, having a principal place of business at Kendall Square, Cambridge, MA, (hereinafter "GENZYME"), through its undersigned attorney of record, hereby disclaims the terminal part of any patent granted on the above-identified application Serial No. 07/515,481, which would extend beyond June 20, 2006, the expiration date of the full statutory term of United States Patent No. 4,840,896, and hereby agrees that any patent so granted on the above-identified application Serial No. 07/515,481 shall be enforceable only for and during such period that the legal title to said patent shall be the same as the legal title to United States Patent No. 4,840,896, United States Patent 4,923,805, and United States Patent No. 5,156,957, this agreement to run with any patent granted on the above-identified application Serial No. 07/515,481 and to be binding upon the grantor, its successors or assigns. GENZYME does not

disclaim any terminal part of any patent granted on the above-identified application Serial No. 07/515,481 prior to the expiration date of the full statutory term of United States Patent No. 4,840,896, in the event that it expires earlier for failure to pay a maintenance fee, is held unenforceable, is found invalid, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321(1), has all claims cancelled by a reexamination certificate, or is otherwise terminated prior to expiration of its full statutory term, except for the separation of legal title stated above. This terminal disclaimer is being made without waiver of petitioner's rights under 35 U.S.C. 156 which may be available to extend the term of any patent granted on the above-identified application beyond the date set by this terminal disclaimer (37 CFR 1.775(a)). A charge form for payment of the fee required by 37 CFR 1.20(d) is attached hereto.

It is GENZYME's intent that the amount of time disclaimed and the scope of the common ownership clause be the minimum required by law and this document is to be construed to effectuate said intent. No admission is made that any claim of the above-identified application is obvious over any claim of Patent No. 4,840,896, Patent No. 4,923,805, or Patent No. 5,156,957.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these

statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

GENZYME CORPORATION

By: Roger L. Browdy
Reg. No. 25,618

March 17, 1994
Date

reddy.tds

Patent 5,639,639
APPLICATION FOR EXTENSION OF PATENT TERM

EXHIBIT C

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PATENT NUMBER	FEE CODE	FEE AMT	SUR CHARGE	APPLICATION NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT	ATTY DKT NUM
5,639,639	183	$850.00	$0.00	08/268,734	06/17/97	06/30/94	04	NO	PAID	

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Patent 5,639,639
APPLICATION FOR EXTENSION OF PATENT TERM

EXHIBIT D

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PATENT NUMBER	FEE CODE	FEE AMT	SUR CHARGE	APPLICATION NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT	ATTY DKT NUM
5,639,639	1552	$2,150.00	$0.00	08/268,734	06/17/97	06/30/94	08	NO	PAID	

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Patent 5,639,639
APPLICATION FOR EXTENSION OF PATENT TERM

EXHIBIT E

3.4. CHEMISTRY, MANUFACTURING, AND CONTROLS SUMMARY

3.4.1. Drug Substance

3.4.1.1. Description and Characterisation of the Active Substance

3.4.1.1.1. Nomenclature

International non-proprietary name

Lutropin alfa.

United States Adopted Name

Lutropin alfa.

Trivial name or chemical description

Recombinant human luteinising hormone (r-hLH).

Chemical Abstracts Service (CAS) Registry Number

CAS-152923-57-4.

Code Designation

ATC G03 GA Gonadotropins

Proposed proprietary name

Luveris™

Identification number of the production strain

V II C6 clone of the original hLH1-111 transfectant cell line.

3.4.1.1.2. Physical and chemical characteristics

Recombinant human luteinising hormone, like its naturally occurring counterpart, is a heterodimer glycoprotein, composed of two non-covalently linked non-identical subunits, designated α and β. The α-subunit, which is common to all the gonadotrophin hormones, is 92 amino acids in length and possesses two sites of N-linked glycosylation (Asn 52 and Asn 78). The β-subunit, which is hormone specific, is 121 amino acids in length and possesses a single site of N-linked glycosylation (Asn 30).

Luteinising hormone is a well understood protein hormone whose pharmacodynamic action is mediated through its binding to its surface-bound receptor on target cells leading to increased adenylate cyclase activity.

Recombinant-hLH has been submitted to extensive and detailed physico-chemical characterisation. The characterisation studies performed on r-hLH have demonstrated that the protein is highly complex being composed of two non-covalently linked α- and β-subunits. Both subunits are N-glycosylated, with two

glycosylation sites on the α-subunit (Asn 52 and Asn 78) and one on the β-subunit (Asn 30). No O-glycosylation site has been detected.

The primary structure of both subunits has been deduced and corresponds to that of the native peptides. N-terminal sequencing and peptide mapping methods have identified N-terminal heterogeneity of both subunits, a feature observed in urinary (u-) and pituitary (p-) hLH also. Sequencing of the C-terminus of the β-subunit identified a full length species corresponding to that predicted by the cDNA sequence and three truncated forms, lacking one, two or four residues. The presence of these truncated forms may be explained by the susceptibility of terminal hydrophobic amino acids (Gly-Leu-Leu-Phe Leu-COOH) to carboxypeptidase cleavage. More extensively truncated forms have been observed in u-hLH and p-hLH (lacking of four, six and seven amino acids). The full length species with an intact C-terminus has not been detected in either u-hLH or p-hLH. From the data presented, it can be concluded that this N-terminal and C-terminal heterogeneity has no relevant impact on the biological activity of the molecule.

The carbohydrate structures attached to the peptide components are important for biological activity for all the gonadotrophin hormones. Deglycosylated variants of LH and the other gonadotrophins bind well to their receptors, however, unlike the glycosylated forms, they are unable to activate significant adenylate cyclase activity.

Conservation of the glycosylation sites on r-hLH has been demonstrated using several methods. The elucidation of the glycan structures collectively and in associated with each glycosylation site has been achieved by a combination of carbohydrate composition analysis, mass spectrometry techniques and glycan mapping. These studies showed that r-hLH carries complex type glycans of bi-, tri-, tetra and penta-antennary form with a variable rate of sialylation. Penta-antenarry forms have also been detected as a minor species. The glycans associated with the β-subunit are fucosylated on the first N-acetylglucosamine of the core.

Analysis of the primary sequence has shown that the C-terminus of the β-subunit displays a significant level of truncation with the lack of one, two or four amino acids. The proportion of full length form (β3 form) versus the truncated ones can be determined by RP-HPLC. No correlation between highly truncated forms and specific bioactivities could be obtained.

An extensive comparative characterisation study has been performed on several r-hLH bulk samples demonstrating the consistency of the data from one bulk to the others.

Physico-chemical characterisation studies have confirmed a low level of amino acid modification in bulk batches:

Absence of deamidated forms (Peptide mapping)

Low oxidation level (RP-HPLC, peptide mapping)

Comparisons have been made between recombinant and natural products, both of urinary and pituitary origins, showing that the main differences are observed at

the glycan level : natural hLH glycans contain N-acetylgalactosamine residues and their sulphated derivatives while r-hLH glycans contain only sialylated species. This is explained by the incapability of CHO cells to produce sulphated residues. Some differences in the N-terminal and C-terminal truncation have also been observed. These differences in the physico-chemical characteristics appear to have limited relevance with regard to the biological activity of the molecule.

The α-subunit and β-subunit of LH possess five and six disulphide bridges, respectively. No attempts have been made to deduce the disulphide bridges for the r-hLH subunits. However, investigations have been performed by the Company on the r-hFSH molecule, which has a common α-subunit with r-hLH. No information could be obtained on the location of the disulphide bridges of the α-subunit. The pairing of the six intrachain bridges of the β-subunit of r-hFSH has been unambiguously identified as being the following : Cys 3 – Cys 51 ; Cys 17 – Cys 66 ; Cys 20 – Cys 104 ; Cys 28 – Cys 82 ; Cys 32 – Cys 84 ; Cys 87 – Cys 94

One of the major difficulties encountered in the location of the disulphide bridges of the α-subunit of r-hFSH, and hence for r-hLH, is the distribution of cystein residues within the amino acid sequence : a few of them are juxtaposed (Cys 31/Cys 32 and Cys 59/Cys 60) or separated by only a few amino acids yielding their accessibility by enzymes difficult. In the β-subunit, several cysteins are also separated by a few amino acids resulting in a poor accessibility by proteolytic enzymes.

Nevertheless, correct pairing of the cysteins in the recombinant molecule can be assumed as the *in vivo* bioactivity and immunoactivity of the r-hLH molecule are maintained.

Information on the secondary structure has been obtained by circular dichroism, identifying random coil and β-sheet conformations.

The extinction coefficient for r-hLH has been determined to be $E_{1\%(276.5nm)}$ = 8.12, according to amino acid analysis, ie. considering the protein moiety only. It is on this correlation that r-hLH protein content is quantified by SE-HPLC in the purified bulk and the final formulated product.

The principal structural characteristics of the r-hLH molecule are summarised in the Table 3.4-1:

Table 3.4-1: Summary of structural features of r-hLH

Characterization Test	r-hLH
Apparent MW by SDS-PAGE under reducing conditions	α-subunit shows a band at about 23 kD β-subunit shows a broad band at about 18 kD
SDS-PAGE under non-reducing conditions	Single broad band at below the 29 kD marker
α-subunit N-terminal amino acid sequence	Full 15 AA sequence Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn - Truncated sequence -2 AA Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn - Almost entirely full sequence plus small amount of truncated sequence (-2 AA)
β-subunit N-terminal amino acid sequence	Full 15 AA sequence Ser Arg Glu Pro Leu Arg Pro Trp Cys His Pro Ile Asn Ala Ile - Sequence + 2 AA Trp Ala Ser Arg Glu Pro Leu Arg Pro Trp Cys His Pro Ile Asn Ala Ile - Full sequence plus trace of sequence + 2 AA
α-subunit C-terminal amino acid sequence	Full 15 AA sequence - Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser-COOH Full sequence only
β-subunit C-terminal amino acid sequence	Full 15 AA sequence (β3 form) - Pro Leu Thr Cys Asp His Pro Gln Leu Ser Gly Leu Leu Phe Leu-COOH Truncated sequence (- 1 AA) (β2 form) - Pro Leu Thr Cys Asp His Pro Gln Leu Ser Gly Leu Leu Phe-COOH Truncated sequence (- 2 AA) (β1 form) - Pro Leu Thr Cys Asp His Pro Gln Leu Ser Gly Leu Leu-COOH Truncated sequence (-4 AA) (β0 form) - Pro Leu Thr Cys Asp His Pro Gln Leu Ser Gly-COOH Full sequence plus some truncated sequences (-1 AA, -2 AA, -4 AA)
Glycosylation sites	α = Asn 52, Asn 78; β = Asn 30
Isoform distribution by IEF	7 principal bands in the range 6 to 10
Carbohydrate content	Fucose, Galactose, GlcNAc, Mannose, Sialic acid
Oligosaccharide structure	Complex, containing Sialic acid α (2-3) - Gal and Gal β (1-4)-GlcNAc β (1-2)-Man trisaccharide
Biospecific activity (Van Hell method)	13 000 - 19 000 IU/mg* of glycoprotein

Key to table: *r-hLH concentration was determined by spectrophotometry at 276.9 nm using the experimentally calculated extinction coefficient for r-hLH (E1% : 5.59 by dry weight)

3.4.1.2. Stability of the drug substance

3.4.1.2.1. Normal test conditions

Stability data are provided on production scale batches and development batches following storage at –20°C.

Methods used to assess stability of the production batches included pH, LH content (SE-HPLC), IEF, glycan mapping, oxidised α-subunit, percent β3-subunit, aggregates, dissociated subunits and biopotency. Up to 30 months data are currently available and demonstrate good stability. Stability testing is planned to continue to 36 months.

Up to 24 months data are available on development batches, however, using a more limited number of tests (β3-subunit, IEF, SE-HPLC purity and biopotency).

Together, these data justify a minimum retest period of 24 months when stored at –20°C.

3.4.1.2.2. Freeze-thaw studies

Freeze–thaw stability studies have been carried out on production batches and development batches. In the case of production batches, up to 20 cycles have been assessed. No trends were apparent in any of the parameters tested.

3.4.1.3. Manufacturers

The production process for r-hLH is composed of two parts: (1) A cell culture process for the production of crude harvest material containing r-hLH and (2) a purification process for the separation of r-hLH from contaminants in the crude harvest. Both operations are conducted at:

Laboratoires Serono S.A (LSA)
Aubonne,
Switzerland.

All drug substance testing (except Bioassay) are performed at :

Laboratoires Serono S.A (LSA)
Aubonne,
Switzerland.

The bioassay is performed at:

Istituto di Ricerche Biomediche "Antoine Marxer" SpA (RBM)
10010 Colleretto Giacosa (TO)
Italy

3.4.1.4. Method of Manufacture

3.4.1.4.1. Development genetics

Standard recombinant DNA techniques were used to isolate and clone the α- and β-subunit genes into expression vectors and transfect into a standard CHO host cell line.

The α-subunit gene was isolated from a bacteriophage lambda library containing human foetal liver genomic DNA. The β-subunit gene was isolated from a pBR322 library containing human pituitary cDNA. In the case of the latter gene, the intact 5' sequence could not be isolated, therefore, the gene was reconstructed in the expression plasmid by insertion of a hCG β-subunit fragment contributing the ATG start codon and the first 12 amino acids of the secretory signal peptide. Both N-terminal sequencing and peptide mapping of the purified product demonstrate that the β-subunit peptide is correctly process and no detectable aberrant sequences arise.

Expression vectors possessed a bacterial origin of replication to allow maintenance in *Esherichia coli* K12, the mouse metallothionein-I gene promoter for constitutive expression of the inserted sequence and the SV40 early polyadenylation signal. The dihydrofolate reductase selectable marker on the α-subunit expression vector was used to isolate the transfectants in the presence of methotrexate. The final clone, designated VIIC6 was obtained by limiting dilution under non-selective conditions. This clone was subsequently used to establish the Master Cell Bank (MCB).

The production cell line has been extensively characterised genotypically and phenotypically. Sequencing of cDNA and genomic 5' and 3' non-transcribed regions, restriction enzyme analysis, mRNA size analysis and fluorescence *in situ* hybridisation confirm stable integration of the transfected sequences into the CHO cell genome and integrity of the r-hLH subunit gene sequences. An approximate 2-fold reduction in gene copy numbers was observed in the Extended Cell Banks (established 12.5 population doubling levels beyond the limit of *in vitro* cell age for production) compared to levels in the MCB. This phenomenon is commonly observed when an initial transfectant has a high copy number and selective pressure is subequently removed; this is not indicative of genetic instability. Indeed, restriction enzyme and DNA sequence analyses of the Extended cell banks confirmed the integrity of the subunit gene sequences and flanking regions and yielded no evidence of rearrangements or aberrant sequences.

3.4.1.4.2. Cell bank system

The r-hLH production cell line used for the establishment of cell banks is an anchorage dependent Chinese hamster ovary (CHO) cell line, transfected with the human genes coding for the LH subunits.

The Master Cell Bank (VII C6 MCB) was established from a single vial of the pre-seed stock by expansion in T-flasks and roller bottles. A total of 330 vials

were cryopreserved, each containing 30 x 10^6 cells at a population doubling level (PDL) of 50.

The Working Cell Bank (VII C6 WCB) was established from a single vial of the MCB in a likewise fashion. A total of 1098 vials were cryopreserved, each containing 26 x 10^6 cells at a population doubling level (PDL) of 61.

The growth medium used throughout was DMEM: Ham F12 (1:1) supplemented with glucose, glutamine and 10% foetal bovine serum (FBS). The cryopreservation medium was DMEM/F12 supplemented with 20% FBS and 10% dimethylsulfoxide.

Both the MCB and WCB are stored at two separate places at the same site, Laboratoires Serono Aubonne S.A., Switzerland.

Characterisation studies of cell banks have been carried out in accordance with the ICH guidelines, Topics Q5A, Q5B and Q5D.

Viral safety studies on the MCB, WCB and an Extended Population Doubling Bank (EPDB) have included tests for retroviruses (reverse transcriptase assays, S^+L^- focus and XC plaque infectivity assays, and transmission EM), *in vivo* assays, *in vitro* assays and antibody production tests. All tests proved negative with the exception of the detection of A-type and C-type defective retroviruses, which are known to be associated with CHO cell lines. Full reports for these cell bank safety tests have been provided in the Microbiology section.

A protocol for the establishment of new WCBs is provided and complies with the above quoted ICH guidelines.

3.4.1.4.3. Production

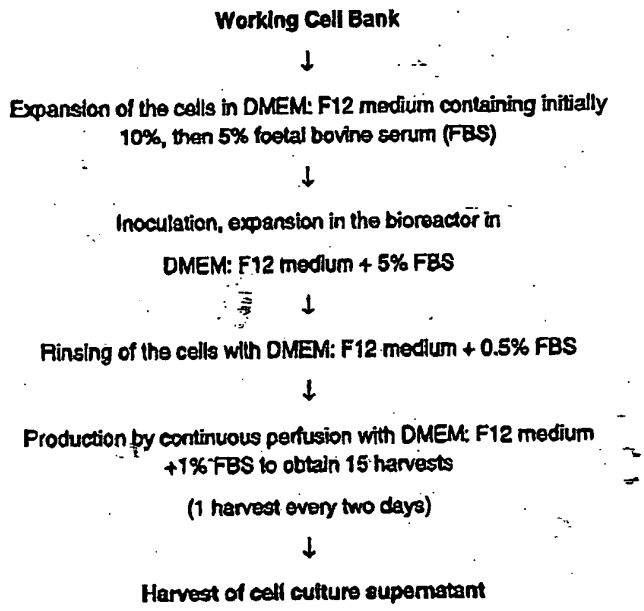
3.4.1.4.3.1. Cell culture process

A production run is initiated from one vial of the WCB or a number of vials which are thawed and pooled prior to expansion in T-flasks and roller bottles. Cell expansion is carried out until a sufficient cell number is obtained for seeding the bioreactor within a defined PDL range (71 to 77). Cell growth continues in a 40 L capacity bioreactor operating in perfusion mode and using a 28 L working volume. Cells are grown and subsequently maintained attached to microcarrier dextran beads composed of porcine collagen (Cytodex® 3) suspended in the culture medium. Once a minimum cell density is obtained within a defined number of days in culture, the production phase is initiated by the collection of harvest material. A total of 15 harvests are collected over a production period of 30 days.

The basal medium used throughout is DMEM:F12. Cell growth from the WCB vial is initiated in media supplemented with 10% FBS, which is reduced to 5% after the first few days of culture. FBS at a concentration of 5% is maintained until two days prior to the initiation of production, at which point it is reduced gradually to 1%. This concentration is maintained throughout the production phase until the end of cell culture.

The flow diagram of the cell culture and harvesting process is presented in Figure 1.

Figure 1: Flow diagram for the r-hLH cell culture and harvesting process



In-process controls of cell culture in the bioreactor include pH, cell density, glucose and lactate concentrations in the supernatant. Mycoplasma tests are performed on the inoculum prior to bioreactor seeding and at the end of the process. A reverse transcriptase test and *in vitro* virology assay are performed at the end of the cell culture process. In case of an equivocal result for the reverse transcriptase test, an S^+L^- focus assay is performed.

Crude harvests are monitored for LH content and bioburden.

Raw materials of animal origin used during the process are FBS, microcarrier beads (porcine collagen) and porcine trypsin. The sourcing and quality control or processing conditions of these materials is adequate to ensure that they pose minimal safety risks.

3.4.1.4.3.2. Purification

Purification of r-hLH from crude harvest material is achieved through a series of six concentration and chromatographic steps.

In brief:

Step I: Crude harvests are clarified, concentrated by ultrafiltration (UF 10 kD) and filtered (0.22 µm). The concentrated harvest material may be stored frozen at NMT –20°C +/- 5°C for up to 6 months.

Step II: The material is thawed, pooled and clarified before ion exchange (DEAE) chromatography. The unbound fraction is then dialysed (UF 100 kD), concentrated (UF 10 kD) and further processed within 3 days.

Step III: The r-hLH solution obtained from step II is loaded onto a second ion exchange (Q-Sepharose) chromatography column. The r-hLH, contained in the unbound fraction, is immediately processed further or stored at NMT –20°C +/- 5°C for up to 6 months.

Steps IV and V: The r-hLH solution obtained is diluted with 2-propanol and subjected to two successive RP-HPLC chromatographic steps at room temperature. The second eluate is concentrated (UF 10 kD) and further processed within 3 days.

Step VI: The r-hLH solution is loaded on a gel permeation chromatography column. The collected fractions containing r-hLH are pooled, ultrafiltered through a Viresolve™ virus removal membrane, concentrated (UF 10 kD) and filtered (0.22 µm) before storage of the purified bulk at NMT –20°C +/- 5°C

Purification equipment is dedicated to the r-hLH purification process.

All starting materials used for the preparation of buffers and sanitisation solutions comply with Pharmacopeias or are of analytical grade. Regeneration and sanitisation conditions for chromatographic columns and ultrafiltration units have been established satisfactorily as well as operational parameters for use and re-use of the columns.

In-process controls performed after each purification step allow the monitoring of the performance of each step with respect to yield and purity.

The flow diagram of the purification process is presented in Figure 2.

Figure 2 : Flow diagram for the r-hLH purification process

Harvest of cell culture supernatant

↓

Prefiltration, dialysis (10 kD) and filtration

↓

Clarification, Chromatography on DEAE Sepharose CL-6B equilibrated with 0.025 M sodium phosphate, ultrafiltration of the eluate on 100 kD then 10 kD membranes

↓

Chromatography on Q Sepharose FF equilibrated with ammonium acetate 0.08 M

↓

Filtration then chromatography on C18 RP-HPLC, elution with 2-propanol/0.05 M ammonium acetate with a linear gradient of 2-propanol

↓

Chromatography on C18 RP-HPLC, elution with 2-propanol/Tris-HCl (0.5M) with a linear gradient of 2-propanol, ultrafiltration on a 10 kD membrane

↓

Chromatography on Sephacryl S100 HR using 0.5 M ammonium hydrogen carbonate

↓

Filtration through Viresolve™ (approx. 70 kD) membrane using 0.5 M ammonium hydrogen carbonate

↓

Ultrafiltration on a 10 kD membrane

↓

Filtration through a 0.22 µm membrane

↓

r-hLH Bulk Solution (stored frozen)

3.4.1.4.3.3. Process validation

Robustness of the cell culture process

A study was carried out to determine the impact of critical parameters (medium feed rate, temperature, dissolved oxygen and pH) on process performance, cell metabolism, product quality and impurity profile of semi-purified bulks. A

Table BAA-1: Batch analysis results (cont.)

	Batch Number	BLCA 9803	BLCA 9804	BLCA 9805	BLCA 9806	BLCA 9808
	Cell culture run	B51-4005-120	B51-4005-120	B51-4005-120	B51-4005-121	B51-4006-122
	Harvest number	H6+H7+H8+H9	H13+H14+H15	H10+H11+H12	H3+H4+H5+H6	H1+H2+H3+H4+H5
	Batch Size (MIU)	22.3.	16.3	16.7	14.2	12.3
	Date of Manufacture	23.01.98	30.01.98	20.02.98	06.03.98.	01.05.98
Parameter	Specification	—	—	—	—	—
Glycan mapping	Z Number: 123-172	151	154	154	162	144
	Neutrals: 6-21%	12	13	12	10	17
	Monosialylated: 30-43%	36	34	35	33	33
	Disialylated: 33-50%	42	42	43	46	42
	Trisialylated: 4-10%	8	8	8	8	6
	Tetrasialylated: 0-3%	2	3	2	2	2
Chemical identity	Retention time of LH monomer relative to that of the Reference House Standard: 0.97 to 1.03	conform	conform	conform	conform	conform
Purity tests						
Dimers and aggregates	Not more than 2 %	< LOD*	< LOD	< LOD	< LOD	< LOD
Dissociated subunits	Not more than 3 %	1 %	2 %	1 %	1 %	2 %
r-hLH monomers	Not less than 95 %	99 %	98 %	99 %	99 %	98 %
Oxidized products	Peak area of oxidised alpha subunit NMT 5% of the sum of peak areas of intact plus oxidised alpha subunit	1 %	1 %	1 %	1 %	1 %
Cell culture derived proteins	Not more than 0.5 ng / 10 µg r-hLH	0.1	0.1	0.1	0.1	0.1
Electrophoretic purity	No additional significant band (Not more than 0.5%) when compared to r-hLH reference house standard	conform	conform	conform	conform	conform
Bacterial endotoxins	Not more than 100 EU per mg r-hLH	conform	conform	conform	conform	conform
Bioburden	Not more than 5 CFU per ml	conform	conform	conform	conform	conform
Mycoplasma	Absence	conform	conform	conform	conform	conform
Assay of active substance						
r-hLH concentration	0.7 to 1.3 mg/ml	0.9	0.8	0.8	0.9	0.8
Specific bioactivity	18'000 – 35'000 IU/mg	25'819	27'473	31'229	26'995	26'279

* LOD = 0.25 %

DEVELOPMENT GENETICS

Overview

The host cell line used for the production of recombinant human luteinising hormone is an anchorage dependent Chinese hamster ovary (CHO) cell line, isolated from the CHO-K1 line and deficient in dihydrofolate reductase (DHFR) activity (Urlaub and Chasin, 1980). This DHFR deficiency makes this cell line auxotrophic for glycine, hypoxanthine and thymine (GHT). The CHO cells were transfected with two plasmids, pHαDHFR and pHLHβODC, each containing two functional genes of interest. The former plasmid possesses the α-hLH gene and DHFR selectable marker, thereby overcoming the auxotrophic GHT requirement and conferring resistance to methotrexate (MTX) whilst the second plasmid contains the β-LH gene and the ornithine decarboxylase (ODC) selectable marker.

A lambda vector Charon 4A clone containing the human α-subunit genomic sequence, within a 17 kilobase pair (kbp) insert, was isolated by screening a Charon 4A library with a full length cDNA probe corresponding to the human chorionic gonadotropin (hCG) α-subunit. The α-subunit is common to hFSH, hLH, hTSH and hCG. The hLH β-subunit cDNA, which is particular to hLH, was isolated from a human pituitary cDNA library in pBR322, using a 88 basepair (bp) *Eco*RII-*Pst*I restriction fragment encoding a conserved region from an hCG β-subunit cDNA clone. This initial h-LH β-subunit cDNA was ligated with two other β-subunit cDNA fragments to give a cDNA fragment of approximately 510 bp. An 11 kbp subfragment of the α-subunit Charon 4A clone and the 510 bp β-subunit cDNA, were introduced into their respective expression vectors, containing the DHFR and ODC selectable markers, respectively.

The α-genomic fragment was inserted into the expression vector pCLH3AXSV2DHFR downstream of and juxtaposed to the mouse metallothionein gene promoter. The α-subunit polyadenylation signal was used for 3' processing of the α-subunit transcript. This expression vector contained the mouse DHFR gene as a selectable and amplifiable marker. The co-transfecting expression construct was of similar design but contained the β-hLH cDNA, in place of the α-subunit gene and the selectable ODC gene in place of the DHFR gene. To enhance expression of the β-hLH cDNA the first intron of the mouse immunoglobulin kappa light chain gene was linked directly upstream of the β-hLH cDNA. Since the endogenous β-subunit polyadenylation signal was removed during construction, the SV40 early polyadenylation signal supplied by the vector was used for 3' processing of the β-subunit transcript.

CHO cells were transfected using a calcium phosphate precipitation procedure, with uncut preparations of the plasmids pHαDHFR and pHLHβODC. Subsequent MTX treatment was used to co-amplify the recombinant genes (separate amplification with an ODC antagonist was not deemed necessary). Eventually, through a process of limiting dilution in the presence and absence of MTX, a single clone, VII C6, was selected on the basis of its expression characteristics as the basis for the development of the r-hLH cell bank system.

Source materials

Genes of interest

α-subunit gene

i) Construction of human fetal liver DNA library

The α-subunit of hFSH, hLH, hCG and hTSH is a common entity to all four hormones. Therefore, during the development of hFSH, hLH and hCG the same α-subunit encoding sequence has been employed. This sequence was originally isolated from a bacteriophage lambda Charon 4A human genomic library. The library was constructed from 18-25 kbp sucrose gradient purified human fetal liver DNA fragments, generated by *AluI*/*Hae*III partial digests (Lawn *et al.*, 1978). *Eco*RI sites of the fragments were blocked with *Eco*RI methylase after which synthetic dodecameric linkers were ligated to both ends. The resulting *Eco*RI fragments were inserted into the *Eco*RI site of bacteriophage lambda Charon 4A vector (Blattner *et al.*, 1977). The resulting DNA constructs were *in vitro* packaged into bacteriophage lambda particles and used to transfect the *Escherichia coli* strain DP50*Sup*F (Leder *et al.*, 1977). The *in vitro* packaging and transfection process selected for inserts up to 20 kbp in size. Approximately 1 x 10^6 plaques (clones) were obtained which were subsequently amplified 10^6 fold by low density growth on agar plates to establish a permanent library of cloned human DNA fragments.

ii) Isolation of α-subunit gene

Approximately 1 x 10^6 recombinant bacteriophages were plated out using the host strain DP50*Sup*F (Leder *et al.*, 1977). Replicas of the plates were made on nirocellulose filters. Since the glycoprotein hormones are a group of structurally related molecules which share a common α-subunit, sequence information from the cDNA clone of αhCG (Fiddes and Goodman, 1979) was utilized to construct suitable probes for the identification of a full-length α-subunit genomic clone. Recombinant phage DNA bound to the filters was hybridized with a 621 bp *Hin*dIII fragment carrying the "full-length" αhCG cDNA. This probe was used to select a recombinant bacteriophage containing a total of 17 kb of human DNA and which has been designated lambda Ch4Aαg. The DNA insert was further analysed with restriction endonuclease enzymes, in combination with Southern blotting against specific probes for the 5' and the 3' ends of the gene, i.e. a 230 bp fragment containing sequences from the 5' untranslated region to codon 34 of the mature protein and a 76 bp fragment containing the most distal part of the 3' untranslated region, respectively. The precise locations of the introns were established by DNA sequencing. The α-subunit gene spans a total of 9.4 kb of human DNA, out of which approximately 700 nucleotides encode the mature transcript (Figure DEG-1). This gene is composed of four exons interrupted by three intron sequences. The largest intron is 6.4 kb long and is located in the 5' untranslated region, 7 nucleotides before the ATG region, separating the leader sequence from the coding portion of the gene. A 1.7 kb intron splits the codon which corresponds to the sixth amino acid of the mature protein, whereas the last intron, 0.4 kb in size is located between codons 67 and 68 (Fiddes and Goodman, 1981).

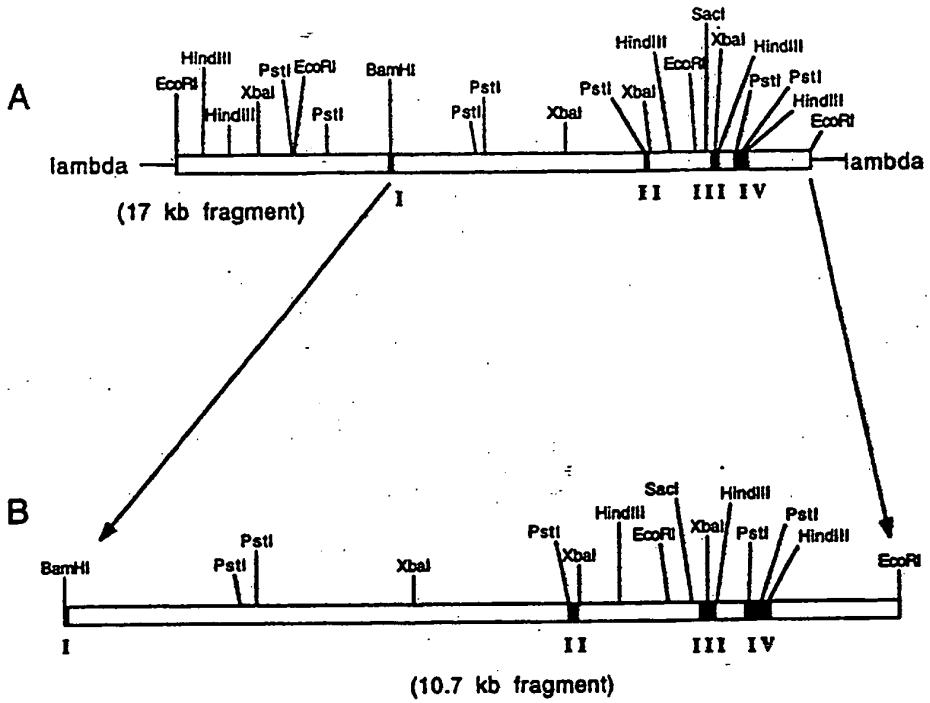


Figure DEG-1: The human gonadotropin alpha subunit gene

iii) Construction of human pituitary cDNA library

The four gonadotropin hormones differ from one another due to the individual ß-subunits. The β-subunit cDNA for r-hLH was isolated from a human pituitary cDNA library, constructed in the commonly used plasmid vector pBR322.

iv) Isolation of β-subunit gene

The LHβ-subunit was constructed from fragments isolated from the LH12, LH6 and hCGβ-subunit cDNA plasmids. A 104 bp EcoRI-DdeI fragment was isolated from the hCGβ cDNA plasmid and was subsequently ligated to a 181 bp DdeI fragment isolated from the LH12 plasmid. This ligation was digested with EcoRI and PstI and the fragment was cloned back into pBR322 digested with EcoRI and PstI; this resulting plasmid was called "LH beta 260". LH beta 260 was digested with EcoRI and NcoI; the resulting 146 bp fragment was used later in the final assembly. In addition, LH12 was also digested with NcoI and PvuII and the isolated 241 bp fragment was used in the final assembly.

The LH6 plasmid was digested with Sau3AI and the 390 bp fragment, whose 3' end is the untranslated portion of the clone, was further digested by HincII and ligated to BamHI linkers. After digestion with BamHI, the ~390 bp fragment was cloned into the plasmid pAPP and the resulting construct was called "LHβ6". pAPP was derived from pBR322 by digestion with AvaI, blunting with Klenow, digesting with PvuII, and ligating to close the plasmid so as to eliminate the PvuII site.

LH6B was digested with EcoRI and PvuII, and was dephosphorylated. The 146 bp EcoRI + NcoI fragment containing the hCGβ signal sequence, the 241bp NcoI + PvuII fragment, and the dephosphorylated LH6B were mixed together in a 3-way ligation. The resulting plasmid, LH520 H/B, contained the complete LHβ coding sequence including the composite CGβ/LHβ secretory signal sequence. This construct contained a single amino acid difference, from leucine in the natural hLH β-subunit to phenylalanine in the recombinant hLH β-subunit, at the fourth amino acid in the secretory signal peptide (due to the 5' hCG sequence). This secretory signal is removed from the protein upon translocation across the endoplasmic reticulum and therefore no sequences derived from the hCG β-subunit cDNA are present in the mature r-hLH β-subunit.

It is unlikely that the leucine to phenylalanine change in the recombinant LHβ signal peptide sequence has any impact on the peptide secretion kinetics and peptide cleavage. Signal peptides consist of three structural regions, a positively charged N-terminal region (n-region), a hydrophobic core region (h-region), and a more polar C-terminal region (c-region). Phenylalanine and leucine are hydrophobic amino acids and are both common components of the h-region. Substitution of one for the other in the same relative position is considered a very conservative structural change which does not significantly affect the signal peptide processing probabilities. In fact, the site of the substitution is too distant (greater than 13 amino acids) from the processing site to be included in the probability calculations (von Heinje, 1986).

The N-terminal sequences of the native LHβ (wt-hLHβ), recombinant hLHβ (rec-hLHβ) and hCGβ are presented below:

	-20	-1	+1
Wt-hLHß	MEMLQGLLLLLLLSMGGAWA		SREPLRP
Rec-hLHß	MEMFQGLLLLLLLSMGGAWA		SREPLRP
hCGß	MEMFQGLLLLLLLSMGGTWA		SKEPLRP

The Company did experiments to replace the hybrid hCG/hLH leader sequence with the native hLHβ leader sequence (GF5995: "Mutagenesis of LHβ subunit signal peptide DNA coding region" – available upon request). LH dimer production from CHO cell isolates co-transfected with the α subunit expression construct and the native LHβ construction was poor. It was concluded that the native LHβ signal sequence was not advantageous with regard to protein production rates.

In summary, the ordering of the components contained within the final hLH β-subunit cDNA is as follows:

- 25 bp which correspond to a portion of the 5' untranslated region of the hCG β-subunit mRNA;
- 39 bp which correspond to the hCG β-subunit start ATG and 12 amino acids of the hCG β-subunit secretory signal peptide;
- 21 bp which correspond to 7 amino acids of the hLH β-subunit secretory signal;
- 363 bp which correspond to 121 amino acids of the mature hLH β-subunit peptide; and
- 62 bp which correspond to a portion of the 3' untranslated region of the hLH β-subunit mRNA.

The size of the composite β-subunit cDNA, without the addition of *Hin*dIII-*Bam*HI linkers, is 510 bp. After the addition of synthetic linkers, the approximate size of the β-subunit DNA fragment is 520 bp.

Figure DEG-2 below shows the construction of the LH β-cDNA.

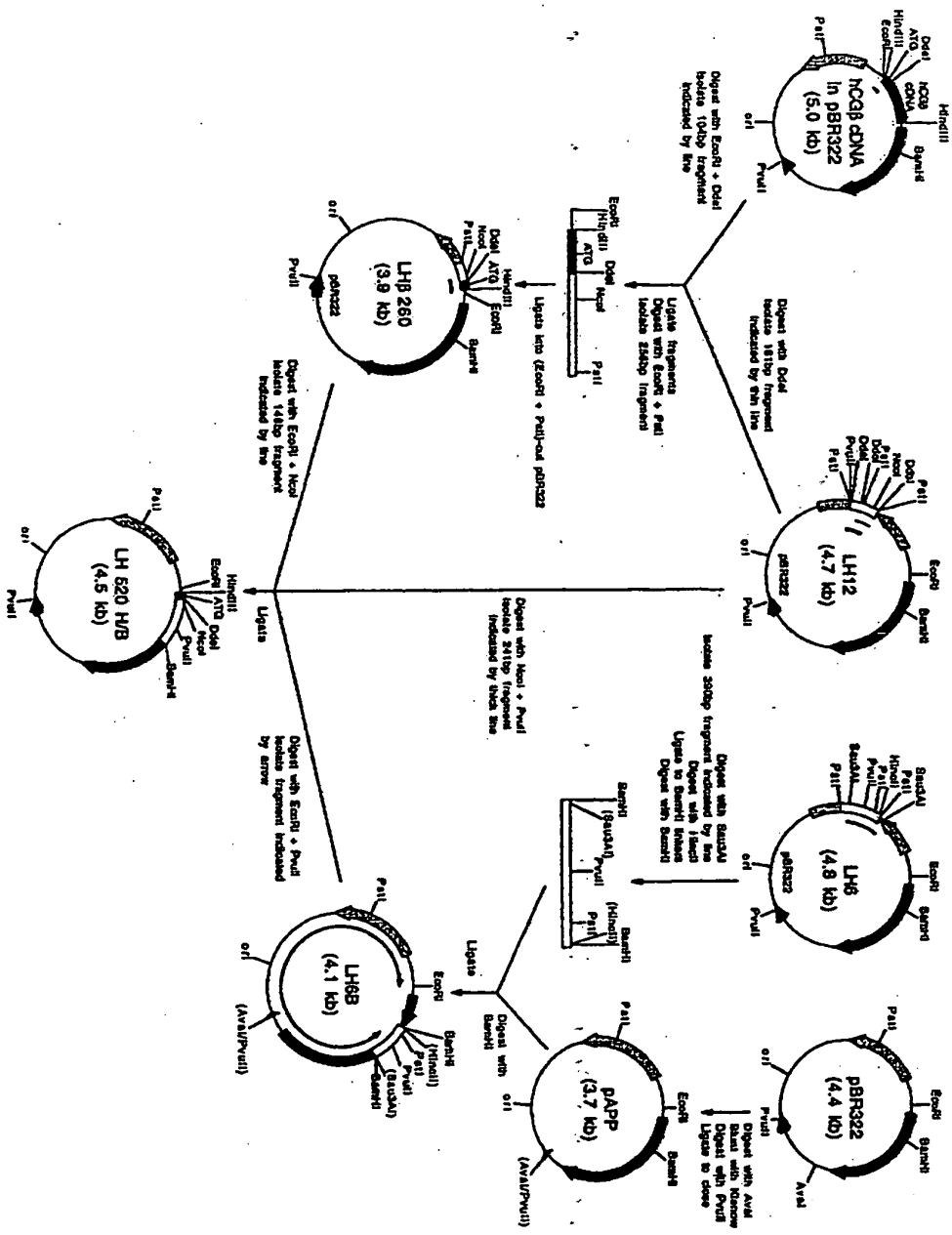


Figure DEG-2: Construction of the LH β-subunit cDNA

Description of the cell line

The starting line, CHO-DUKX, which was employed to produce r-hLH lacks activity of the enzyme DHFR (tetrahydrofolate dehydrogenase, 7, 8-dihydrofolate: $NADP^+$ oxidoreductase; EC 1.5.1.3). The CHO-DUKX cell line was isolated following a two step procedure. Mutagenesis of the parental CHO-K1 cell line with ethylmethanesulfonate and exposure to high specific activity [^{3}H] deoxyuridine resulted in a partially DHFR-deficient clone (Urlaub and Chasin, 1980). This clone was then subjected to gamma irradiation to yield the fully *dhfr*-deficient cell line, DXB11 (Graf and Chasin, 1982), also known as CHO-DUKX. DHFR-deficient mutants require glycine, hypoxanthine and thymidine for growth. The central role of DHFR in the synthesis of nucleic acid precursors, together with the sensitivity of DHFR-expressing cells to tetrahydrofolate analogs such as methotrexate (MTX), present two major advantages. Firstly, transfection of DHFR-deficient cells by plasmids containing a DHFR gene allows the selection of recombinant DHFR-expressing cells. Secondly, culture of these cells in selective media containing increasing concentrations of MTX results in amplification of the DHFR gene and the associated DNA (Kaufman and Sharp, 1982).

Preparation of the production cell line

The employed CHO cell line was co-transfected with two expression plasmids, encoding the α- and β-subunits. One plasmid was based on the vector pCLH3AXSV2DHFR and contained the α-subunit gene, this vector allowed growth in the presence of MTX. The second plasmid was based on the vector pCLH3AXSV2ODC and contained the β-subunit cDNA. To amplify the transfected genes, selected transfected cells were subjected to MTX treatment. Cell line hLH1-111 was subjected to limited dilution cloning yielding transfectant VII C6 which was subsequently employed for the production of the Master and Working Cell Banks.

A flow diagram giving an overview of the cloning of the α- and β-subunit sequences, the construction of the expression plasmids, the transfection and subsequent isolation of a r-hLH producing clone is shown in Figure DEG-3.

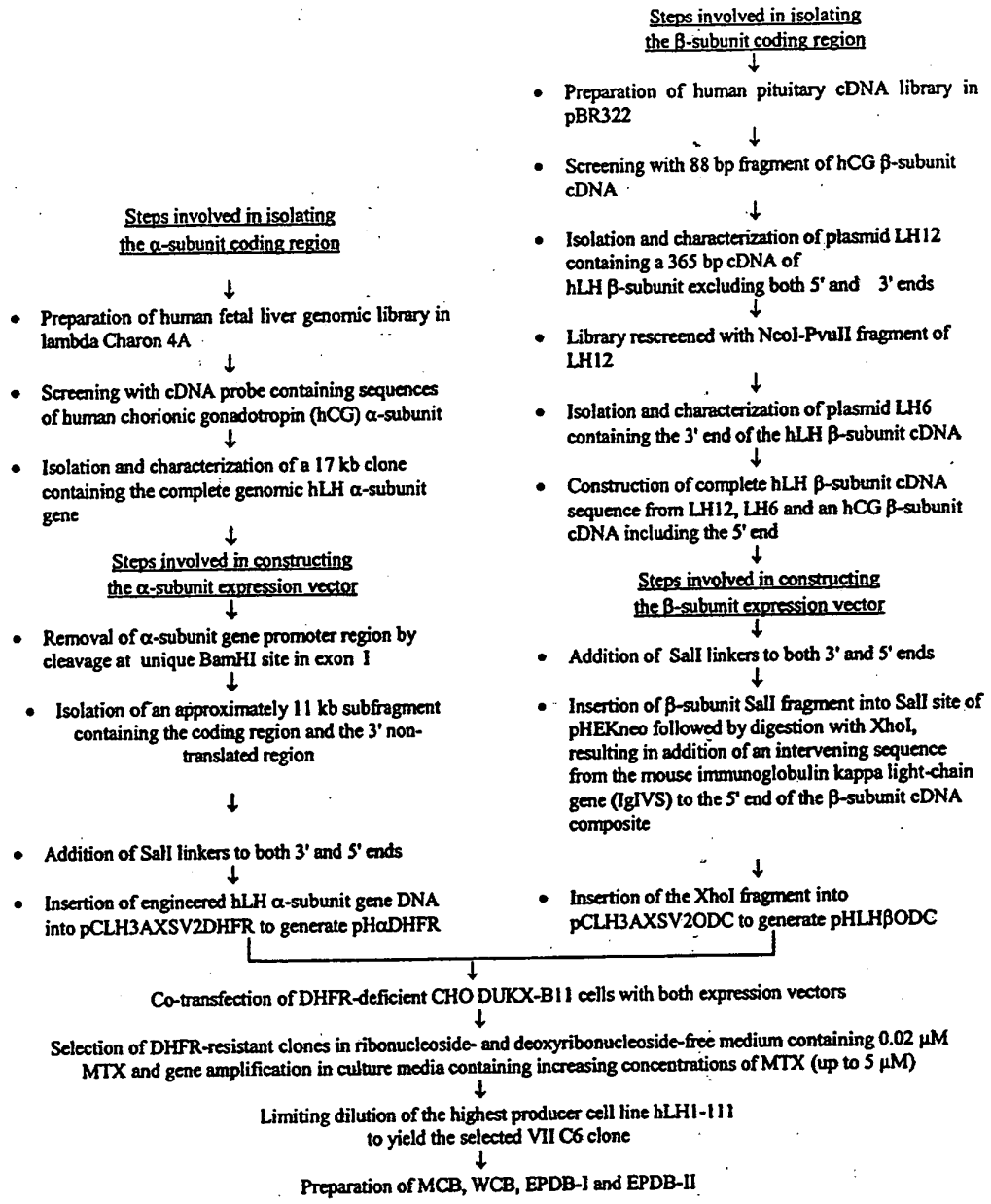


Figure DEG-3: Outline of the construction of CHO cells expressing human LH.

Construction of the expression plasmids

The expression vectors for both the α-and β-subunits (pCLH3AXSV2DHFR and pCLH3AXSV2ODC, respectively) are based on the parent vector pCLH3A. Figure DEG-4 details the original construction of pCLH3A. Subsequent introduction of either the mouse DHFR or ODC gene gave rise to the vectors pCLH3AXSV2DHFR and pCLH3AXSV2ODC, respectively (as described in the following sections).

In addition, the Company initiated and completed a project to determine the DNA sequences of the expression vectors, pαDHFR and pHLHβODC. The work was contracted to Lark Technologies, Inc. (Houston, TX). The sequence data was determined from at least two gel readings, preferably on opposite strands, without any ambiguities.

The complete report "Expression Vector DNA Sequence Analysis: Human α Subunit Genomic Gene inpCHL3AXSV2DHFR and ODC Coding Region" (GF 6157) is available upon request.

The main features of pCLH3A are:

- 2616 bp from the plasmid pML-1 (Lusky and Botchan, 1981), derived from pBR322, carrying a bacterial ampicillin resistance gene and the ColEl bacterial origin of replication,
- 971 bp from the mouse metallothionein-I 3' flanking region (Hamer and Walling, 1982)
- 2000 bp from the mouse metallothionein-I gene promoter and 5' flanking region (Hamer and Walling, 1982) followed by the unique *Xho*I restriction endonuclease site,
- 242 bp containing the SV40 early polyadenylation region.

Construction of the α-subunit expression plasmid

The vector employed for the expression of the α-subunit was the plasmid pCLH3AXSV2DHFR. The construction of pCLH3AXSV2DHFR from pCLH3A is shown in Figure DEG-5.

The main feature of this vector, in addition to the features of pCLH3A, is:

- a transcription unit derived from pSV2-*dhfr* (Subramani *et al.*, 1981) composed of the SV40 promoter (342 bp), the mouse DHFR cDNA (734 bp) and the small t intron and early polyadenylation signal (847 bp).

For the construction of the α-expression plasmid a *Bam*HI-*Eco*RI (3' terminal) subfragment of approximately 10.7 kb in size was derived from the α-genomic λclone and contained part of exon I (less the first 35 nucleotides of the 5' untranslated region of the mRNA), exons II, III and IV, the intervening sequences and approximately 2 kb of 3' flanking sequence (Figure DEG-1). This fragment was initially introduced into the plasmid pBR322. The 10.7 kb α-subunit fragment was modified to produce a fragment with *Sal*I sites at the extremities and was cloned in the plasmid pUC18 (Figure DEG-6). The fragment was subsequently cloned into the unique *Xho*I site of CLH3AXSV2DHFR (Figure DEG-7).

The resulting recombinant plasmid, internally designated pHαDHFR, contained the genomic α-hFSH gene fused to the promoter of the mouse metallothionein-I gene. The genomic α-gene is followed by SV40 sequences carrying the early polyadenylation region. This plasmid contains also a DHFR gene used as a selectable and amplifiable marker. Figure DEG-8 depicts the restriction enzyme map of pHαDHFR.

Construction of the β-subunit expression plasmid

The vector employed for the expression of the β-subunit was the plasmid pCLH3AXSV2ODC. This vector is similar to pCLH3AXSV2DHFR except that a 1.65 kbp cDNA coding for mouse ODC (Chiang and McConlogue, 1988; Gupta and Coffino, 1985) was inserted between the *Hin*dIII and *Bgl*II sites of pCLH3AXSV2DHFR in place of the DHFR cDNA. Figure DEG-9 schematically depicts the construction of pCLH3AXSV2ODC.

For assembly of the hLH β-subunit expression plasmid, the hybrid β-subunit cDNA, originally engineered as a 520 bp *Hin*dIII-*Bam*HI fragment, was converted to a *Sal*I fragment by the addition of *Sal*I linkers to both ends.

The resulting 540 bp β-subunit *Sal*I fragment was inserted into the *Sal*I site of pHEKneo, a myeloma cell expression vector which contained the first intron of the mouse immunoglobulin kappa light-chain gene 5' to the β-subunit composite DNA fragment. This approximately 130 bp intron, which is referred to as the immunoglobulin intervening sequence (IgIVS), was derived from a mouse mopc41 myeloma kappa light-chain clone and was added to the β-subunit transcription unit to ensure a high efficiency of mRNA accumulation. Enhancement of mRNA accumulation by the addition of introns is well documented in the literature (see e.g. Huang and Gorman, 1990). Serono has previously observed that mRNA accumulation can be improved when this heterologous intron is placed upstream of the coding region of gonadotropin subunit genes (Chappel et al., 1992).

The complete mouse kappa light-chain gene was thus not included in the β-subunit expression vector. Instead, a fragment of the kappa light-chain gene, which contains a small intron in addition to sequence corresponding to about 12 amino acids of the leader sequence, was incorporated into the vector utilized for expression of the LH β-subunit. The sequence corresponding to the leader is not preceded by an ATG start codon, and is therefore not translated; it is part of the 5' untranslated region of the β-subunit mRNA. The intron is removed from the nuclear RNA transcript by splicing. Therefore, the kappa gene protein is not expressed in the LH production cell line.

The 670 bp IgIVS/β-subunit XhoI DNA fragment was inserted into the unique *Xho*I site of the pCLH3AXSV2ODC expression vector so that transcription was directed by the MMT-I promoter (Figure DEG-10). This insertion resulted in the generation of the hLH β-subunit expression plasmid pCLH3AXSV2ODCIgIVS hLHβ (abbreviated to pHLHβODC). Since the endogenous β-subunit polyadenylation signal was removed during engineering, the SV40 early polyadenylation signal supplied by the vector was used for 3' processing of the β-subunit transcript. Figure DEG-10 depicts the construction of pHLHβODC.

The β-subunit expression plasmid, depicted in Figure DEG-11, shares the same important features of the α-subunit expression vector except that the DHFR gene has been replaced by the mouse ornithine decarboxylase (ODC) gene.

ODC is the initial enzyme of a pathway involved in the synthesis of polyamines and thus is essential for cell growth. Gene amplification can be achieved by growing the CHO cells in media containing stepwise increases in concentrations of the ODC inhibitor, difluoromethylornithine. As discussed in the section dedicated to "Data and cloning of an r-hLH producing cell line", direct and separate amplification of the β-subunit plasmid using ODC antagonist was found not to be necessary.

Figure DEG-4: Construction of parent expression vector pCLH3A

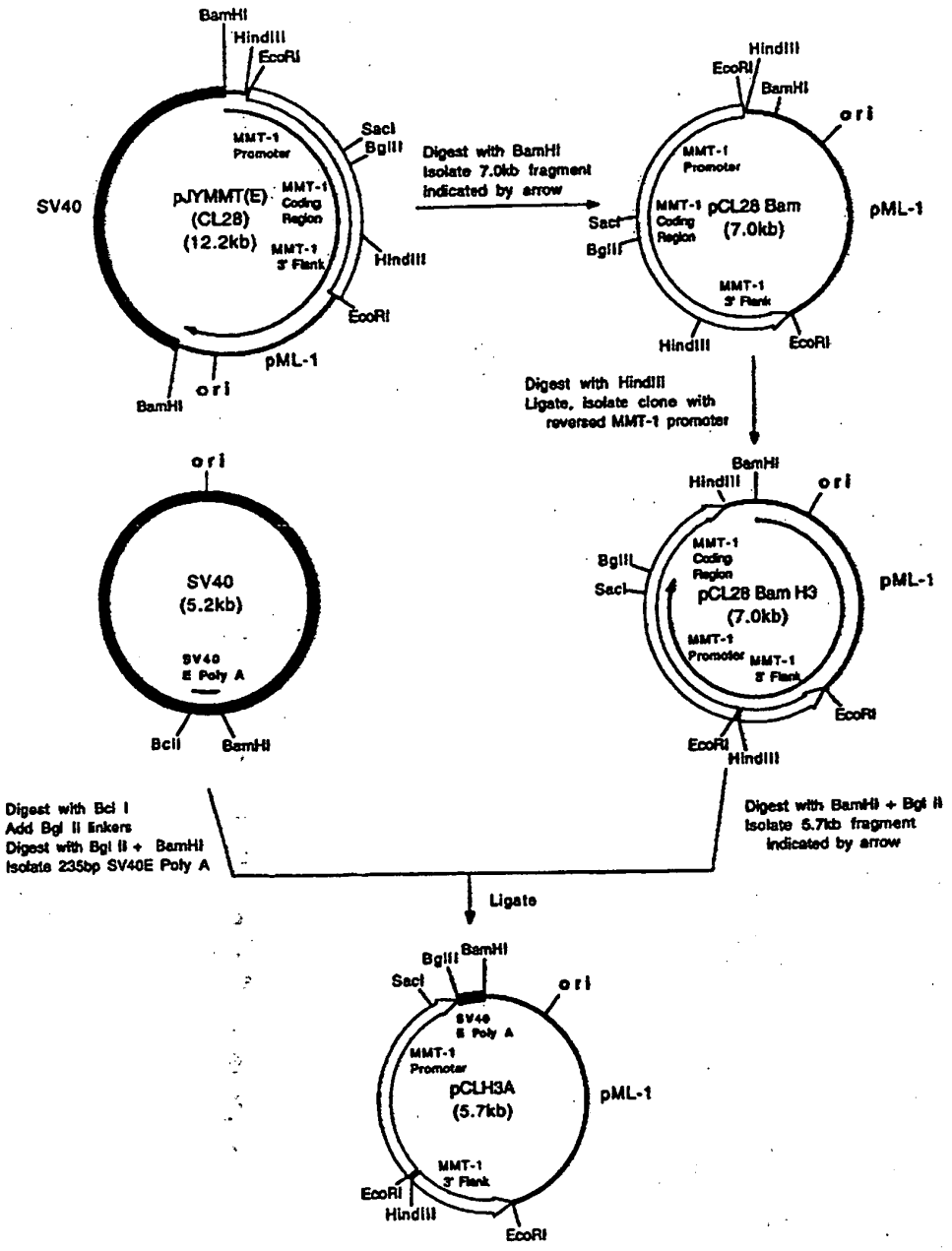


Figure DEG-5: Construction of expression vector pCLH3AXSV2DHFR

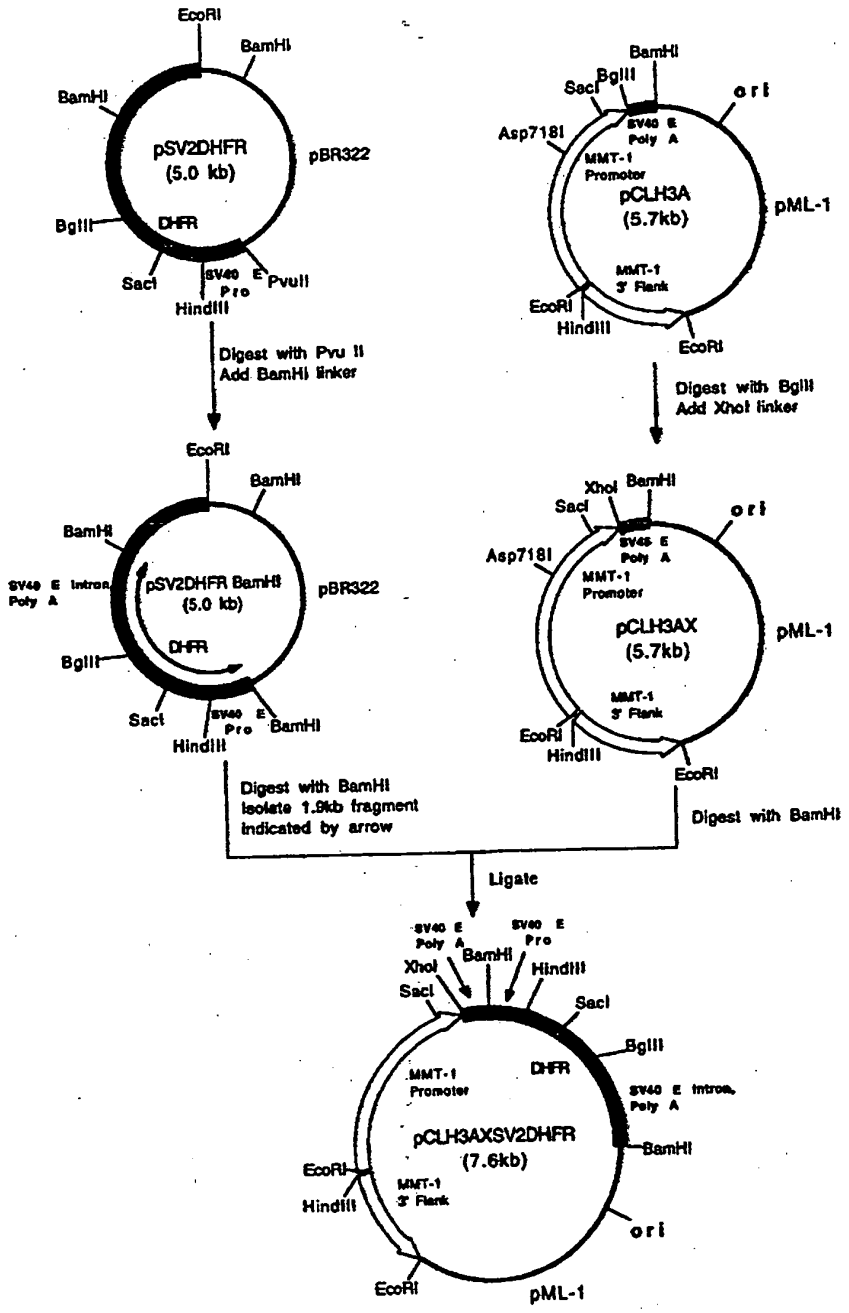


Figure DEG-6: Construction of α-subunit expression plasmid pHαDHFR (Step 1)

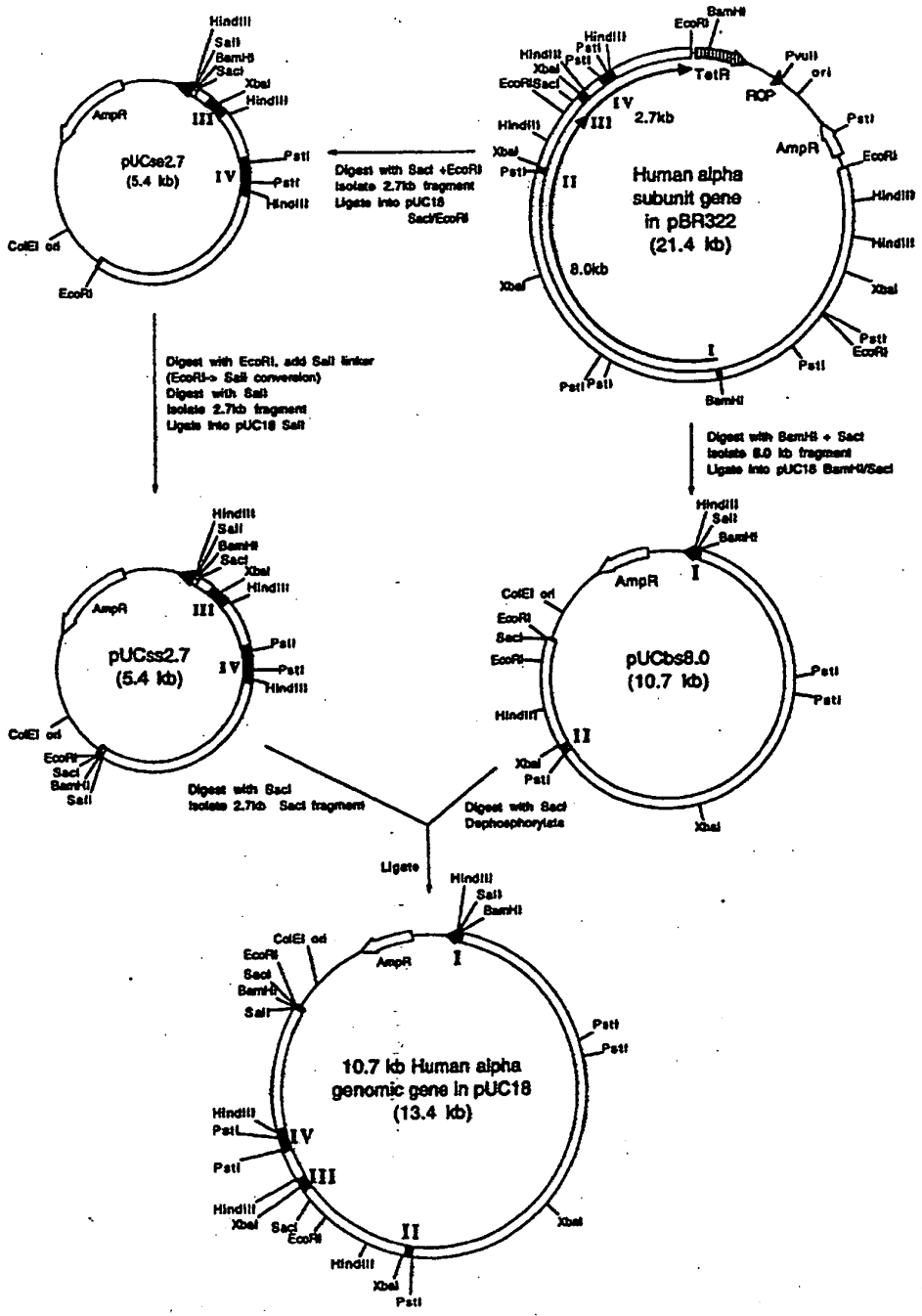


Figure DEG-7: Construction of α-subunit expression plasmid pHαDHFR (Step 2)

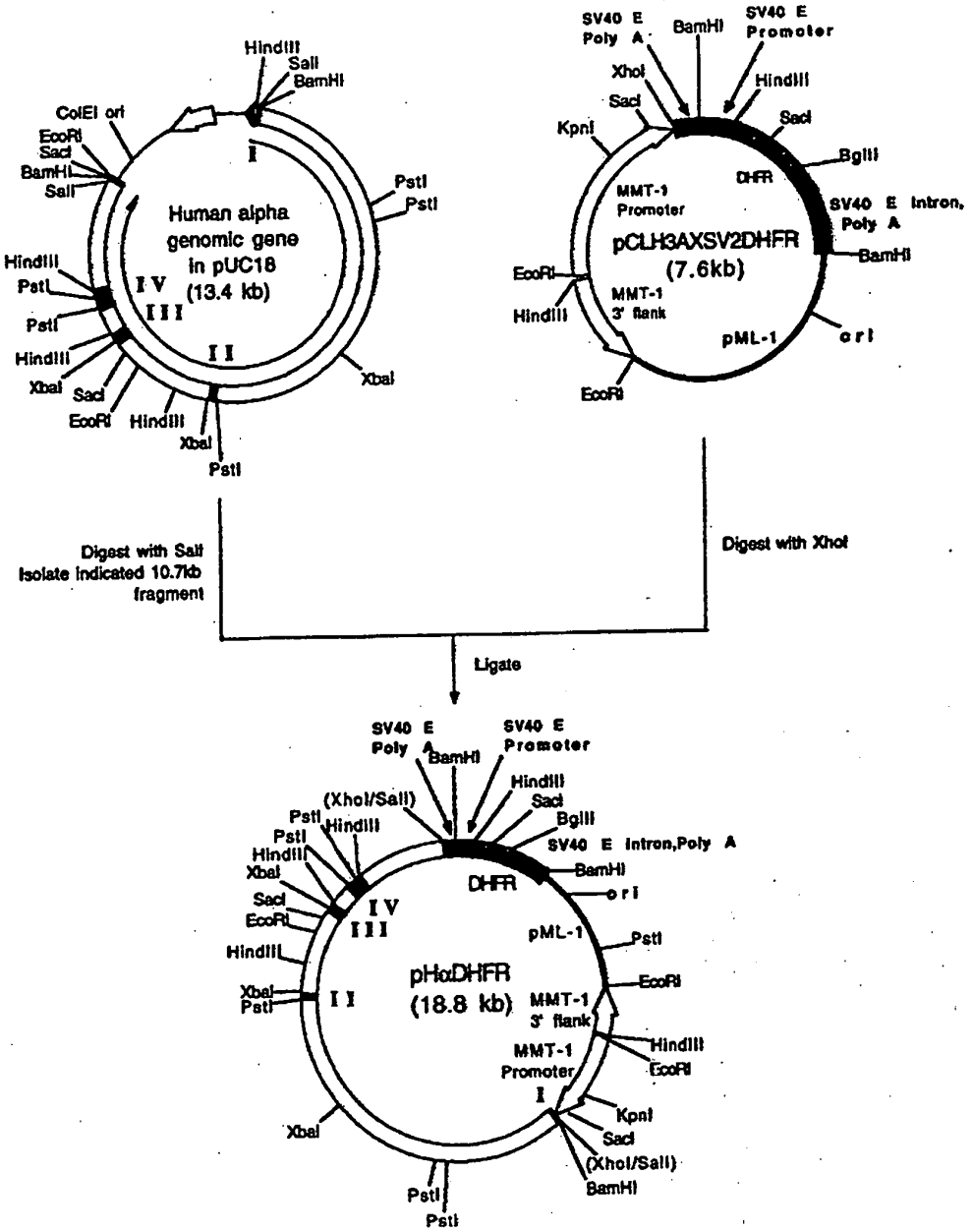


Figure DEG-8: Restriction enzyme map of pHαDHFR

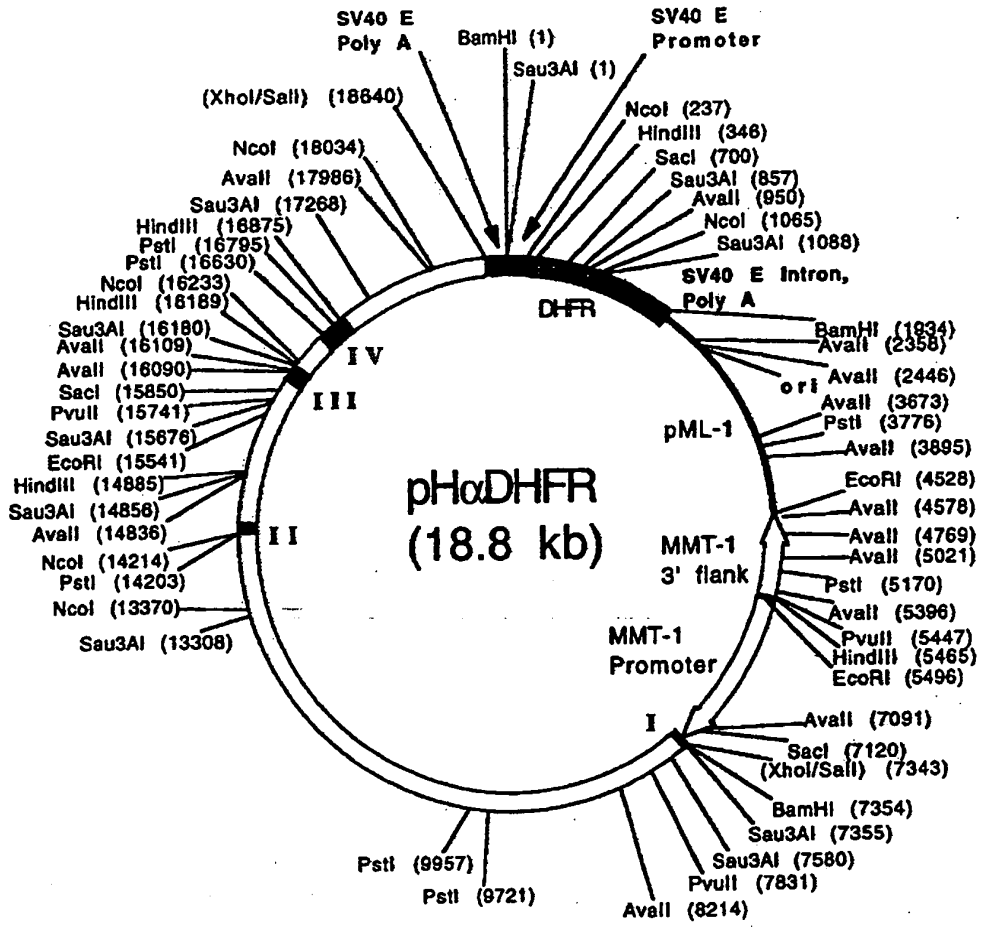


Figure DEG-9: Construction of expression vector pCLH3AXSV2ODC

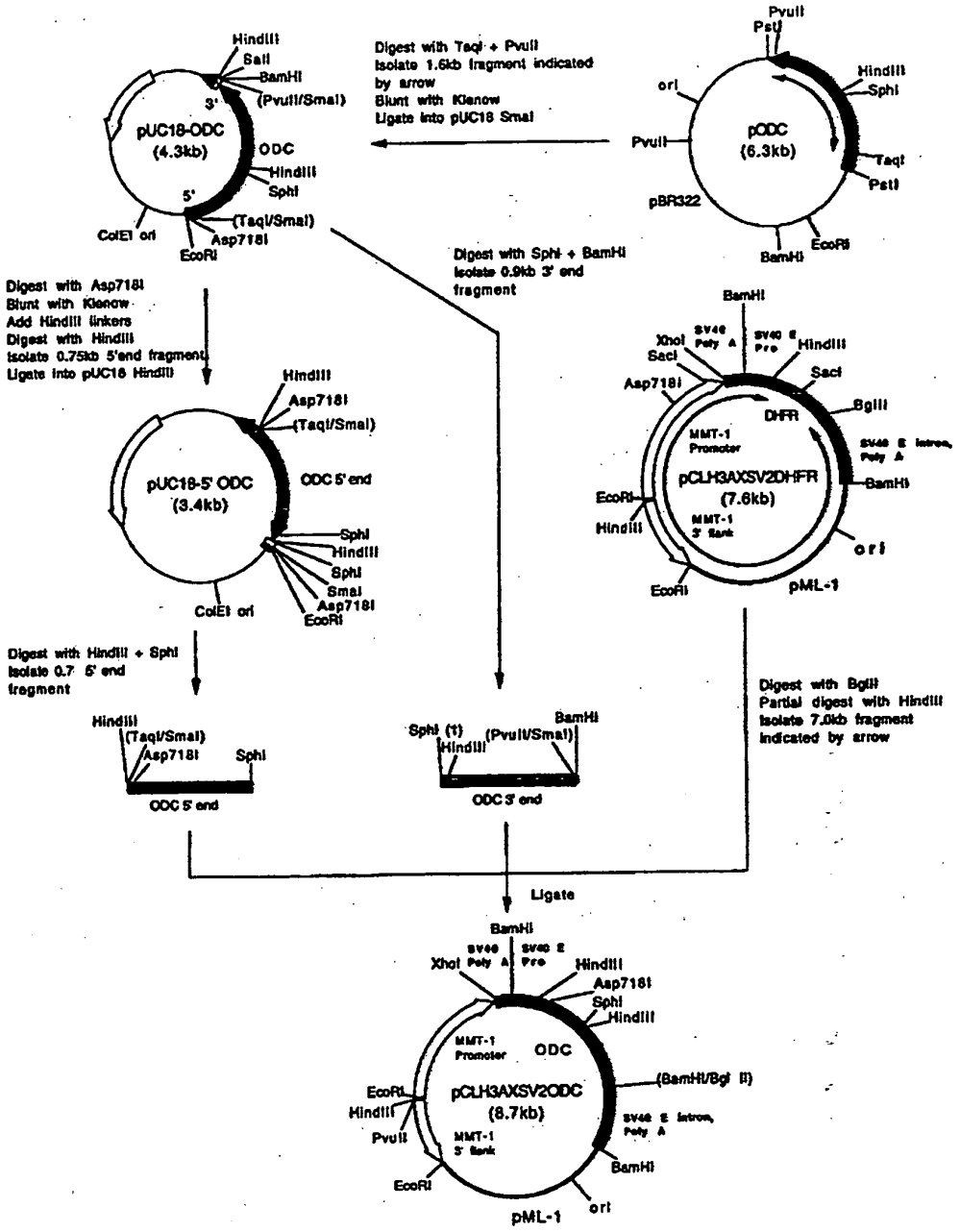


Figure DEG-10: Construction of β-subunit expression plasmid pHLHβODC

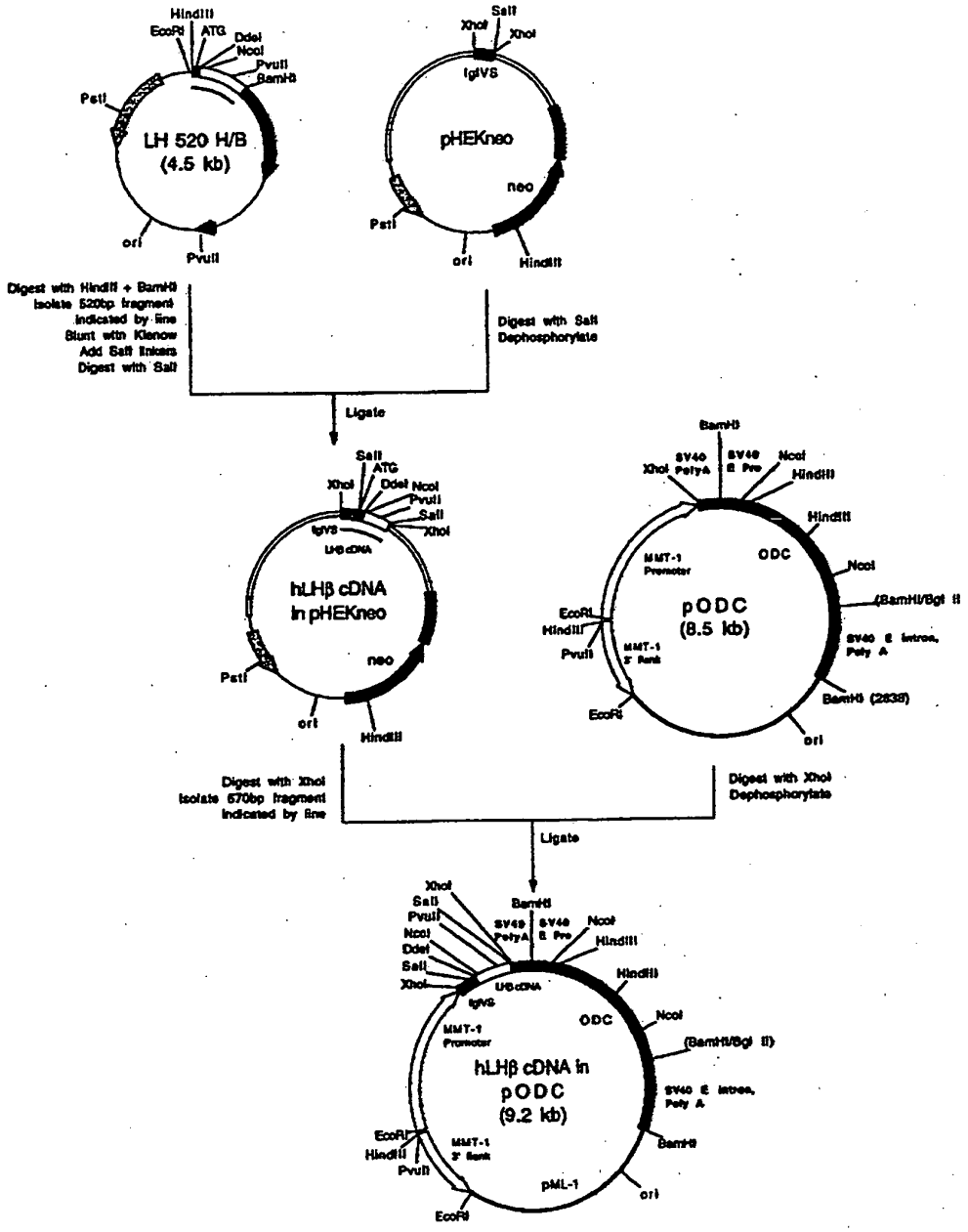
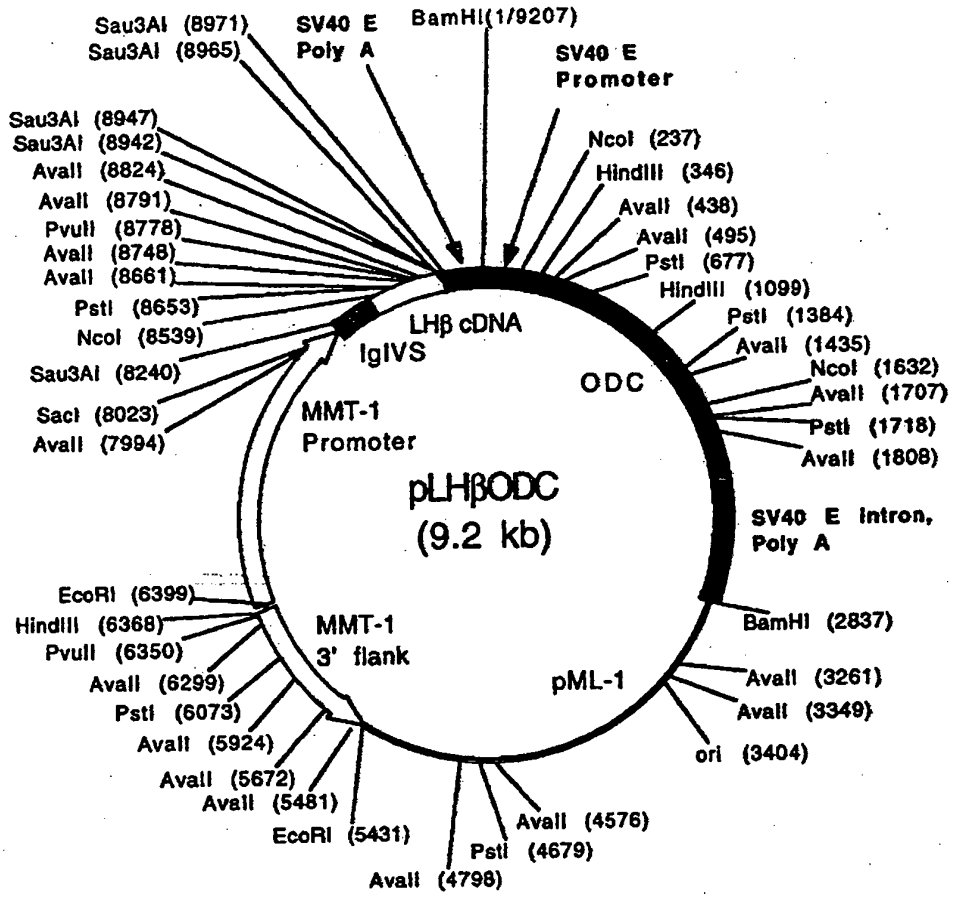


Figure-DEG-11: Restriction enzyme map of pHLHβODC



Mode of introduction into the production strain

The DHFR deficient CHO cell line DUKX-B11, was initially cultured in alpha-Minimum Essential Medium (α-MEM) containing ribonucleosides and deoxyribonucleosides and was supplemented with 2mM L-glutamine (final concentration is 4 mM) and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Cells were co-transfected using a modified calcium phosphate precipitation procedure (Graham and van der Eb, 1973) with uncut prepartions of the expression constructs pHαDHFR and pHLHβODC. The vectors were co-transfected into the CHO cells at a mass:mass ratio of 1α:3β. After 6 hours the cells were osmotically shocked by adding transfection solution containing 15% glycerol for 3.5 min. The cells were then rinsed and re-fed with fresh growth medium.

Data on cloning of an r-hLH producing cell line

Two days after transfection, cells were subcultured in selective medium consisting of α-MEM lacking ribonucleosides and deoxyribonucleosides. The α-MEM was supplemented with 2mM L-glutamine, 10% dialyzed fetal bovine serum (dFBS) and 0.02 μM MTX. After selection for 10-14 days with 0.02 μM MTX, approximately 120 individual transfectants were isolated by trypsinization with cloning chambers and were transferred to multi-well plates. When wells were ~50-80% confluent, cells were refed to collect media samples 24hrs later for immunoradiometric assay. Approximately 50 of the highest expressing isolates from the transfection were subsequently transferred to separate 25cm^2 tissue culture (T25) flasks in which the levels of MTX were increased to a final concentration of 0.1 μM. Culture medium was replaced as the cells approached 70-90% confluency so that production of r-hLH could be measured on a pg/cell/24 hr basis.

A similar procedure was followed for each subsequent stepwise increase in MTX. The steps used were 0.02 → 0.1 → 0.5 → 1.0 → 5.0 μM MTX, with cells maintained at each step for a minimum of 7-10 days. Co-amplification of the α- and β-subunit plasmids was a frequent occurrence when the cells were exposed to stepwise increases in MTX selection; therefore direct and separate amplification of the β-subunit plasmid using ODC antagonist was found not to be necessary.

Production rates of r-hLH in the culture medium of these amplified transfectants were determined by a radioimmunoassay kit (LH MAIAclone™, Serono Diagnostics) containing ^{125}I- and fluoroscein-labeled monoclonal antibodies which bind at discrete sites on the LH molecule. Subsequent incubation of the LH monoclonal antibody complex with an anti-fluorescein antibody coupled to a magnetic solid phase (MAIA) allows the quantitative determination of LH in the culture supernatant. Two individual cell lines, hLH1-109 and hLH1-111, displayed a wild-type CHO morphology and exhibited mean LH production rates of 1-2 pg/cell/24hr after amplification to 5.0 μM MTX (Table DEG-1). Upon further evaluation, these two fully amplified cell lines exhibited similar growth and stability characteristics. However, in the absence of MTX, hLH1-111 displayed higher productivity of r-hLH than hLH1-109 (Table DEG-2) and hLH1-111 was chosen for further cloning by limiting dilution.

Wurm (1990) encourages the cloning and expansion of DHFR-selected and amplified recombinant CHO cell lines in the absence of MTX. In his experience, the culture of transfected cell lines in the presence of MTX caused variability in levels of expression, location and amount of integrated sequences. This contributed to the overall instability of transfected sequences. In the absence of MTX, clonal cell lines were characterized by genetically stable, single, unique, identifiable integrations of amplified sequences.

Wurm suggests that MTX is unnecessary for the maintenance of transfected sequences as long as the amplified sequence and/or its expression are not harmful to the cells. This information reinforced the concern for maintaining selective pressure on the clonal lines by using MTX in the media.

The cell line hLH1-111 was thus cloned by limiting dilution in the presence and absence of 5.0 μM MTX in order to determine whether MTX was a requirement for obtaining a high producing clone. A trial cloning experiment, in which seeding densities of 4, 2, 1, 0.5 and 0.25 cells per well were utilized, showed that the dilution plating of 0.5 cells per well resulted in fewer than 33% of the wells exhibiting growth. This dilution was selected for the eventual cloning.

Ten 96 well plates were seeded with hLH1-111 cell suspension in the presence of 5.0 μM MTX and ten plates were seeded in the absence of MTX. As the cultures became confluent, 104 clones were transferred to 24-well culture plates. Spent medium was removed and replaced with fresh medium every 3-4 days. On day 16 the spent medium was removed and replaced with medium containing 1% FBS. Forty eight hours later, wells were sampled for r-hLH by MAIAClone™ immunoradiometric assay and the 25 highest expressing clones were harvested and passed to T25 flasks. Similar expansion/r-hLH productivity analysis steps were performed and 8 clones were further expanded in T75 flasks and these same 8 clones were finally expanded in T150 flasks. On the basis of their productivities of r-hLH in medium containing 1% FBS, uniformity of morphology and growth characteristics (e.g. ease of subpassage), these 8 clones grown in the absence of MTX were selected and redesignated IA11, IIB12, IIIC10, VD5, VIG7, VIIC6, VIIIF7, VIIIG8. Their respective production rates are presented in Table DEG-3. Cells cloned in the presence of MTX displayed equivalent growth and expression characteristics (Table DEG-4) as those cloned under non-selective conditions. The VIIC6 subclone, subcloned in the absence of MTX, was chosen for further process development and was used to establish the Master Cell Bank.

Table DEG-1: Production levels (pg/cell/24hr) of r-hLH by hLH1 cell lines in T25 flasks following gene amplication with up to 5.0 µM MTX.

Cell Line	r-hLH Production Levels (picograms/cell/24hr.)				
	0.02 µM MTX	0.1 µM MTX	0.5 µM MTX	1.0 µM MTX	5.0 µM MTX
hLH1-2	0.10	0.09	0.19	0.05	0.23
hLH1-26	0.37	0.17	0.22	0.006	0.003
hLH1-73	0.16	0.39	0.38	1.10	0.95
hLH1-84	0.09	0.19	0.56	0.63	0.96
hLH1-109	0.18	0.25	0.47	1.05	1.61
hLH1-111	0.14	0.17	0.35	1.03	1.20
hLH1-113	0.15	0.16	0.49	0.76	0.59

Table DEG-2: Comparative (48 hr.) productivities of r-hLH by hLH1-109 and hLH1-111 cells grown in T75 flasks in presence and absence of 5.0 µM MTX.

Cell Line	Absence or Presence of MTX	PDL	r-hLH Production IU/flask
hLH1-109	ABSENCE	19.10	249.74
		25.60	110.67
		32.90	147.95
		35.50	200.02
	PRESENCE	7.80	144.12
		17.00	319.94
		21.70	288.15
		28.30	274.28
		30.80	407.14
		47.70	77.66
hLH1-111	ABSENCE	20.60	280.87
		28.00	292.48
		30.40	301.34
		37.70	441.86
		62.90	142.97
	PRESENCE	7.60	154.02
		20.80	353.24
		25.70	266.11
		26.20	348.06
		36.70	578.76
		57.60	97.38

Table DEG-3: Clonal growth and productivity in T150 flasks of clones from the limited dilution cloning of hLH1-111 (-MTX group)

Clone	Titer	Cell Yield	r-hLH production	
-MTX	IU/L	cells/cm^2	IU/10^{6}cells/day	pg/cell/day
IAII	18,475	2.9×10^5	10.7	1.53
IIB12	19,399	1.4×10^5	23.1	3.30
IIIC10	19,286	2.9×10^5	11.2	1.60
VD5	17,401	2.6×10^5	11.1	1.59
VIG7	17,417	1.9×10^5	15.5	2.22
VIIC6	18,197	2.6×10^5	11.7	1.67
VIIIF7	18,381	2.5×10^5	12.4	1.77
VIIIG8	14,898	1.5×10^5	16.9	2.42

Table DEG-4: Clonal growth and productivity in T150 flasks of clones from the limited dilution cloning of hLH1-111 (+MTX group)

Clone	Titer	Cell Yield	r-hLH production	
+MTX	IU/L	cells/cm^2	IU/10^{6}cells/day	pg/cell/day
IF8	18,398	1.9×10^5	16.4	2.34
IIB10	17,823	1.7×10^5	17.8	2.54
IVH7	17,924	1.8×10^5	16.6	2.37
VC5	16,052	1.7×10^5	16.0	2.29
VIDI	17,972	1.6×10^5	18.7	2.67
VIIG6	18,370	1.8×10^5	17.0	2.43
XE5	18,402	1.6×10^5	19.2	2.74
XF9	16,233	1.7×10^5	16.2	2.32

Description of the production cell line

The hLH1-111 clone described in "Data on cloning of an r-hLH producing cell line" was used to produce by limited dilution cloning in the absence of MTX a further clone, VII C6. This clone was subsequently used for the production of the MCB and WCB. In addition, cells from two production fermentations were used to produce two extended population doubling banks (EPDB-I and -II). In this section will be described the following experiments which were performed to characterize the clone VII C6 by analyzing the MCB, WCB and the two EPDBs.

i. Cell growth kinetics.

ii. Specific productivity.

iii. Cellular morphology.

iv. Isoenzyme analysis.