CAVELIER
ABOGADOS

Señor Director
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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Act. 523 RESPUESTA Folios: 69

Trámite : 011
Evento : 379
Actuación : 523

Referencia : Expediente **No. 07077615 A**
Asunto : Solicitud de patente para: **"Composiciones inmunogénicas PCV-2".**
Solicitante : **BOEHRINGER INGELHEIM VETMEDICA, INC**
Fecha de solicitud: 30 de julio de 2007.

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio **No. 23764** notificado por fijación en lista del 31 de diciembre de 2013.

2. Mediante memorial radicado el día 26 de marzo de 2014, solicité plazo de treinta (30) días para atender el oficio No. **23764.**

3. Mediante oficio No. **23764** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

3.1 <u>Pliego reivindicatorio</u>

De manera resumida, el Examinador ha expuesto lo siguiente:

- Reivindicación 1: debe definir las características esenciales que contribuyen a la obtención de una composición inmunogénica que es efectiva en reducir la severidad de síntomas clínicos asociados con la infección PCV2.
- Se sugiere incluir el listado de secuencias con el fin de suportar el polipéptido de la reivindicación 1.
- Reivindicación 4: Debe reemplazar la expresión "BEI" por su denominación común.
- Reivindicación 3: debe definir el vector recombinante por la secuencia recombinante que contiene.
- Reivindicación 6: debe limitarse a la materia debidamente soportada en la descripción.
- Existe falta de concisión a lo largo del pliego reivindicatorio.

3.2 Nivel inventivo

De acuerdo con el Examinador, las enseñanzas de los documentos US6497883 (D1) y "FENAUX M. ET AL, Journal of Clinical Microbiology, Vol.38, No. 7, Págs. 2494-2503" (D2) afectan el nivel inventivo de las reivindicaciones 1 a 8 de la presente solicitud de patente.

4. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

4.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 7 reivindicaciones.

Así, siguiendo las amables sugerencias del Examinador se ha modificado el pliego reivindicatorio limitando el alcance del mismo a aquella materia debidamente soportada en la descripción. En particular, (i) la reivindicación 1 ha sido modificada definiendo los elementos que hacen parte de la composición y que permiten que la misma sea efectiva para reducir la severidad de síntomas clínicos asociados con la infección PCV2, (ii) se presenta anexo al presente memorial el listado de secuencias en forma separada de la descripción en formato físico y digital, (iii) se ha remplazado la expresión BEI por "etilenimina binaria" y (iv) la reivindicación 3 ha sido eliminada del pliego reivindicatorio.

Con respecto a los términos objetados: "portadores adyuvante", "medio", "inactivadotes víricos", "diluyentes", "agentes isotónicos", "agentes inmunomoduladores", "antibióticos", mi representada desea poner de presente que dichos términos se encuentra definidos de forma clara y precisa en la descripción de la presente solicitud y por lo tanto, el alcance del pliego reivindicatorio también se encuentra claramente definido. Lo anterior, si se tiene en cuenta que de conformidad con lo señalado en el artículo 51 de la Decisión 486 la descripción de una solicitud sirve para interpretar las reivindicaciones: *"El alcance de la protección conferida por la patente estará determinado por el tenor de las reivindicaciones. La descripción y los dibujos, o en su caso, el material biológico depositado, servirán para interpretarlas*". De modo que, la objeción formulada a este respecto se debe tener por superada.

Por lo anterior, el pliego reivindicatorio cumple con los requerimientos de claridad, concisión y soporte establecidos en el Artículo 30 de la Decisión 486. Igualmente, define un único concepto común inventivo, por lo que las objeciones formuladas por el Examinador en este sentido se ven superadas.

4.2 <u>Argumentos a favor de la patentabilidad de la solicitud</u>

De acuerdo con el artículo 18 de la Decisión 486 señala lo siguiente:

*"**Artículo 18.-** Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica*

correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."

Una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

A este respecto, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta el nivel inventivo, con base en la siguiente información:

De acuerdo con el Examinador, el documento D1 divulga una composición inmunogénica que comprende, proteína ORFs seleccionada del grupo que comprende 1,2,3,4,5,6,7,8,9,10,11,12 y 13 de circovirus porcina tipo II y también divulga que la composición comprende un vector vírico baculovirs recombinante. Sin embargo, luego de revisar el documento D1, mi representada desea poner de presente que en contraste con lo afirmado por el Examinador, en D1 no existe enseñanza o sugerencia alguna en relación con un vector vírico baculovirus recombinante o un virus PRRS (ver documento D1 anexo al presente memorial).

En efecto, el documento D1 en la columna 1, líneas 31-54 revela que la invención allí divulgada se refiere a:

"La presente invención se refiere a vectores, tales como vectores recombinantes; por ejemplo, los virus recombinantes, tales como virus de la viruela , por ejemplo , virus de la viruela modificado y a métodos para fabricar y usar la misma . En algunas formas de realización, la invención se refiere a los virus de la viruela aviar recombinante, tales como los virus de la viruela del canario , por ejemplo , ALVAC . La invención se refiere además a tales vectores , por ejemplo , poxvirus , que expresan productos génicos , por ejemplo , antígeno (s) , ORF (s) , y / o epítopo (s) de interés de la misma, de circovirus porcino tipo 2 (PCV2); a composiciones inmunológicas o vacunas . La invención todavía se refiere además a tales vectores , por ejemplo , virus de la viruela, que inducen una respuesta inmune dirigida a o contra productos génicos PCV y / o PCV2 ; y , a ventajosamente , dichas composiciones que son composiciones inmunológicas , inmunogénicas o de vacuna y / o conferir inmunidad protectora contra la infección por PCV2 . La invención todavía se refiere además a los usos de y métodos para preparar y usar tales vectores y composiciones, así como intermedios de los mismos, y dichos intermedios. Y, la invención se refiere a los productos de los mismos, por ejemplo , a partir de los usos y métodos que implican la recombinante de la invención o virus de la viruela , tales como anticuerpos de expresión".

De tal manera que, D1 se refiere a un campo técnico distinto al de la presente invención, toda vez que en dicho documento no enseña o sugiere sub-unidades de vacunas proteicas. Asimismo, a diferencia de la presente solicitud, en los ejemplos de D1 únicamente se usa y menciona un virus (in vivo) atenuado de viruela recombinante (ALVAC) que contiene el PCV2 ORF2 y ORF1 insertado en el locus C6 de ALVAC, en una orientación de cabeza a cabeza (ver Ejemplos 6, 8 y 9 de D1).

En cuanto al virus ALVAC se describe adicionalmente en el Ejemplo 1 de D1:

"El virus de la viruela del canario parental (cepa Rentschler) es una cepa de vacuna para canarios. La cepa de vacuna se obtuvo a partir un tipo aislado y atenuada a través de más de 200 pases seriados en fibroblastos de embrión de pollo salvaje. Una semilla viral se sometió a cuatro purificaciones en placa sucesivas bajo agar y un clon de placa se

amplificó a través de cinco pasajes adicionales, tras lo cual se utilizó el virus de reserva como virus parental en pruebas de recombinación in vitro. La placa purificada aislado se designa ALVAC . El ALVAC se depositó el 14 de noviembre 1996 , en los términos del Tratado de Budapest en la American Type Culture Collection , ATCC número de acceso VR -2547".

A este respecto, vale la pena resaltar que la diferencia entre las vacunas de vectores de virus recombinantes de D1 y la subunidad de vacunas de la presente invención es fundamental : En comparación con el uso de virus vivo atenuado, que generalmente tiene la ventaja de una eficacia superior pero la desventaja de que puede cambiar o mutar (y por lo tanto el virus ALVAC utilizado de acuerdo a D1 tendría por lo menos el potencial de volver a una forma virulenta y causar enfermedad en aves) , la ventaja de una vacuna de subunidad es que se basa en una (o más) proteína (s) que no es / son capaz de replicar o mutar. Además, debido a que las vacunas de subunidades contienen sólo el antígeno esencial (s) y no todas las otras moléculas que componen el microbio (en particular, que no contiene un viral activo del genoma) , las posibilidades de reacciones adversas a la vacuna son menores.

Sin embargo, una desventaja clave de vacunas de subunidades es por lo general que tienen que ser administras al menos dos veces y que requieren adyuvantes fuertes que a menudo inducen reacciones tisulares. En consecuencia, fue un hallazgo sorprendente de acuerdo con la presente invención que la composición inmunogénica como se reivindica permita la vacunación segura y eficaz con una sola dosis (ver página 41, línea 24 hasta página 42, línea 4 de la solicitud internacional).

Con respecto al documento D2, mi representada desea poner de presente que dicho documento no enseña la secuencia SEQ ID NO: 6. En su lugar, D2 sólo se refiere a la caracterización genética de PCV2, en el que "Para determinar el grado de heterogeneidad genética de PCV-2 aislados, se amplificó el genoma completo de seis PCV-2 aislados de diferentes regiones de América del Norte por PCR y se secuenciaron " y "Análisis filogenéticos y de secuencias confirmaron que existen dos genotipos distintos de PCV: genotipo no patógeno PCV-1 y genotipo PMWS asociado PCV-2 " (ver Resumen de D2).

Por lo tanto, a partir de las enseñanzas de los documentos D1 y/o D2 por si solos o combinados, no se derivaría de manera obvia u evidente la materia de invención objeto de protección y recitada en el nuevo pliego reivindicatorio, toda vez que dichos documentos no enseñan ni sugieren como un todo las características técnicas esenciales de la reivindicación 1. Es decir, dichos documentos no enseñan ni sugieren por si solos o combinados:

> *Una composición inmunogénica que comprende* ***la proteína baculovirus ORF2 de PCV2 expresada de forma recombinante y un adyuvante****,*
>
> *en donde dicha proteína ORF2 de PCV2 es:*
>
> *(i) un polipéptido que comprende la secuencia* ***SEQ ID NO:6; o***
>
> *(ii) un polipéptido que se codifica por un ADN que comprende SEQ ID NO:4; y*
>
> *en donde el adyuvante seleccionado del grupo que consiste de polímeros de ácido acrílico que se reticulan con éteres de polialquenilo de azúcares o polialcoholes.*

De otro lado, el efecto técnico inesperado que provee el método reivindicado se demuestra con los ejemplos de la descripción y particularmente con el ejemplo 1, tabla 1.

Por consiguiente, el método reivindicado cumple con los requerimientos de patentabilidad del artículo 18 de la Decisión 486.

4.3 Conclusión

Con las modificaciones y los argumentos acá presentados, se superan las objeciones formuladas por el Examinador en el oficio 23764.

5. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente para la reivindicación 1. En este sentido, respetuosamente recordamos al Examinador que

la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 23764, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

6. <u>Anexos</u>

-Nuevo pliego reivindicatorio conformado por 7 reivindicaciones.
-Listado de secuencias en formato físico y digital.
-Documento D1: US6497883B1

Señor Director,

LUZ CLEMENCIA DE PAEZ
C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

COLO-15149-0841-017-5
MGM\MGM\ID-264848\WPC15149
No. M-2014-264848\MGM

REIVINDICACIONES

1. Una composición inmunogénica CARACTERIZADA PORQUE comprende la proteína ORF2 de PCV2 expresada de forma recombinante y un adyuvante,

en donde dicha proteína ORF2 de PCV2 es:

(i) un polipéptido que comprende la secuencia de SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:10 o SEQ ID NO:11; o

(ii) un polipéptido que se codifica por un ADN que comprende la secuencia ID NO:3 o SEQ ID NO:4; y

en donde el adyuvante seleccionado del grupo que consiste de polímeros de ácido acrílico que se reticulan con éteres de polialquenilo de azúcares o polialcoholes.

2. La composición inmunogénica de la reivindicación 1, en donde dicha composición adicional comprende un vector vírico inactivo y sobrenadante de cultivo celular.

3. La composición inmunogénica de la reivindicación 2 o 3, en donde dicha composición comprende etilenimina binaria.

4. La composición inmunogénica de la reivindicación 3, en donde dicha composición comprende tiosulfato de sodio.

5. La composición inmunogénica de acuerdo con cualquiera de las reivindicaciones 1 a 4, en donde dicha composición comprende un ingrediente adicional seleccionado del grupo que consiste de portadores, adyuvantes, medio, inactivadores víricos, diluyentes, agentes isotónicos, agentes inmunomoduladores, antibióticos y combinaciones de estos.

6. La composición inmunogénica de la reivindicación 5, en donde dicha composición comprende una sal farmacéuticamente aceptable, preferiblemente solución salina.

7. Un kit que comprende i) un contenedor conteniendo al menos una dosis de la composición inmunogénica de cualquiera de las reivindicaciones 1 a 6, y ii) un contenedor conteniendo una composición inmunogénica comprendiendo el virus vivo PRRS modificado (MLV PRRS) y un manual de instrucción, incluyendo la información para administrar ambas composiciones farmacéuticas.

MGM\MGM\ID-264771\WPC15149

34816-CIP1.ST25
SEQUENCE LISTING

<110> Roof, Mike
Eichmeyer, Mark
Nitzel, Greg
Schaeffer, Merrill
Hayes, Phillip

<120> PCV2 IMMUNOGENIC COMPOSITIONS AND METHODS OF PRODUCING SUCH COMPOSITIONS

<130> 34816-CIP1

<140> UNKNOWN
<141> 2005-01-10

<150> Unknown
<151> 2004-12-30

<160> 11

<170> PatentIn version 3.3

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<211> 8
<212> DNA
<213> Artificial

<220>
<223> This is a modified Kozak's sequence.

<400> 1
ccgccatg 8

<210> 2
<211> 6
<212> DNA
<213> Artificial

<220>
<223> This is a recombinant Eco R1 sequence.

<400> 2
gaattc 6

<210> 3
<211> 713
<212> DNA
<213> Porcine circovirus

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ttggccagat cctccgccgc cgcccctggc tcgtccaccc ccgccaccgc taccgttgga 120

gaaggaaaaa tggcatcttc aacacccgcc tctcccgcac cttcggatat actgtggaga 180

aggaaaaatg gcatcttcaa cacccgcctc tcccgcacct tcggatatac tgtgacgact 240

ttgttccccc gggaggggag accaacaaaa tctctatacc ctttgaatac tacagaataa 300

gaaaggttaa ggttgaattc tggccctgct cccccatcac ccagggtgat aggggagtgg 360

gctccactgc tgttattcta gatgataact ttgtaacaaa ggccacagcc ctaacctatg 420

acccatatgt aaactactcc tcccgccata caatccccca acccttctcc taccactccc 480

gttacttcac acccaaacct gttcttgact ccactattga ttacttccaa ccaaataaca 540

aaaggaatca gctttggctg aggctacaaa cctctagaaa tgtggaccac gtaggcctcg 600

gcactgcgtt cgaaaacagt aaatacgacc aggactacaa tatccgtgta accatgtatg 660

tacaattcag agaatttaat cttaaagacc ccccacttaa accctaaatg aat 713

<210> 4
<211> 713
<212> DNA
<213> Porcine circovirus

<400> 4
ccgccatgac gtatccaagg aggcgttacc gcagaagaag acaccgcccc cgcagccatc 60

ttggccagat cctccgccgc cgcccctggc tcgtccaccc ccgccaccgc taccgttgga 120

gaaggaaaaa tggcatcttc aacacccgcc tctcccgcac cttcggatat actgtcaagg 180

ctaccacagt cacaacgccc tcctgggcgg tggacatgat gagatttaat attgacgact 240

ttgttccccc gggaggggggg accaacaaaa tctctatacc ctttgaatac tacagaataa 300

gaaaggttaa ggttgaattc tggccctgct cccccatcac ccagggtgat aggggagtgg 360

gctccactgc tgttattcta gatgataact ttgtaacaaa ggccacagcc ctaacctatg 420

acccatatgt aaactactcc tcccgccata caatccccca acccttctcc taccactccc 480

gttacttcac acccaaacct gttcttgact ccactattga ttacttccaa ccaaataaca 540

aaaggaatca gctttggctg aggctacaaa cctctagaaa tgtggaccac gtaggcctcg 600

gcactgcgtt cgaaaacagt aaatacgacc aggactacaa tatccgtgta accatgtatg 660

tacaattcag agaatttaat cttaaagacc ccccacttga accctaagaa ttc 713

<210> 5
<211> 233
<212> PRT
<213> Porcine circovirus

<400> 5

Met Thr Tyr Pro Arg Arg Arg Tyr Arg Arg Arg Arg His Arg Pro Arg
1 5 10 15

Ser His Leu Gly Gln Ile Leu Arg Arg Arg Pro Trp Leu Val His Pro
20 25 30

Arg His Arg Tyr Arg Trp Arg Arg Lys Asn Gly Ile Phe Asn Thr Arg
35 40 45

Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys Ala Thr Thr Val Thr Thr
50 55 60

Pro Ser Trp Ala Val Asp Met Met Arg Phe Asn Ile Asp Asp Phe Val
65 70 75 80

Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser Ile Pro Phe Glu Tyr Tyr
85 90 95

Arg Ile Arg Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr
100 105 110

Gln Gly Asp Arg Gly Val Gly Ser Thr Ala Val Ile Leu Asp Asp Asn
115 120 125

Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro Tyr Val Asn Tyr
130 135 140

Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
145 150 155 160

Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
165 170 175

Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg Leu Gln Thr Ser Arg Asn
180 185 190

Val Asp His Val Gly Leu Gly Thr Ala Phe Glu Asn Ser Lys Tyr Asp
195 200 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
210 215 220

Asn Leu Lys Asp Pro Pro Leu Lys Pro
225 230

<210> 6
<211> 233
<212> PRT
<213> Porcine circovirus

<400> 6

Met Thr Tyr Pro Arg Arg Arg Tyr Arg Arg Arg Arg His Arg Pro Arg
1 5 10 15

Ser His Leu Gly Gln Ile Leu Arg Arg Arg Pro Trp Leu Val His Pro
20 25 30

Arg His Arg Tyr Arg Trp Arg Arg Lys Asn Gly Ile Phe Asn Thr Arg
35 40 45

Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys Ala Thr Thr Val Thr Thr
50 55 60

Pro Ser Trp Ala Val Asp Met Met Arg Phe Asn Ile Asp Asp Phe Val
65 70 75 80

Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser Ile Pro Phe Glu Tyr Tyr
85 90 95

Arg Ile Arg Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr
100 105 110

Gln Gly Asp Arg Gly Val Gly Ser Thr Ala Val Ile Leu Asp Asp Asn
115 120 125

Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro Tyr Val Asn Tyr
130 135 140

Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
145 150 155 160

Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
165 170 175

Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg Leu Gln Thr Ser Arg Asn
180 185 190

Val Asp His Val Gly Leu Gly Thr Ala Phe Glu Asn Ser Lys Tyr Asp
195 200 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
210 215 220

Asn Leu Lys Asp Pro Pro Leu Glu Pro
225 230

<210> 7
<211> 756
<212> DNA
<213> Artificial

<220>
<223> This sequence is from porcine circovirus type 2, open reading frame 2, together with a portion from the pGEM T-easy vector.

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gcggccgcgg gaattcgatc cgccatgacg tatccaagga ggcgttaccg cagaagaaga 60

caccgccccc gcagccatct tggccagatc ctccgccgcc gcccctggct cgtccacccc 120

cgccaccgct accgttggag aaggaaaaat ggcatcttca acacccgcct ctcccgcacc 180

ttcggatata ctgtcaaggc taccacagtc acaacgccct cctgggcggt ggacatgatg 240

agatttaata ttgacgactt tgttcccccg ggaggggga ccaacaaaat ctctataccc 300

tttgaatact acagaataag aaaggttaag gttgaattct ggccctgctc ccccatcacc 360

cagggtgata ggggagtggg ctccactgct gttattctag atgataactt tgtaacaaag 420

gccacagccc taacctatga cccatatgta aactactcct cccgccatac aatcccccaa 480

cccttctcct accactcccg ttacttcaca cccaaacctg ttcttgactc cactattgat 540

tacttccaac caaataacaa aaggaatcag ctttggctga ggctacaaac ctctagaaat 600

gtggaccacg taggcctcgg cactgcgttc gaaaacagta aatacgacca ggactacaat 660

atccgtgtaa ccatgtatgt acaattcaga gaatttaatc ttaaagaccc cccacttgaa 720

ccctaagaat tctatcacta gtgaattcgc ggccgc 756

<210> 8
<211> 10387
<212> DNA
<213> Artificial

<220>
<223> This is the porcine circovirus type 2, ORF2 construct, which includes baculovirus and pGEM T-easy coding sequences.

<400> 8
aagctttact cgtaaagcga gttgaaggat catatttagt tgcgtttatg agataagatt 60

gaaagcacgt gtaaaatgtt tcccgcgcgt tggcacaact atttacaatg cggccaagtt 120

ataaaagatt ctaatctgat atgttttaaa acacctttgc ggcccgagtt gtttgcgtac 180

gtgactagcg aagaagatgt gtggaccgca gaacagatag taaaacaaaa ccctagtatt 240

ggagcaataa tcgatttaac caacacgtct aaatattatg atggtgtgca ttttttgcgg 300

gcgggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc tgaaagcata 360

gttcaagaat ttattgacac ggtaaaagaa tttacagaaa agtgtcccgg catgttggtg 420

ggcgtgcact gcacacacgg tattaatcgc accggttaca tggtgtgcag atatttaatg 480

cacaccctgg gtattgcgcc gcaggaagcc atagatagat tcgaaaaagc cagaggtcac 540

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<213> Porcine circovirus

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His Leu Gly Gln
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<213> Porcine circovirus

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Thr Leu Ser

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<220>
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1 5 10 15

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20 25 30

Arg His Arg Tyr Arg Trp Arg Arg Lys Asn Gly Ile Phe Asn Thr Arg
35 40 45

Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys Ala Thr Thr Val Arg Thr

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Pro Ser Trp Ala Val Asp Met Met Arg Phe Asn Ile Asp Asp Phe Val
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Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser Ile Pro Phe Glu Tyr Tyr
85 90 95

Arg Ile Lys Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr
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Gln Gly Asp Arg Gly Val Gly Ser Thr Ala Val Ile Leu Asp Asp Asn
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Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro Tyr Val Asn Tyr
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Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
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Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
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Val Asp His Val Gly Leu Gly Thr Ala Phe Glu Asn Ser Ile Tyr Asp
195 200 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
210 215 220

Asn Leu Lys Asp Pro Pro Leu Lys Pro
225 230

Industria y Comercio
SUPERINTENDENCIA

PLANILLA DE RADICACIÓN - DIGITALIZACIÓN DIARIA
RELACIÓN DE ANEXOS

Dependencia: 2020	Fecha: ____/____/____ AAAA MM DD 2014 8 8	Recorrido: 4
Tipo de Radicación: Entrada: X Salida: ☐ Traslado: ☐ Convenio: ☐		
Radicador:		Planilla:

ID	No. Radicación	Consecutivo	Item
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2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			

Item	Unidad de Conservación
1	Bolsa
2	Caja
3	Libro
4	Sobre
5	Tomo
6	Otro
Item	***Soporte Documental***
7	CD
8	Disquete
9	DVD
10	Folleto
11	Foto
12	Periódico
13	Plano
14	Publicación Periódica
15	USB
16	Otro

Observaciones:
EL CD ANEXO CONTIENE UN TOTAL DE 1 ARCHIVO:
1 EN FORMATO PDF, EL CUAL FUE PROCESADO:
EL CD SE ENCUENTRA EN EL FOLIO (23).

US006497883B1

(12) **United States Patent**
Bublot et al.

(10) **Patent No.: US 6,497,883 B1**
(45) **Date of Patent: Dec. 24, 2002**

(54) **PORCINE CIRCOVIRUS RECOMBINANT POXVIRUS VACCINE**

(75) Inventors: **Michel Bublot**, Delmar, NY (US); **Jennifer M. Perez**, East Nassau, NY (US); **Catherine E. Charreyre**, St.-Laurent De Mure (FR)

(73) Assignee: **Merial**, Lyons (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/583,545**

(22) Filed: **Jun. 1, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/138,478, filed on Jun. 10, 1999.

(51) **Int. Cl.**[7] **A61K 39/12**; C12N 15/00

(52) **U.S. Cl.** **424/204.1**; 424/199.1; 424/209.1; 424/218.1; 435/320.1; 435/235.1; 536/23.72

(58) **Field of Search** 424/199.1, 204.1, 424/209.1, 218.1; 536/23.72; 435/320.1, 235.1

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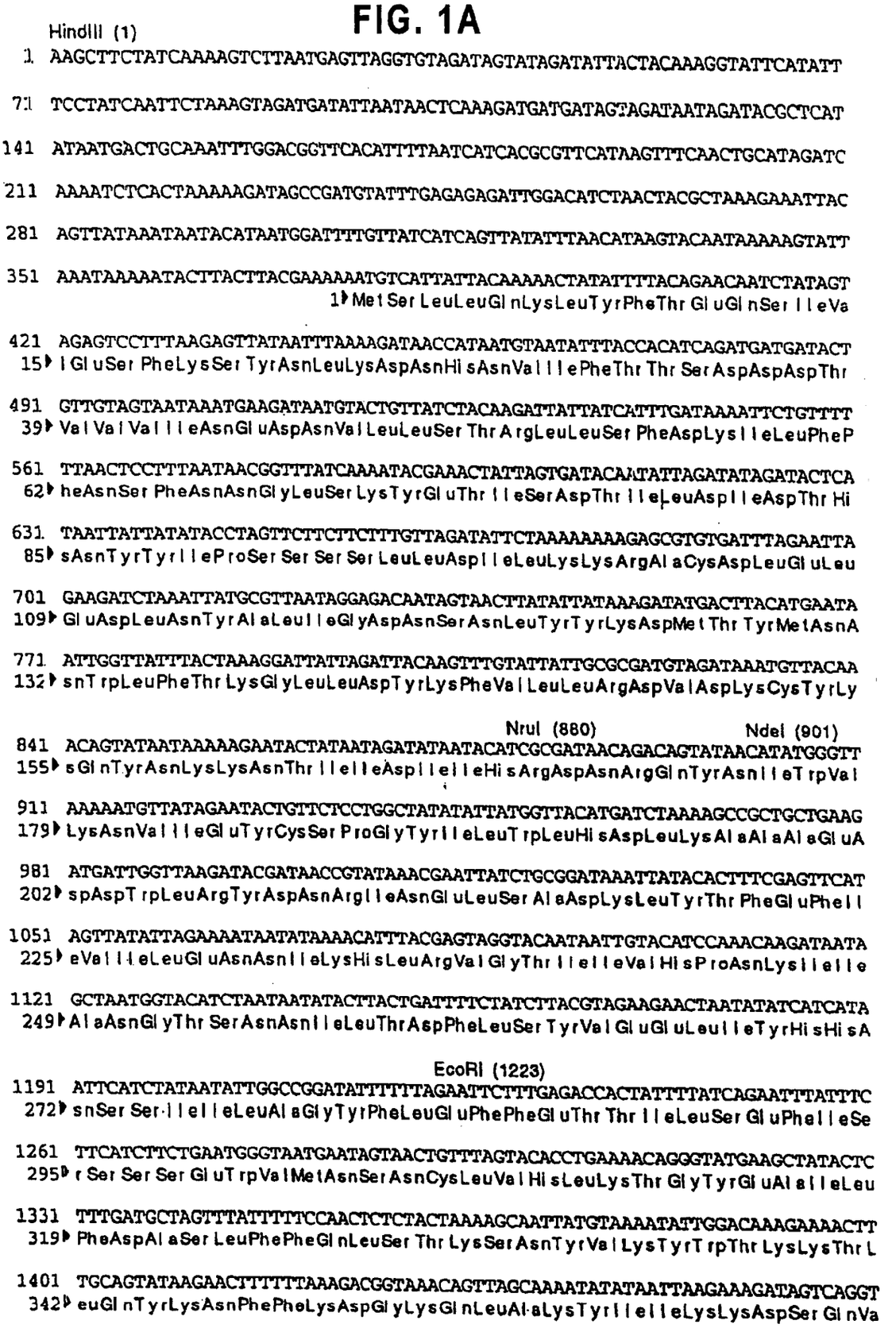
Primary Examiner—Ali R. Salimi
(74) *Attorney, Agent, or Firm*—Frommer Lawrence & Haug, LLP; William S. Frommer; Thomas J. Kowalski

(57) **ABSTRACT**

What is described is a recombinant poxvirus, such as avipox virus, containing foreign DNA from porcine circovirus 2. What are also described are immunological compositions containing the recombinant poxvirus for inducing an immunological response in a host animal to which the immunological composition is administered. Also described are methods of treating or preventing disease caused by porcine circovirus 2 by administering the immunological compositions of the invention to an animal in need of treatment or susceptible to infection by porcine circovirus 2.

26 Claims, 11 Drawing Sheets

FIG. 1A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1 Met Ser LeuLeuGlnLysLeuTyrPheThr GluGlnSer IleVa
421 AGAGTCCTTTAAGAGTTATAATTTAAAAGATAACCATAATGTAATATTTACCACATCAGATGATGATACT
15 IGluSer PheLysSer TyrAsnLeuLysAspAsnHisAsnVal IlePheThr Thr SerAspAspAspThr
491 GTTGTAGTAATAAATGAAGATAATGTACTGTTATCTACAAGATTATTATCATTTGATAAAATTCTGTTTT
39 Val Val Val IleAsnGluAspAsnVal LeuLeuSer Thr ArgLeuLeuSer PheAspLys IleLeuPheP
561 TTAACTCCTTTAATAACGGTTTATCAAAATACGAAACTATTAGTGATACAATATTAGATATAGATACTCA
62 heAsnSer PheAsnAsnGlyLeuSer LysTyrGluThr IleSerAspThr IleLeuAsp IleAspThr Hi
631 TAATTATTATATACCTAGTTCTTCTTCTTTGTTAGATATTCTAAAAAAAAGAGCGTGTGATTTAGAATTA
85 sAsnTyrTyr IleProSer Ser Ser Ser LeuLeuAsp IleLeuLysLysArgAlaCysAspLeuGluLeu
701 GAAGATCTAAATTATGCGTTAATAGGAGACAATAGTAACTTATATTATAAAGATATGACTTACATGAATA
109 GluAspLeuAsnTyrAlaLeu IleGlyAspAsnSerAsnLeuTyrTyrLysAspMet Thr TyrMetAsnA
771 ATTGGTTATTTACTAAAGGATTATTAGATTACAAGTTTGTATTATTGCGCGATGTAGATAAATGTTACAA
132 snTrpLeuPheThr LysGlyLeuLeuAspTyrLysPheVal LeuLeuArgAspVal AspLysCysTyrLy
NruI (880) NdeI (901)
841 ACAGTATAATAAAAAGAATACTATAATAGATATAATACATCGCGATAACAGACAGTATAACATATGGGTT
155 sGlnTyrAsnLysLysAsnThr IleIleAsp IleIleHisArgAspAsnArgGlnTyrAsn IleTrpVal
911 AAAAATGTTATAGAATACTGTTCTCCTGGCTATATATTATGGTTACATGATCTAAAAGCCGCTGCTGAAG
179 LysAsnVal IleGluTyrCysSer ProGlyTyr IleLeuTrpLeuHisAspLeuLysAlaAlaAlaGluA
981 ATGATTGGTTAAGATACGATAACCGTATAAACGAATTATCTGCGGATAAATTATACACTTTCGAGTTCAT
202 spAspTrpLeuArgTyrAspAsnArg IleAsnGluLeuSer AlaAspLysLeuTyrThr PheGluPhe I
1051 AGTTATATTAGAAAATAATATAAAACATTTACGAGTAGGTACAATAATTGTACATCCAAACAAGATAATA
225 eVal IleLeuGluAsnAsn IleLysHisLeuArgVal GlyThr IleIleVal HisProAsnLys IleIle
1121 GCTAATGGTACATCTAATAATATACTTACTGATTTTCTATCTTACGTAGAAGAACTAATATATCATCATA
249 AlaAsnGlyThr SerAsnAsn IleLeuThrAspPheLeuSer TyrVal GluGluLeu IleTyrHisHisA
EcoRI (1223)
1191 ATTCATCTATAATATTGGCCGGATATTTTTTAGAATTCTTTGAGACCACTATTTTATCAGAATTTATTTC
272 snSer Ser IleIleLeuAlaGlyTyrPheLeuGluPhePheGluThr Thr IleLeuSer GluPhe IleSe
1261 TTCATCTTCTGAATGGGTAATGAATAGTAACTGTTTAGTACACCTGAAAACAGGGTATGAAGCTATACTC
295 r Ser Ser Ser GluTrpVal MetAsnSer AsnCysLeuVal HisLeuLysThr GlyTyrGluAla IleLeu
1331 TTTGATGCTAGTTTATTTTTCCAACTCTCTACTAAAAGCAATTATGTAAAATATTGGACAAAGAAAACTT
319 PheAspAlaSer LeuPhePheGlnLeuSer Thr LysSerAsnTyrVal LysTyrTrpThr LysLysThr L
1401 TGCAGTATAAGAACTTTTTTAAAGACGGTAAACAGTTAGCAAAATATATAATTAAGAAAGATAGTCAGGT
342 euGlnTyrLysAsnPhePheLysAspGlyLysGlnLeuAlaLysTyr IleIleLysLysAspSer GlnVa

FIG. 1B

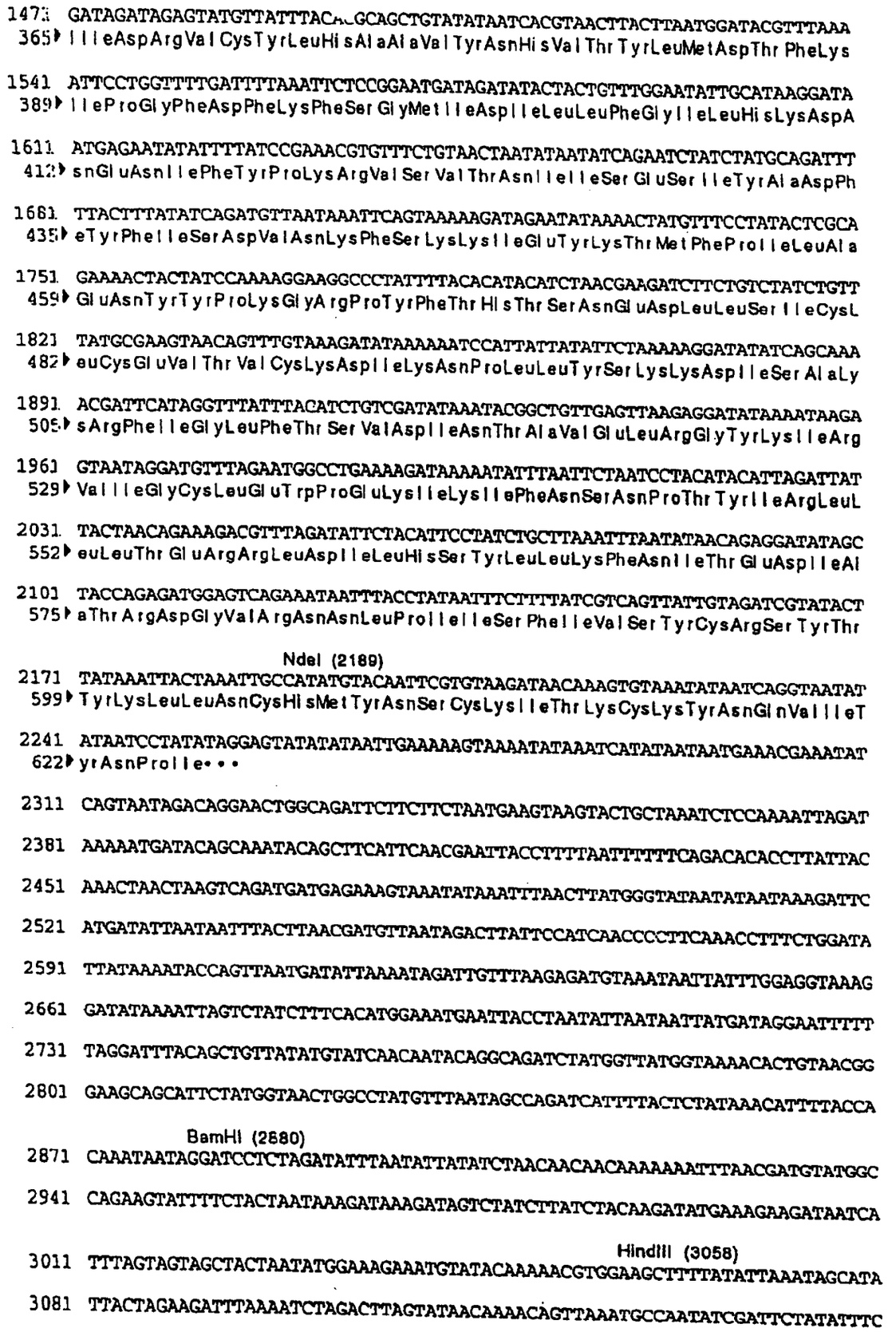

1471 GATAGATAGAGTATGTTATTTACAAGCAGCTGTATATAATCACGTAACTTACTTAATGGATACGTTTAAA
365 IleAspArgValCysTyrLeuHisAlaAlaValTyrAsnHisValThrTyrLeuMetAspThrPheLys
1541 ATTCCTGGTTTTGATTTTAAATTCTCCGGAATGATAGATATACTACTGTTTGGAATATTGCATAAGGATA
389 IleProGlyPheAspPheLysPheSerGlyMetIleAspIleLeuLeuPheGlyIleLeuHisLysAspA
1611 ATGAGAATATATTTTATCCGAAACGTGTTTCTGTAACTAATATAATATCAGAATCTATCTATGCAGATTT
413 snGluAsnIlePheTyrProLysArgValSerValThrAsnIleIleSerGluSerIleTyrAlaAspPh
1681 TTACTTTATATCAGATGTTAATAAATTCAGTAAAAAGATAGAATATAAAACTATGTTTCCTATACTCGCA
435 eTyrPheIleSerAspValAsnLysPheSerLysLysIleGluTyrLysThrMetPheProIleLeuAla
1751 GAAAACTACTATCCAAAAGGAAGGCCCTATTTTACACATACATCTAACGAAGATCTTCTGTCTATCTGTT
459 GluAsnTyrTyrProLysGlyArgProTyrPheThrHisThrSerAsnGluAspLeuLeuSerIleCysL
1821 TATGCGAAGTAACAGTTTGTAAAGATATAAAAAATCCATTATTATATTCTAAAAAGGATATATCAGCAAA
482 euCysGluValThrValCysLysAspIleLysAsnProLeuLeuTyrSerLysLysAspIleSerAlaLy
1891 ACGATTCATAGGTTTATTTACATCTGTCGATATAAATACGGCTGTTGAGTTAAGAGGATATAAAATAAGA
505 sArgPheIleGlyLeuPheThrSerValAspIleAsnThrAlaValGluLeuArgGlyTyrLysIleArg
1961 GTAATAGGATGTTTAGAATGGCCTGAAAAGATAAAAATATTTAATTCTAATCCTACATACATTAGATTAT
529 ValIleGlyCysLeuGluTrpProGluLysIleLysIlePheAsnSerAsnProThrTyrIleArgLeuL
2031 TACTAACAGAAAGACGTTTAGATATTCTACATTCCTATCTGCTTAAATTTAATATAACAGAGGATATAGC
552 euLeuThrGluArgArgLeuAspIleLeuHisSerTyrLeuLeuLysPheAsnIleThrGluAspIleAl
2101 TACCAGAGATGGAGTCAGAAATAATTTACCTATAATTTCTTTTATCGTCAGTTATTGTAGATCGTATACT
575 aThrArgAspGlyValArgAsnAsnLeuProIleIleSerPheIleValSerTyrCysArgSerTyrThr
NdeI (2189)
2171 TATAAATTACTAAATTGCCATATGTACAATTCGTGTAAGATAACAAAGTGTAAATATAATCAGGTAATAT
599 TyrLysLeuLeuAsnCysHisMetTyrAsnSerCysLysIleThrLysCysLysTyrAsnGlnValIleT
2241 ATAATCCTATATAGGAGTATATATAATTGAAAAAGTAAAATATAAATCATATAATAATGAAACGAAATAT
622 yrAsnProIle•••
2311 CAGTAATAGACAGGAACTGGCAGATTCTTCTTCTAATGAAGTAAGTACTGCTAAATCTCCAAAATTAGAT
2381 AAAAATGATACAGCAAATACAGCTTCATTCAACGAATTACCTTTTAATTTTTTCAGACACACCTTATTAC
2451 AAACTAACTAAGTCAGATGATGAGAAAGTAAATATAAATTTAACTTATGGGTATAATATAATAAAGATTC
2521 ATGATATTAATAATTTACTTAACGATGTTAATAGACTTATTCCATCAACCCCTTCAAACCTTTCTGGATA
2591 TTATAAAATACCAGTTAATGATATTAAAATAGATTGTTTAAGAGATGTAAATAATTATTTGGAGGTAAAG
2661 GATATAAAATTAGTCTATCTTTCACATGGAAATGAATTACCTAATATTAATAATTATGATAGGAATTTTT
2731 TAGGATTTACAGCTGTTATATGTATCAACAATACAGGCAGATCTATGGTTATGGTAAAACACTGTAACGG
2801 GAAGCAGCATTCTATGGTAACTGGCCTATGTTTAATAGCCAGATCATTTTACTCTATAAACATTTTACCA
BamHI (2680)
2871 CAAATAATAGGATCCTCTAGATATTTAATATTATATCTAACAACAACAAAAAAATTTAACGATGTATGGC
2941 CAGAAGTATTTTCTACTAATAAAGATAAAGATAGTCTATCTTATCTACAAGATATGAAAGAAGATAATCA
HindIII (3058)
3011 TTTAGTAGTAGCTACTAATATGGAAAGAAATGTATACAAAAACGTGGAAGCTTTTATATTAAATAGCATA
3081 TTACTAGAAGATTTAAAATCTAGACTTAGTATAACAAAACAGTTAAATGCCAATATCGATTCTATATTTC

FIG. 1C

3151 ATCATAACAGTAGTACATTAATCAGTGATATACTGAAACGATCTACAGACTCAACTATGCAAGGAATAAG

3221 CAATATGCCAATTATGTCTAATATTTTAACTTTAGAACTAAAACGTTCTACCAATACTAAAAATAGGATA

3291 CGTGATAGGCTGTTAAAAGCTGCAATAAATAGTAAGGATGTAGAAGAAATACTTTGTTCTATACCTTCGG

3361 AGGAAAGAACTTTAGAACAACTTAAGTTTAATCAAACTTGTATTTATGAACACTATAAAAAAATTATGGA

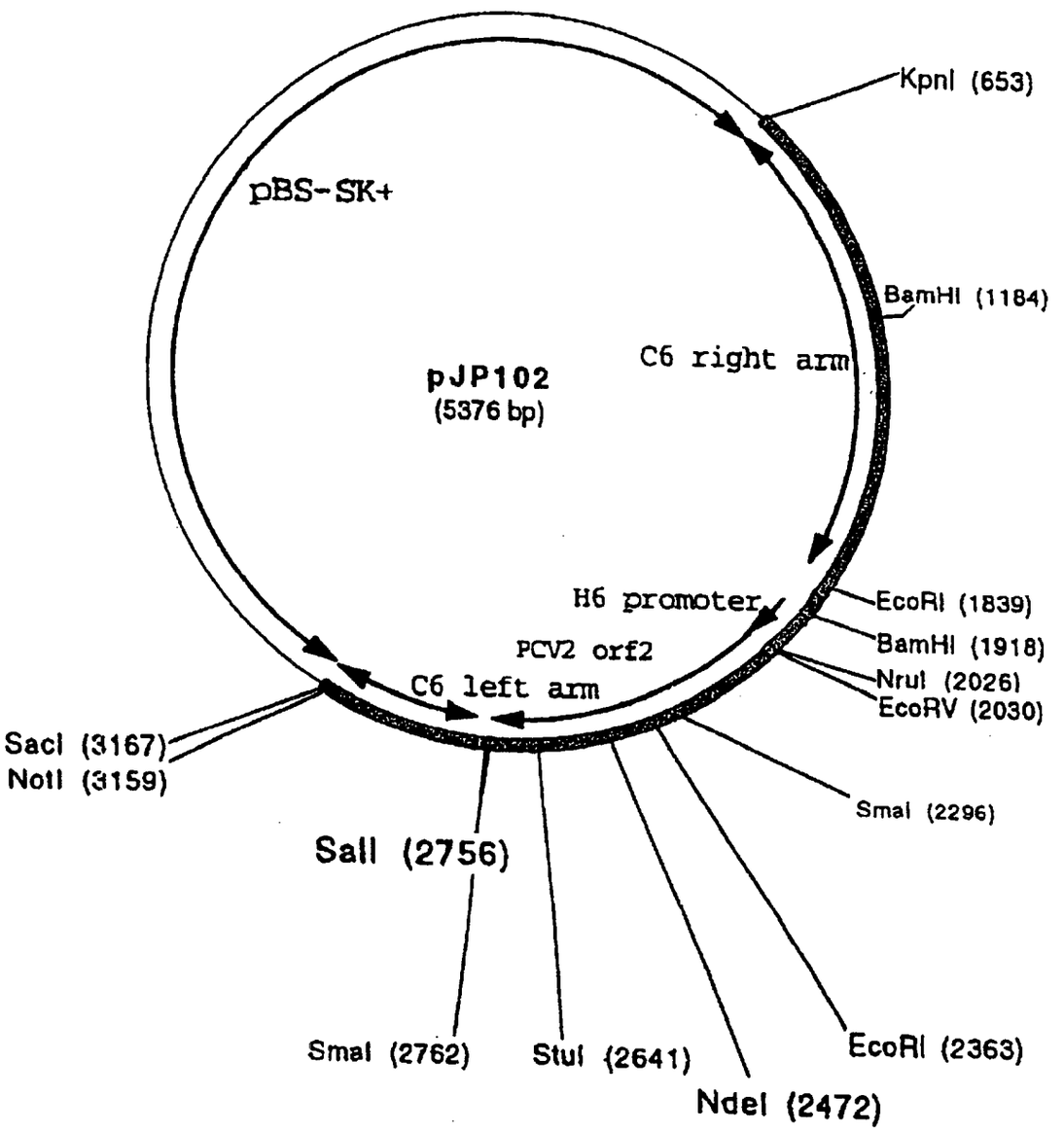
3431 AGATACAAGTAAAAGAATGGATGTTGAATGTCGTAGTTTAGAACATAACTATACGGCTAACTTATATAAA

3501 GTGTACGGACAAAACGAATATATGATTACTTATATACTAGCTCTCATAAGTAGGATTAATAATATTATAG

3571 AAACTTTAAAATATAATCTGGTGGGGCTAGACGAATCTACAATACGTAATATAAATTATATAATTTCACA

3641 AAGAACAAAAAAAAATCAAGTTTCTAATACCTTATAGATAAACTATATTTTTTACCACTGA

FIG. 2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pJP102
(5376 bp)
KpnI (653)
BamHI (1184)
C6 right arm
H6 promoter
EcoRI (1839)
BamHI (1918)
PCV2 orf2
NruI (2026)
EcoRV (2030)
C6 left arm
SacI (3167)
NotI (3159)
SmaI (2296)
SalI (2756)
SmaI (2762)
StuI (2641)
EcoRI (2363)
NdeI (2472)

FIG. 3A

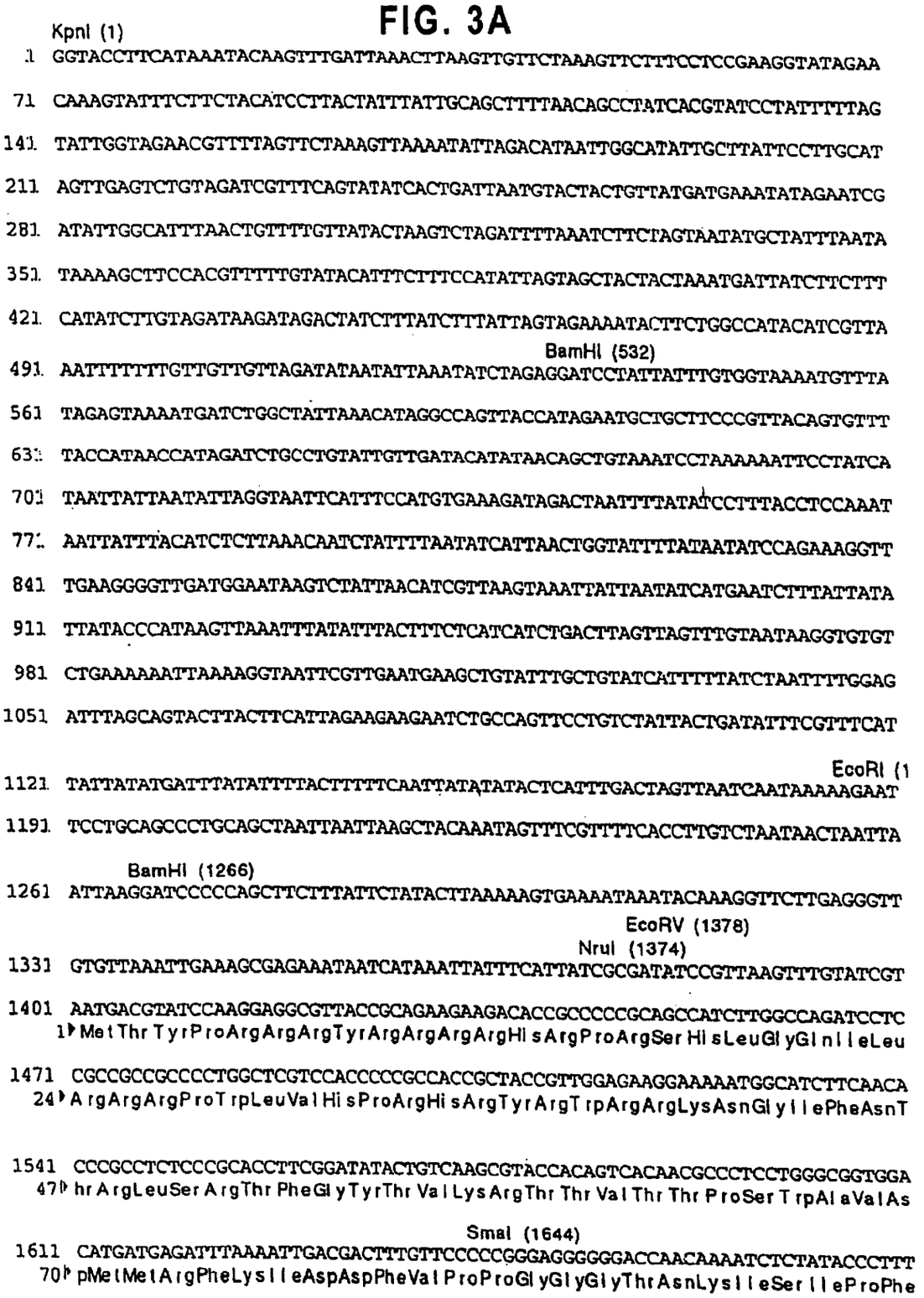

KpnI (1)
1 GGTACCTTCATAAATACAAGTTTGATTAAACTTAAGTTGTTCTAAAGTTCTTTCCTCCGAAGGTATAGAA
71 CAAAGTATTTCTTCTACATCCTTACTATTTATTGCAGCTTTTAACAGCCTATCACGTATCCTATTTTTAG
141 TATTGGTAGAACGTTTTAGTTCTAAAGTTAAAATATTAGACATAATTGGCATATTGCTTATTCCTTGCAT
211 AGTTGAGTCTGTAGATCGTTTCAGTATATCACTGATTAATGTACTACTGTTATGATGAAATATAGAATCG
281 ATATTGGCATTTAACTGTTTTGTTATACTAAGTCTAGATTTTAAATCTTCTAGTAATATGCTATTTAATA
351 TAAAAGCTTCCACGTTTTTGTATACATTTCTTTCCATATTAGTAGCTACTACTAAATGATTATCTTCTTT
421 CATATCTTGTAGATAAGATAGACTATCTTTATCTTTATTAGTAGAAAATACTTCTGGCCATACATCGTTA
BamHI (532)
491 AATTTTTTTGTTGTTGTTAGATATAATATTAAATATCTAGAGGATCCTATTATTTGTGGTAAAATGTTTA
561 TAGAGTAAAATGATCTGGCTATTAAACATAGGCCAGTTACCATAGAATGCTGCTTCCCGTTACAGTGTTT
631 TACCATAACCATAGATCTGCCTGTATTGTTGATACATATAACAGCTGTAAATCCTAAAAAATTCCTATCA
701 TAATTATTAATATTAGGTAATTCATTTCCATGTGAAAGATAGACTAATTTTATATCCTTTACCTCCAAAT
771 AATTATTTACATCTCTTAAACAATCTATTTTAATATCATTAACTGGTATTTTATAATATCCAGAAAGGTT
841 TGAAGGGGTTGATGGAATAAGTCTATTAACATCGTTAAGTAAATTATTAATATCATGAATCTTTATTATA
911 TTATACCCATAAGTTAAATTTATATTTACTTTCTCATCATCTGACTTAGTTAGTTTGTAATAAGGTGTGT
981 CTGAAAAAATTAAAAGGTAATTCGTTGAATGAAGCTGTATTTGCTGTATCATTTTTATCTAATTTTGGAG
1051 ATTTAGCAGTACTTACTTCATTAGAAGAAGAATCTGCCAGTTCCTGTCTATTACTGATATTTCGTTTCAT
EcoRI (1
1121 TATTATATGATTTATATTTTACTTTTTCAATTATATATACTCATTTGACTAGTTAATCAATAAAAAGAAT
1191 TCCTGCAGCCCTGCAGCTAATTAATTAAGCTACAAATAGTTTCGTTTTCACCTTGTCTAATAACTAATTA
BamHI (1266)
1261 ATTAAGGATCCCCCAGCTTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGTT
EcoRV (1378)
NruI (1374)
1331 GTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCGTTAAGTTTGTATCGT
1401 AATGACGTATCCAAGGAGGCGTTACCGCAGAAGAAGACACCGCCCCCGCAGCCATCTTGGCCAGATCCTC
1 MetThrTyrProArgArgArgTyrArgArgArgArgHisArgProArgSerHisLeuGlyGlnIleLeu
1471 CGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGCTACCGTTGGAGAAGGAAAAATGGCATCTTCAACA
24 ArgArgArgProTrpLeuValHisProArgHisArgTyrArgTrpArgArgLysAsnGlyIlePheAsnT
1541 CCCGCCTCTCCCGCACCTTCGGATATACTGTCAAGCGTACCACAGTCACAACGCCCTCCTGGGCGGTGGA
47 hrArgLeuSerArgThrPheGlyTyrThrValLysArgThrThrValThrThrProSerTrpAlaValAs
SmaI (1644)
1611 CATGATGAGATTTAAAATTGACGACTTTGTTCCCCCGGGAGGGGGGACCAACAAAATCTCTATACCCTTT
70 pMetMetArgPheLysIleAspAspPheValProProGlyGlyGlyThrAsnLysIleSerIleProPhe

FIG. 3B

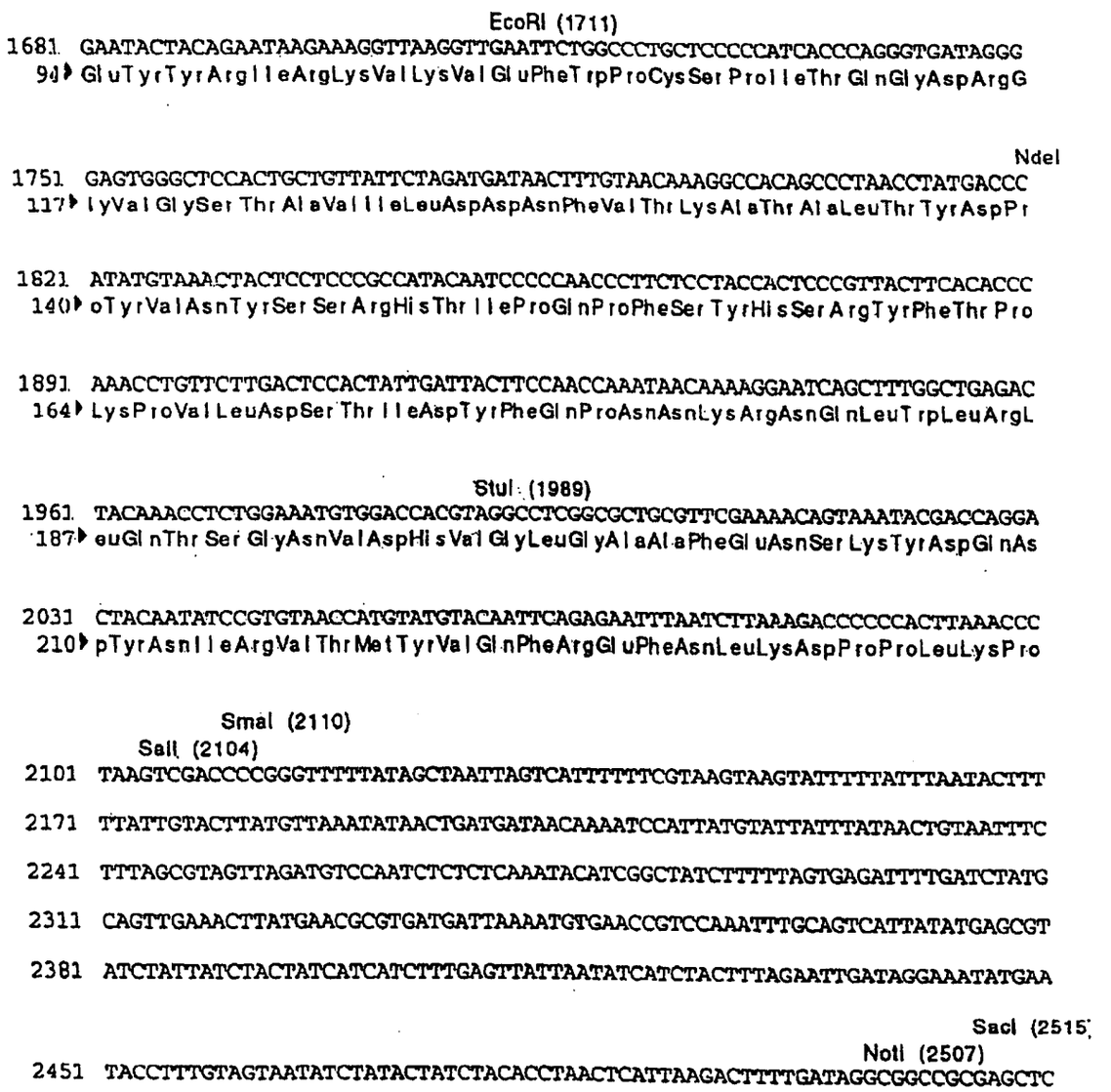

EcoRI (1711)
1681 GAATACTACAGAATAAGAAAGGTTAAGGTTGAATTCTGGCCCTGCTCCCCCATCACCCAGGGTGATAGGG
94 GluTyrTyrArgIleArgLysValLysValGluPheTrpProCysSerProIleThrGlnGlyAspArgG
NdeI
1751 GAGTGGGCTCCACTGCTGTTATTCTAGATGATAACTTTGTAACAAAGGCCACAGCCCTAACCTATGACCC
117 lyValGlySerThrAlaValIleLeuAspAspAsnPheValThrLysAlaThrAlaLeuThrTyrAspPr
1821 ATATGTAAACTACTCCTCCCGCCATACAATCCCCCAACCCTTCTCCTACCACTCCCGTTACTTCACACCC
140 oTyrValAsnTyrSerSerArgHisThrIleProGlnProPheSerTyrHisSerArgTyrPheThrPro
1891 AAACCTGTTCTTGACTCCACTATTGATTACTTCCAACCAAATAACAAAAGGAATCAGCTTTGGCTGAGAC
164 LysProValLeuAspSerThrIleAspTyrPheGlnProAsnAsnLysArgAsnGlnLeuTrpLeuArgL
StuI (1989)
1961 TACAAACCTCTGGAAATGTGGACCACGTAGGCCTCGGCGCTGCGTTCGAAAACAGTAAATACGACCAGGA
187 euGlnThrSerGlyAsnValAspHisValGlyLeuGlyAlaAlaPheGluAsnSerLysTyrAspGlnAs
2031 CTACAATATCCGTGTAAGCATGTATGTACAATTCAGAGAATTTAATCTTAAAGACCCCCCACTTAAACCC
210 pTyrAsnIleArgValThrMetTyrValGlnPheArgGluPheAsnLeuLysAspProProLeuLysPro
SmaI (2110)
SalI (2104)
2101 TAAGTCGACCCCGGGTTTTTATAGCTAATTAGTCATTTTTTCGTAAGTAAGTATTTTTATTTAATACTTT
2171 TTATTGTACTTATGTTAAATATAACTGATGATAACAAAATCCATTATGTATTATTTATAACTGTAATTTC
2241 TTTAGCGTAGTTAGATGTCCAATCTCTCTCAAATACATCGGCTATCTTTTTAGTGAGATTTTGATCTATG
2311 CAGTTGAAACTTATGAACGCGTGATGATTAAAATGTGAACCGTCCAAATTTGCAGTCATTATATGAGCGT
2381 ATCTATTATCTACTATCATCATCTTTGAGTTATTAATATCATCTACTTTAGAATTGATAGGAAAATATGAA
SacI (2515)
NotI (2507)
2451 TACCTTTGTAGTAATATCTATACTATCTACACCTAACTCATTAAGACTTTTGATAGGCGGCCGCGAGCTC

FIG. 4

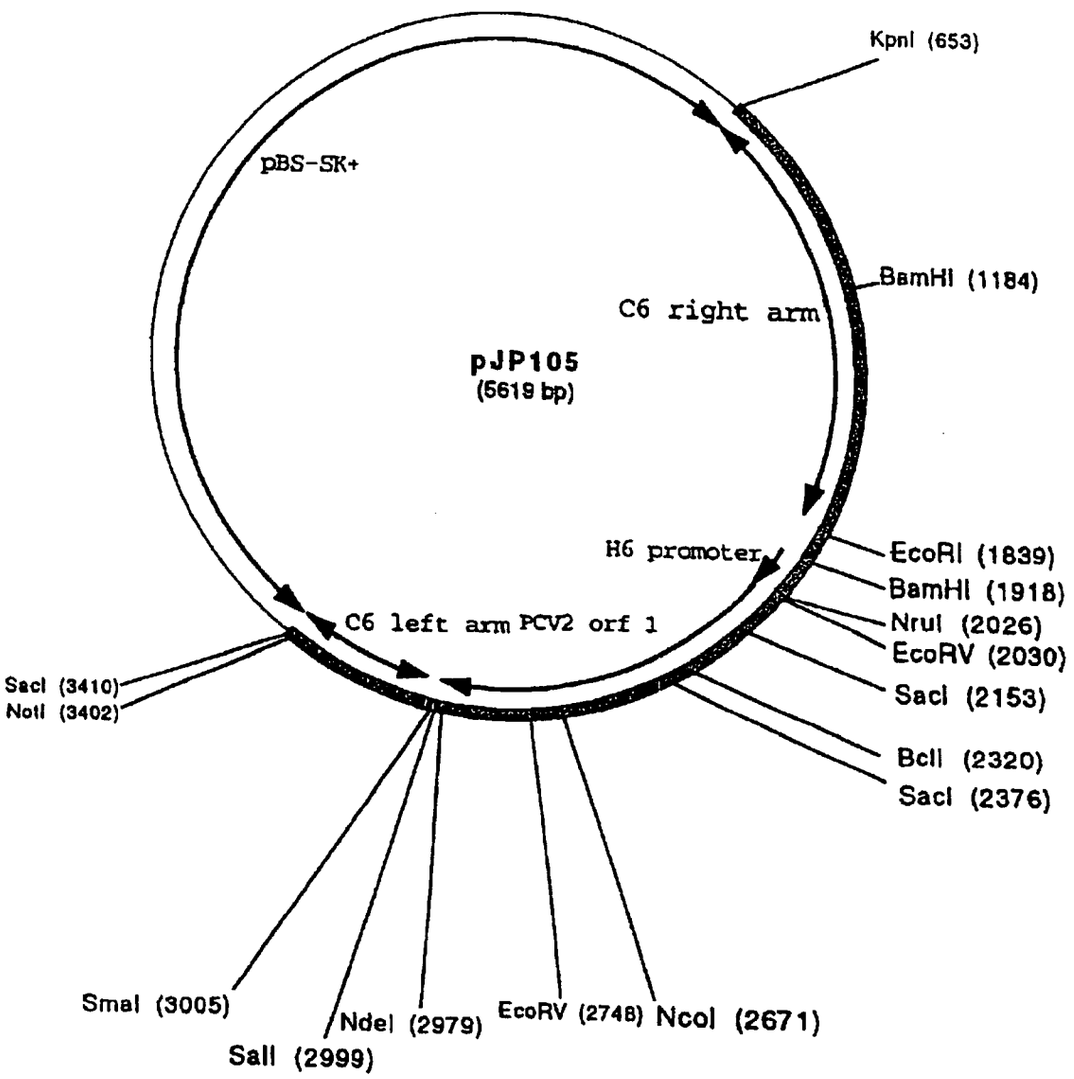
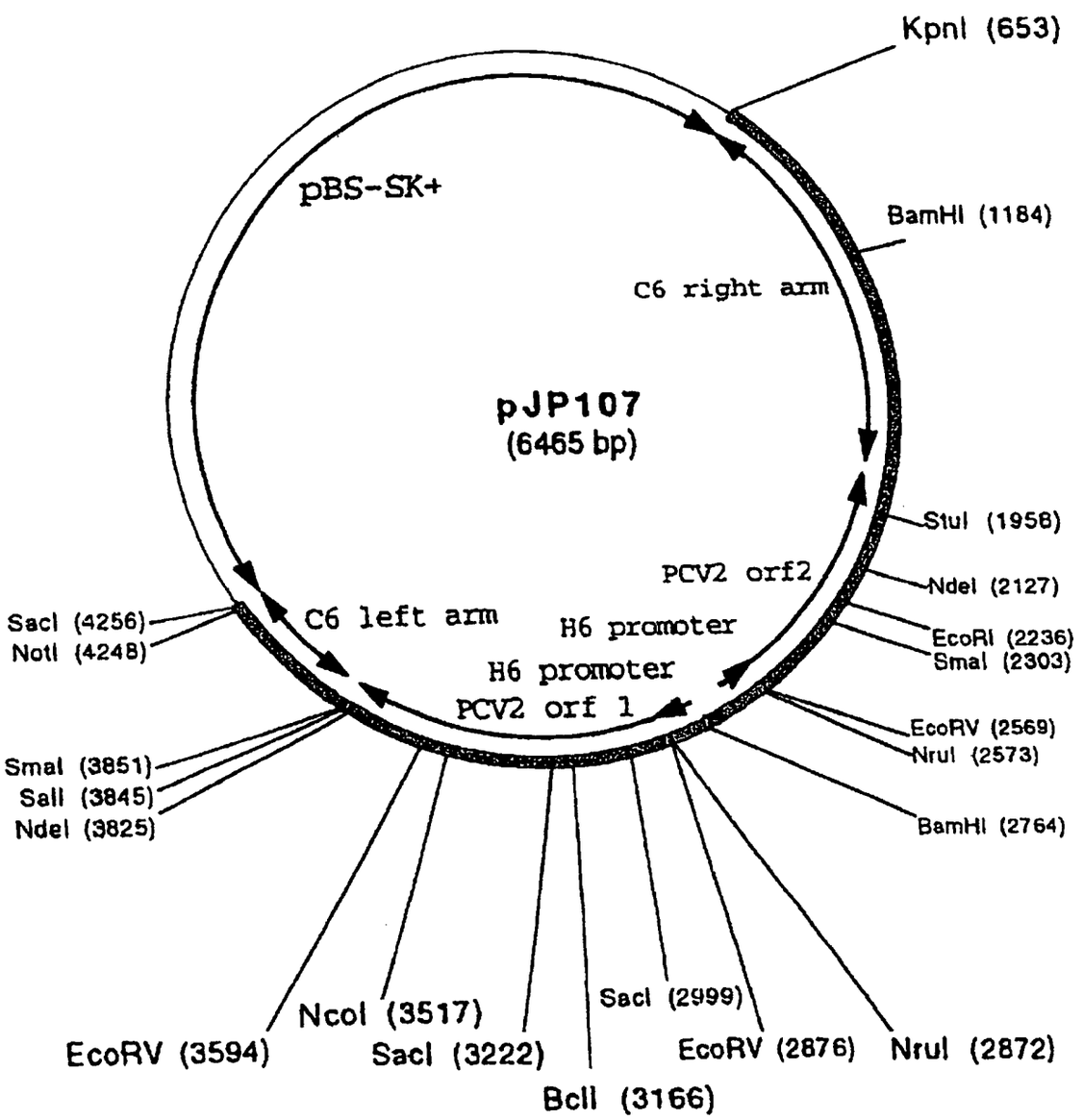

KpnI (653)
pBS-SK+
BamHI (1184)
C6 right arm
pJP105
(5619 bp)
H6 promoter
EcoRI (1839)
BamHI (1918)
NruI (2026)
EcoRV (2030)
SacI (2153)
BclI (2320)
SacI (2376)
C6 left arm PCV2 orf 1
SacI (3410)
NotI (3402)
SmaI (3005)
SalI (2999)
NdeI (2979)
EcoRV (2748)
NcoI (2671)

FIG. 5


KpnI (653)
pBS-SK+
BamHI (1184)
C6 right arm
pJP107
(6465 bp)
StuI (1958)
PCV2 orf2
NdeI (2127)
SacI (4256)
NotI (4248)
C6 left arm
H6 promoter
EcoRI (2236)
SmaI (2303)
H6 promoter
PCV2 orf 1
EcoRV (2569)
NruI (2573)
SmaI (3851)
SalI (3845)
NdeI (3825)
BamHI (2764)
SacI (2999)
NcoI (3517)
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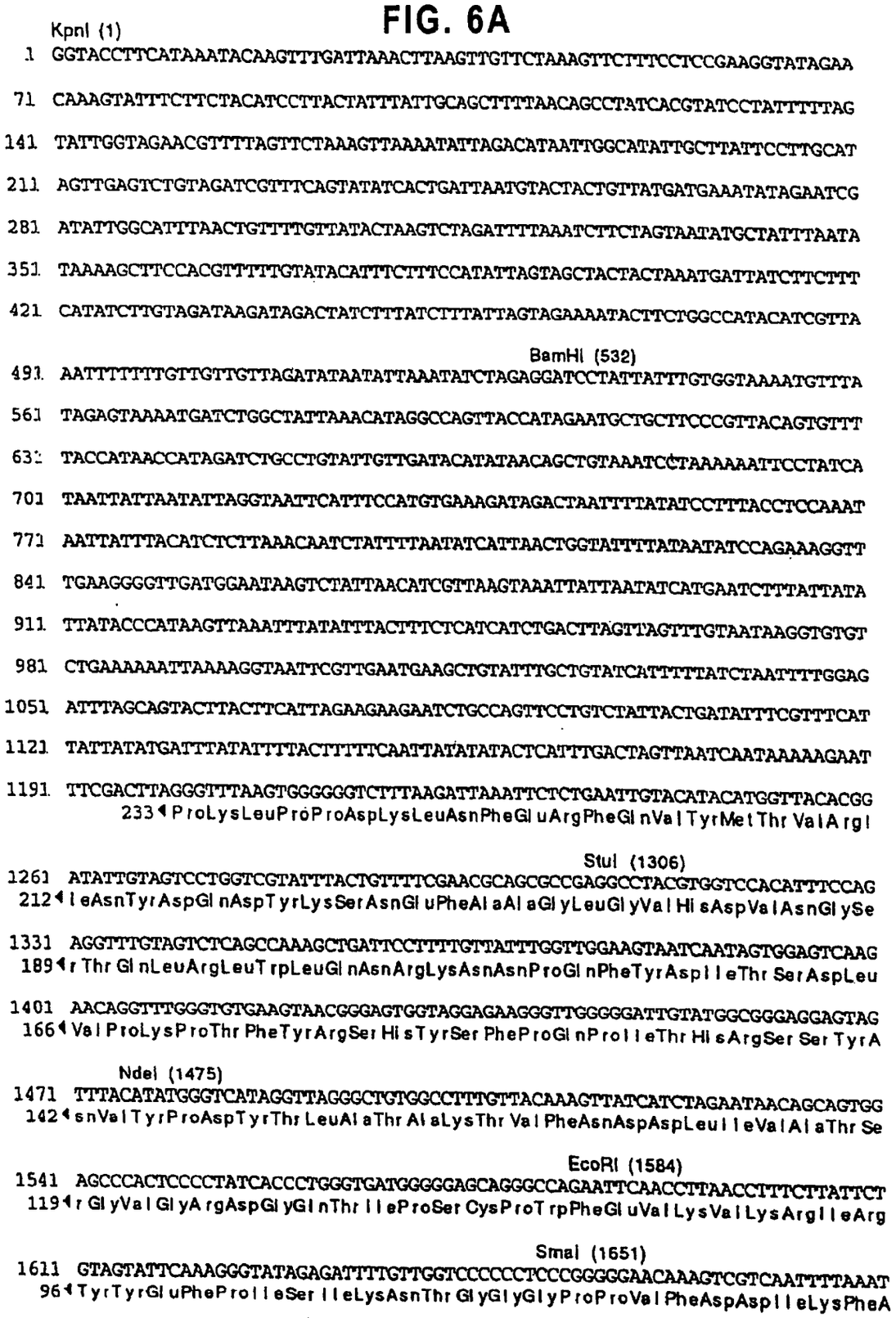
EcoRV (2876)
NruI (2872)
BclI (3166)

FIG. 6A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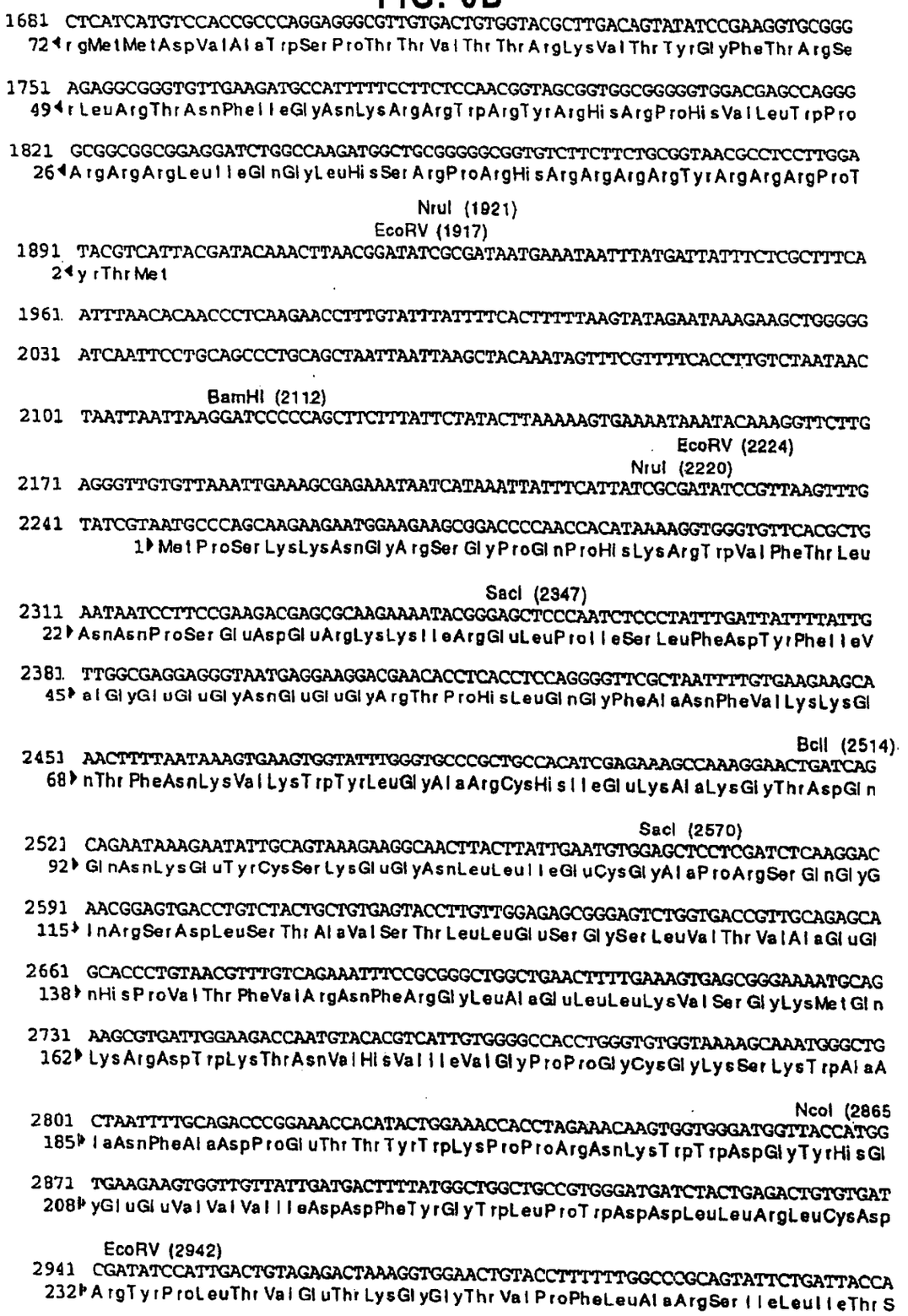
1 GGTACCTTCATAAATACAAGTTTGATTAAACTTAAGTTGTTCTAAAGTTCTTTCCTCCGAAGGTATAGAA
71 CAAAGTATTTCTTCTACATCCTTACTATTTATTGCAGCTTTTAACAGCCTATCACGTATCCTATTTTTAG
141 TATTGGTAGAACGTTTTAGTTCTAAAGTTAAAATATTAGACATAATTGGCATATTGCTTATTCCTTGCAT
211 AGTTGAGTCTGTAGATCGTTTCAGTATATCACTGATTAATGTACTACTGTTATGATGAAATATAGAATCG
281 ATATTGGCATTTAACTGTTTTGTTATACTAAGTCTAGATTTTAAATCTTCTAGTAATATGCTATTTAATA
351 TAAAAGCTTCCACGTTTTTGTATACATTTCTTTCCATATTAGTAGCTACTACTAAATGATTATCTTCTTT
421 CATATCTTGTAGATAAGATAGACTATCTTTATCTTTATTAGTAGAAAATACTTCTGGCCATACATCGTTA
BamHI (532)
491 AATTTTTTTGTTGTTGTTAGATATAATATTAAATATCTAGAGGATCCTATTATTTGTGGTAAAATGTTTA
561 TAGAGTAAAATGATCTGGCTATTAAACATAGGCCAGTTACCATAGAATGCTGCTTCCCGTTACAGTGTTT
631 TACCATAACCATAGATCTGCCTGTATTGTTGATACATATAACAGCTGTAAATCCTAAAAAATTCCTATCA
701 TAATTATTAATATTAGGTAATTCATTTCCATGTGAAAGATAGACTAATTTTATATCCTTTACCTCCAAAT
771 AATTATTTACATCTCTTAAACAATCTATTTTAATATCATTAACTGGTATTTTATAATATCCAGAAAGGTT
841 TGAAGGGGTTGATGGAATAAGTCTATTAACATCGTTAAGTAAATTATTAATATCATGAATCTTTATTATA
911 TTATACCCATAAGTTAAATTTATATTTACTTTCTCATCATCTGACTTAGTTAGTTTGTAATAAGGTGTGT
981 CTGAAAAAATTAAAAGGTAATTCGTTGAATGAAGCTGTATTTGCTGTATCATTTTTATCTAATTTTGGAG
1051 ATTTAGCAGTACTTACTTCATTAGAAGAAGAATCTGCCAGTTCCTGTCTATTACTGATATTTCGTTTCAT
1121 TATTATATGATTTATATTTTACTTTTTCAATTATATATACTCATTTGACTAGTTAATCAATAAAAAGAAT
1191 TTCGACTTAGGGTTTAAGTGGGGGGTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACACGG
233 ProLysLeuProProAspLysLeuAsnPheGluArgPheGlnValTyrMetThrValArgI
StuI (1306)
1261 ATATTGTAGTCCTGGTCGTATTTACTGTTTTCGAACGCAGCGCCGAGGCCTACGTGGTCCACATTTCCAG
212 IeAsnTyrAspGlnAspTyrLysSerAsnGluPheAlaAlaGlyLeuGlyValHisAspValAsnGlySe
1331 AGGTTTGTAGTCTCAGCCAAAGCTGATTCCTTTTGTTATTTGGTTGGAAGTAATCAATAGTGGAGTCAAG
189 rThrGlnLeuArgLeuTrpLeuGlnAsnArgLysAsnAsnProGlnPheTyrAspIleThrSerAspLeu
1401 AACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGAGAAGGGTTGGGGGATTGTATGGCGGGAGGAGTAG
166 ValProLysProThrPheTyrArgSerHisTyrSerPheProGlnProIleThrHisArgSerSerTyrA
NdeI (1475)
1471 TTTACATATGGGTCATAGGTTAGGGCTGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCAGTGG
142 snValTyrProAspTyrThrLeuAlaThrAlaLysThrValPheAsnAspAspLeuIleValAlaThrSe
EcoRI (1584)
1541 AGCCCACTCCCCTATCACCCTGGGTGATGGGGGAGCAGGGCCAGAATTCAACCTTAACCTTTCTTATTCT
119 rGlyValGlyArgAspGlyGlnThrIleProSerCysProTrpPheGluValLysValLysArgIleArg
SmaI (1651)
1611 GTAGTATTCAAAGGGTATAGAGATTTTGTTGGTCCCCCCTCCCGGGGGAACAAAGTCGTCAATTTTAAAT
96 TyrTyrGluPheProIleSerIleLysAsnThrGlyGlyGlyProProValPheAspAspIleLysPheA

FIG. 6B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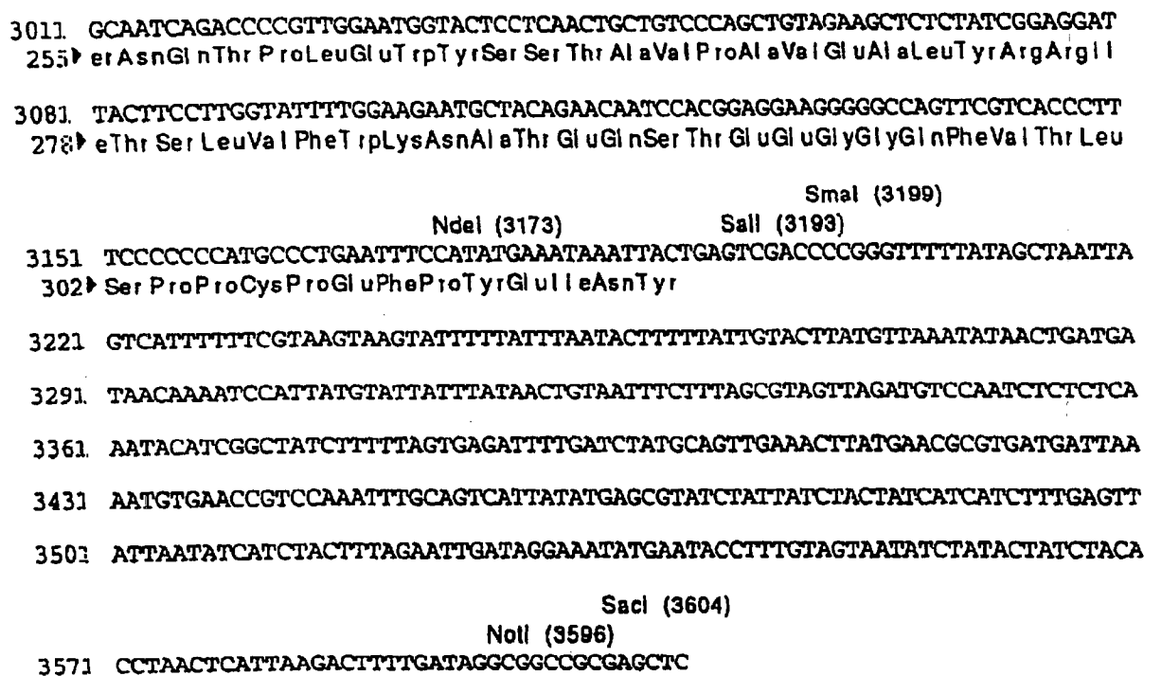
72 rgMetMetAspValAlaTrpSerProThrThrValThrThrArgLysValThrTyrGlyPheThrArgSe
1751 AGAGGCGGGTGTTGAAGATGCCATTTTTCCTTCTCCAACGGTAGCGGTGGCGGGGGTGGACGAGCCAGGG
49 rLeuArgThrAsnPheIleGlyAsnLysArgArgTrpArgTyrArgHisArgProHisValLeuTrpPro
1821 GCGGCGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTTCTGCGGTAACGCCTCCTTGGA
26 ArgArgArgLeuIleGlnGlyLeuHisSerArgProArgHisArgArgArgArgTyrArgArgArgProT
NruI (1921)
EcoRV (1917)
1891 TACGTCATTACGATACAAACTTAACGGATATCGCGATAATGAAATAATTTATGATTATTTCTCGCTTTCA
2 yrThrMet
1961 ATTTAACACAACCCTCAAGAACCTTTGTATTTATTTTCACTTTTTAAGTATAGAATAAAGAAGCTGGGGG
2031 ATCAATTCCTGCAGCCCTGCAGCTAATTAATTAAGCTACAAATAGTTTCGTTTTCACCTTGTCTAATAAC
BamHI (2112)
2101 TAATTAATTAAGGATCCCCCAGCTTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTG
EcoRV (2224)
NruI (2220)
2171 AGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCGTTAAGTTTG
2241 TATCGTAATGCCCAGCAAGAAGAATGGAAGAAGCGGACCCCAACCACATAAAAGGTGGGTGTTCACGCTG
1 MetProSerLysLysAsnGlyArgSerGlyProGlnProHisLysArgTrpValPheThrLeu
SacI (2347)
2311 AATAATCCTTCCGAAGACGAGCGCAAGAAAATACGGGAGCTCCCAATCTCCCTATTTGATTATTTTATTG
22 AsnAsnProSerGluAspGluArgLysLysIleArgGluLeuProIleSerLeuPheAspTyrPheIleV
2381 TTGGCGAGGAGGGTAATGAGGAAGGACGAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCA
45 alGlyGluGluGlyAsnGluGluGlyArgThrProHisLeuGlnGlyPheAlaAsnPheValLysLysGl
BclI (2514)
2451 AACTTTTAATAAAGTGAAGTGGTATTTGGGTGCCCGCTGCCACATCGAGAAAGCCAAAGGAACTGATCAG
68 nThrPheAsnLysValLysTrpTyrLeuGlyAlaArgCysHisIleGluLysAlaLysGlyThrAspGln
SacI (2570)
2521 CAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTTATTGAATGTGGAGCTCCTCGATCTCAAGGAC
92 GlnAsnLysGluTyrCysSerLysGluGlyAsnLeuLeuIleGluCysGlyAlaProArgSerGlnGlyG
2591 AACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTTGGAGAGCGGGAGTCTGGTGACCGTTGCAGAGCA
115 InArgSerAspLeuSerThrAlaValSerThrLeuLeuGluSerGlySerLeuValThrValAlaGluGl
2661 GCACCCTGTAACGTTTGTCAGAAATTTCCGCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAG
138 nHisProValThrPheValArgAsnPheArgGlyLeuAlaGluLeuLeuLysValSerGlyLysMetGln
2731 AAGCGTGATTGGAAGACCAATGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTG
162 LysArgAspTrpLysThrAsnValHisValIleValGlyProProGlyCysGlyLysSerLysTrpAlaA
NcoI (2865
2801 CTAATTTTGCAGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGG
185 laAsnPheAlaAspProGluThrThrTyrTrpLysProProArgAsnLysTrpTrpAspGlyTyrHisGl
2871 TGAAGAAGTGGTTGTTATTGATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAGACTGTGTGAT
208 yGluGluValValValIleAspAspPheTyrGlyTrpLeuProTrpAspAspLeuLeuArgLeuCysAsp
EcoRV (2942)
2941 CGATATCCATTGACTGTAGAGACTAAAGGTGGAACTGTACCTTTTTTGGCCCGCAGTATTCTGATTACCA
232 ArgTyrProLeuThrValGluThrLysGlyGlyThrValProPheLeuAlaArgSerIleLeuIleThrS

FIG. 6C


3011 GCAATCAGACCCCGTTGGAATGGTACTCCTCAACTGCTGTCCCAGCTGTAGAAGCTCTCTATCGGAGGAT
255 erAsnGlnThrProLeuGluTrpTyrSerSerThrAlaValProAlaValGluAlaLeuTyrArgArgIl
3081 TACTTCCTTGGTATTTTGGAAGAATGCTACAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTT
278 eThrSerLeuValPheTrpLysAsnAlaThrGluGlnSerThrGluGluGlyGlyGlnPheValThrLeu
SmaI (3199)
NdeI (3173) SalI (3193)
3151 TCCCCCCCATGCCCTGAATTTCCATATGAAATAAATTACTGAGTCGACCCCGGGTTTTTATAGCTAATTA
302 SerProProCysProGluPheProTyrGluIleAsnTyr
3221 GTCATTTTTTCGTAAGTAAGTATTTTTATTTAATACTTTTTATTGTACTTATGTTAAATATAACTGATGA
3291 TAACAAAATCCATTATGTATTATTTATAACTGTAATTTCTTTAGCGTAGTTAGATGTCCAATCTCTCTCA
3361 AATACATCGGCTATCTTTTTAGTGAGATTTTGATCTATGCAGTTGAAACTTATGAACGCGTGATGATTAA
3431 AATGTGAACCGTCCAAATTTGCAGTCATTATATGAGCGTATCTATTATCTACTATCATCATCTTTGAGTT
3501 ATTAATATCATCTACTTTAGAATTGATAGGAAATATGAATACCTTTGTAGTAATATCTATACTATCTACA
SacI (3604)
NotI (3596)
3571 CCTAACTCATTAAGACTTTTGATAGGCGGCCGCGAGCTC

PORCINE CIRCOVIRUS RECOMBINANT POXVIRUS VACCINE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. application Ser. No. 60/138,478, filed Jun. 10, 1999. Reference is made to WO-A-99 18214, 1998, French applications Nos. 97/12382, 98/00873, 98/03707, filed Oct. 3, 1997, Jan. 22, 1998, and Mar. 20, 1998, and WO99/29717. Each of the aforementioned U.S., PCT and French applications, and each document cited in the text and the record or prosecution of each of the aforementioned U.S., PCT and French applications ("application cited documents") and each document referenced or cited in each of the application cited documents, is hereby incorporated herein by reference; and, technology in each of the aforementioned U.S., PCT and French applications, and each document cited in the text and the record or prosecution of each of the aforementioned U.S., PCT and French applications can be used in the practice of this invention.

Several publications are referenced in this application. Full citation to these documents is found at the end of the specification preceding the claims, and/or where the document is cited. These documents pertain to the field of this invention; and, each of the documents cited or referenced in this application ("herein cited documents") and each document cited or referenced in herein cited documents are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to vectors, such as recombinant vectors; for instance, recombinant viruses, such as poxviruses, e.g., modified poxviruses and to methods of making and using the same. In some embodiments, the invention relates to recombinant avipox viruses, such as canarypox viruses, e.g., ALVAC. The invention further relates to such vectors, e.g., poxviruses, that express gene products, e.g., antigen(s), ORF(s), and/or epitope(s) of interest therefrom, of porcine circovirus 2 (PCV2); to immunological compositions or vaccines. The invention yet further relates to such vectors, e.g., poxviruses, that induce an immune response directed to or against PCV2 gene products and/or PCV2; and, to advantageously, such compositions that are immunological, immunogenic or vaccine compositions and/or confer protective immunity against infection by PCV2. The invention yet further relates to the uses of and methods for making and using such vectors and compositions, as well as intermediates thereof, and said intermediates. And, the invention relates to the products therefrom, e.g., from the uses and methods involving the inventive recombinant or poxvirus, such as antibodies from expression.

BACKGROUND OF THE INVENTION

Postweaning multisystemic wasting syndrome (PMWS) is a recently recognized disease of young pigs. PMWS is characterized clinically by progressive weight loss and other symptoms such as tachypnea, dyspnea and jaundice. Pathologically, lymphocytic and granulomatous infiltrates, lymphadenopathy, and, more rarely, lymphocytic and granulomatous hepatitis and nephritis have been observed (Clark, 1997; Harding, 1997).

This disease has been described in different European countries as well as in North America. Treatment and prevention of this disease are not currently available.

Several lines of evidence point to porcine circovirus as the etiologic agent of PMWS (Ellis et al., 1998). Circoviruses have been recovered from pigs with PMWS, and antibodies to porcine circovirus have been demonstrated in pigs with the disease.

Circoviruses are single stranded circular DNA viruses found in a range of animal and plant species. Porcine circovirus was originally isolated as a contaminant from a continuous pig kidney cell line. The cell culture isolate has been designated PK-15 (Meehan et al., 1997). More recently, porcine circovirus obtained from pigs with PMWS has been compared to PK-15. Such viruses differ substantially from PK-15 at the nucleotide and protein sequence level, and have been designated PCV2 (Meehan et al., 1998; Hamel et al., 1998).

As many as thirteen open reading frames (ORFs) have been identified in the PCV2 genome (COL1 to COL13 in the French patent application 98 03707). Four of these ORFs share substantial homology with analogous ORFs within the genome of PK-15. ORF1 (Meehan et al., 1998; corresponding to COL4 in the French patent application 98 03707), comprising nt 398-1342 (GenBank accession number AF055392), has the potential to encode a protein with a predicted molecular weight of 37.7 kD. ORF2 (Meehan et al., 1998; corresponding to COL13 in the French patent application 98 03707), comprising nt 1381-1768 joined to 1-314 (GenBank accession number AF055392), may encode a protein with a predicted molecular weight of 27.8 kD. ORF3 (Meehan et al., 1998; corresponding to COL7 in the French patent application 98 03707), comprising nt 1018-704 (GenBank accession number AF055392), may encode a protein with a predicted molecular weight of 11.9 kD. ORF4 (Meehan et al., 1998; corresponding to COL10 in the French patent application 98 03707), comprising nt 912-733 (GenBank accession number AF055392), may encode a protein with a predicted molecular weight of 6.5 kD.

ORF1 of PCV2 is highly homologous (86% identity) to the ORF1 of the PK-15 isolate (Meehan et al., 1998). The ORF1 protein of PK-15 has been partially characterized (Meehan et al., 1997; Mankertz et al., 1998a). It is known to be essential for virus replication, and is probably involved in the viral DNA replication.

Protein sequence identity between the respective ORF2s was lower (66% identity) than that of the ORF1s but each of the ORF2s shared a highly conserved basic N-terminal region, similar to that observed in the N-terminal region of the major structural protein of the avian circovirus chicken anemia virus (CAV) (Meehan et al., 1998). Recently, Mankertz et al. (1998b) has suggested that the ORF2 of the PK-15 isolate (designated ORF 1 in Mankertz et al., 1998b) codes for a capsid protein.

Greater differences were observed between the respective ORF3s and ORF4s of the PK-15 isolate and PCV2. In each case, there was a deletion of the C-terminal region of PCV2 ORF4 and ORF3 compared to the corresponding ORFs present in the genome of the PK-15 isolate. The highest protein sequence homology was observed at the N-terminal regions of both ORF3 and ORF4 (Meehan et al., 1998).

The transcription analysis of the genome of PCV2 has not been published yet. Recent data obtained with the PK-15 isolate indicated that the ORF2 transcript is spliced (Mankertz et al., 1998b).

Vaccinia virus has been used successfully to immunize against smallpox, culminating in the worldwide eradication of smallpox in 1980. With the eradication of smallpox, a new role for poxviruses became important, that of a genetically

engineered vector for the expression of foreign genes (Panicali and Paoletti, 1982; Paoletti et al., 1984). Genes encoding heterologous antigens have been expressed in vaccinia, often resulting in protective immunity against challenge by the corresponding pathogen (reviewed in Tartaglia et al., 1990). A highly attenuated strain of vaccines, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Pat. No. 5,185,146.

Two additional vaccine vector systems involve the use of naturally host-restricted poxviruses, avipox viruses. Both fowlpoxvirus (FPV; Taylor et al. 1988a, b) and canarypoxvirus (CPV; Taylor et al., 1991 & 1992) have been engineered to express foreign gene products. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. The virus causes an economically important disease of poultry which has been well controlled since the 1920's by the use of live attenuated vaccines. Replication of the avipox viruses is limited to avian species (Matthews, 1982) and there are no reports in the literature of avipoxvirus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipoxvirus based vaccine vectors in veterinary and human applications an attractive proposition.

FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant (Taylor et al., 1988c). After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor et al., 1988c). FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor et al., 1990; Edbauer et al., 1990).

Other attenuated poxvirus vectors have been prepared by genetic modifications of wild type strains of virus. The NYVAC vector, derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia et al., 1992) has proven useful as a recombinant vector in eliciting a protective immune response against an expressed foreign antigen.

Another engineered poxvirus vector is ALVAC, derived from canarypox virus (ALVAC was deposited with American Type Culture Collection, P.O. Box 1549, Manassas, Va. 20108, USA, under the terms of the Budapest Treaty on Nov. 14, 1996, and is designated as Accession Number VR-2547). ALVAC does not productively replicate in non-avian hosts, a characteristic thought to improve its safety profile (Taylor et al., 1991 & 1992). Both ALVAC and NYVAC are BSL-1 vectors.

One approach to the development of a subunit PCV2 vaccine is the use of live viral vectors to express relevant PCV2 ORFs. Recombinant poxviruses can be constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described in U.S. Pat. Nos. 4,769,330; 4,722,848; 4,603,112; 5,110,587; 5,174,993; 5,494,807; and 5,505,941, the disclosures of which are incorporated herein by reference. It can thus be appreciated that provision of a PCV2 recombinant poxvirus, and of compositions and products therefrom particularly ALVAC based PCV2 recombinants and compositions and products therefrom, especially such recombinants containing ORFs 1 and/or 2 of PCV2, and compositions and products therefrom would be a highly desirable advance over the current state of technology.

OBJECTS AND SUMMARY OF THE INVENTION

It is therefore an object of this invention to provide compositions and methods for treatment and prophylaxis of infection with PCV2. It is also an object to provide a means to treat or prevent PMWS.

In one aspect, the present invention relates to an antigenic, immunological, immunogenic, or vaccine composition or a therapeutic composition for inducing an antigenic, immunogenic or immunological response in a host animal inoculated with the composition. The composition advantageously includes a carrier or diluent and a recombinant virus, such as a recombinant poxvirus. The recombinant virus or poxvirus contains and expresses an exogenous nucleic acid molecule encoding an ORF, antigen, immunogen, or epitope of interest from PCV2, or a protein that elicits an immunological response against PCV2 or conditions caused by PCV2, such as PMWS. For instance, the recombinant virus can be a modified recombinant virus or poxvirus; for example, such a virus or poxvirus that has inactivated therein virus-encoded genetic functions, e.g., nonessential virus-encoded genetic functions, so that the recombinant virus has attenuated virulence and enhanced safety. And, the invention further provides the viruses used in the composition, as well as methods for making and uses of the composition and virus.

The virus used in the composition according to the present invention is advantageously a poxvirus, particularly a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus and more advantageously, ALVAC. The modified recombinant virus can include, e.g., within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein, e.g., derived from PCV2 ORFs, e.g., PCV2 ORF 1 and/or 2.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety. The modified recombinant virus includes, e.g., within a non-essential region of the virus genome, a heterologous DNA sequence which encodes an antigenic protein (e.g., derived from PCV2 ORFs, especially ORFS 1 and/or 2) wherein the composition, when administered to a host, is capable of inducing an immunological response specific to the antigen.

In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further contains DNA from a heterologous source, e.g., in a nonessential region of the virus genome. The DNA can code for PCV2 genes such as any or all of PCV2 ORF1, ORF2, ORF3, or ORF4 (Meehan et al., 1998), or epitope(s) of interest therefrom. The genetic functions can be inactivated by deleting an open reading frame encoding a virulence factor or by utilizing naturally host-restricted viruses. The virus used according to the present invention is advantageously a poxvirus, e.g., a vaccinia virus or an avipox virus, such as fowlpox virus or canarypox virus.

Advantageously, the open reading frame that is deleted from the poxvirus or virus geneome is selected from the group consisting of J2R, B13R+B14R, A26L, A56R, C7L–K1L, and I4L (by the terminology reported in Goebel et al., 1990); and, the combination thereof. In this respect, the open reading frame comprises a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemag-

glutinin gene, a host range gene region or a large subunit, ribonucleotide reductase; or, the combination thereof.

A suitable modified Copenhagen strain of vaccinia virus is identified as NYVAC (Tartaglia et al., 1992), or a vaccinia virus from which has been deleted J2R, B13R+B14R, A26L, A56R, C7L–K11 and 14L or a thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range region, and a large subunit, ribonucleotide reductase (See also U.S. Pat. Nos. 5,364,773, 5,494,807, and 5,762,938, with respect to NYVAC and vectors having additional deletions or inactivations from those of NYVAC that are also useful in the practice of this invention).

Preferably, the poxvirus vector is an ALVAC or, a canarypox virus which was attenuated, for instance, through more than 200 serial passages on chick embryo fibroblasts (Rentschler vaccine strain), a master seed therefrom was subjected to four successive plague purifications under agar from which a plague clone was amplified through five additional passages. (See also U.S. Pat. Nos. 5,756,103 and 5,766,599 with respect to ALVAC and TROVAC (an attenuated fowlpox virus useful in the practice of this invention); and U.S. Pat. Nos. 6,004,777, 5,990,091, 5,770,212, 6,033, 904, 5,869,312, 5,382,425, and WO 95/30018, with respect to vectors that also can be used in the practice of this invention, such as vectors having enhanced expression, vectors having functions deleted therefrom and vectors useful with respect to porcine hosts (for instance, vectors useful with porcine hosts can include a poxvirus, including a vaccinia virus, an avipox virus, a canarypox virus, and a swinepox virus), as well as with respect to terms used and teachings herein such as "immunogenic composition", "immunological composition", "vaccine", and "epitope of interest", and dosages, routes of administration, formulations, adjuvants, and uses for recombinant viruses and expression products therefrom).

The invention in yet a further aspect relates to the product of expression of the inventive recombinant poxvirus and uses therefor, such as to form antigenic, immunological or vaccine compositions for treatment, prevention, diagnosis or testing; and, to DNA from the recombinant poxvirus which is useful in constructing DNA probes and PCR primers.

These and other embodiments are disclosed or are obvious from and encompassed by the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

A better understanding of the present invention will be had by referring to the accompanying drawings, incorporated herein by reference, in which:

FIG. **1** (SEQ ID NOS:1 and 2) shows the nucleotide sequence of a 3.7 kilobase pair fragment of ALVAC DNA containing the C6 open reading frame.

FIG. **2** shows the map of pJP102 donor plasmid.

FIG. **3** (SEQ ID NOS:8 9 and 10) shows the nucleotide sequence of the 2.5 kilobase pair fragment from pJP 102 donor plasmid from the KpnI (position 653) to the SacI (position 3166) restriction sites.

FIG. **4** shows the map of pJP105 donor plasmid.

FIG. **5** shows the map of pJP107 donor plasmid.

FIG. **6** (SEQ ID NOs: 14 and 15) shows the nucleotide sequence of the 3.6 kilobase pair fragment from pJP107 donor plasmid from the KpnI (position 653) to the SacI (position 4255) restriction sites.

DETAILED DESCRIPTION

In one aspect, the present invention relates to a recombinant virus, such as a recombinant poxvirus, containing therein a DNA sequence from PCV2, e.g., in a non-essential region of the poxvirus genome. The poxvirus is advantageously an avipox virus, such as fowlpox virus, especially an attenuated fowlpox virus, or a canarypox virus, especially an attenuated canarypox virus, such as ALVAC.

According to the present invention, the recombinant poxvirus expresses gene products of the foreign PCV2 gene. Specific ORFs of PCV2 are inserted into the poxvirus vector, and the resulting recombinant poxvirus is used to infect an animal. Expression in the animal of PCV2 gene products results in an immune response in the animal to PCV2. Thus, the recombinant poxvirus of the present invention may be used in an immunological composition or vaccine to provide a means to induce an immune response which may, but need not be, protective.

The administration procedure for recombinant poxvirus-PCV2 or expression product thereof, compositions of the invention such as immunological, antigenic or vaccine compositions or therapeutic compositions, can be via a parenteral route (intradermal, intramuscular or subcutaneous). Such an administration enables a systemic immune response, or humoral or cell-mediated responses.

More generally, the inventive poxvirus-PCV2 recombinants, antigenic, immunological or vaccine poxvirus-PCV2 compositions or therapeutic compositions can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical or veterinary art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical or veterinary arts taking into consideration such factors as the age, sex, weight, species and condition of the particular patient, and the route of administration. The compositions can be administered alone, or can be co-administered or sequentially administered with compositions, e.g., with "other" immunological, antigenic or vaccine or therapeutic compositions thereby providing multivalent or "cocktail" or combination compositions of the invention and methods employing them. Again, the ingredients and manner (sequential or co-administration) of administration, as well as dosages can be determined taking into consideration such factors as the age, sex, weight, species and condition of the particular patient, and, the route of administration. In this regard, reference is made to U.S. Pat. No. 5,843,456, incorporated herein by reference, and directed to rabies compositions and combination compositions and uses thereof.

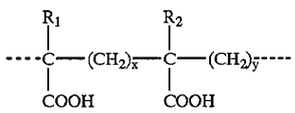
Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, peroral, intragastric, etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the recombinant poxvirus or antigens may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, adjuvants, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation. Suitable dosages can also be based upon the Examples below.

The compositions can contain at least one adjuvant compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative.

The preferred adjuvant compounds are the polymers of acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These compounds are known by the term carbomer (Phameuropa Vol. 8, No. 2, June 1996). Persons skilled in the art can also refer to U.S. Pat. No. 2,909,462 (incorporated herein by reference) which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than 8, the hydrogen atoms of at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name Carbopol® (BF Goodrich, Ohio, USA) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among then, there may be mentioned Carbopol® 974P, 934P and 971P.

Among the copolymers of maleic anhydride and alkenyl derivative, the copolymers EMA® (Monsanto) which are copolymers of maleic anhydride and ethylene, linear or cross-linked, for example cross-linked with divinyl ether, are preferred. Reference may be made to J. Fields et al., Nature, 186: 778–780, 4 June 1960, incorporated herein by reference.

From the point of view of their structure, the polymers of acrylic or methacrylic acid and the copolymers EMA® are preferably formed of basic units of the following formula:



in which:

R_1 and R_2, which are identical or different, represent H or CH_3

x=0or 1, preferably x=1

y=1 or 2, with x+y=2

For the copolymers EMA®, x=0 and y=2. For the carbomers, x=y=1.

The dissolution of these polymers in water leads to an acid solution which will be neutralized, preferably to physiological pH, in order to give the adjuvant solution into which the vaccine itself will be incorporated. The carboxyl groups of the polymer are then partly in COO^- form.

Preferably, a solution of adjuvant according to the invention, especially of carbomer, is prepared in distilled water, preferably in the presence of sodium chloride, the solution obtained being at acidic pH. This stock solution is diluted by adding it to the desired quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, preferably physiological saline (NaCL 9 g/l) all at once in several portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is for mixing with the vaccine, which may be especially stored in freeze-dried, liquid or frozen form.

The polymer concentration in the final vaccine composition will be 0.01% to 2% w/v, more particularly 0.06 to 1% w/v, preferably 0.1 to 0.6% w/v. The immunological compositions according to the invention may be associated to at least one live attenuated, inactivated, or sub-unit vaccine, or recombinant vaccine (e.g. poxvirus as vector or DNA plasmid) expressing at least one immunogen from another pig pathogen.

The invention encompasses vectors encoding and expressing equivalent nucleotide sequences, that is to say the sequences which change neither the functionality or the strain specificity (say strain of type 1 and strain of type 2) of the gene considered or those of the polypeptides encoded by this gene. The sequences differing through the degeneracy of the code are, of course, included.

The PCV-2 sequences used in the examples are derived from Meehan et al. (Strain Imp.1010; ORF1 nucleotides 398–1342; ORF2 nucleotides 1381–314; and correspond respectively to ORF4 and ORF13 in U.S. application Ser. No. 09/161,092 of Sep. 25 1998 and to COL4 and COL13 in WO-A-9918214). Other PCV-2 strains and their sequences have been published in WO-A-9918214 and are called Imp1008, Imp999, Imp1011-48285 and Imp1011-48121, as well as in A.L. Hamel et al. J. Virol. June 1998, vol 72, 6: 5262–5267 (GenBank AF027217) and in I. Morozov et al. J. Clinical Microb. September 1998 vol. 36, 9: 2535–2541, as well as GenBank AF086834, AF086835 and AF086836, and give access to equivalent ORF sequences. These sequences, or ORFs therefrom, or regions thereof encoding an antigen or epitope of interest can also be used in the practice of this invention.

The invention also encompasses the equivalent sequences to those used herein and in documents cited herein; for instance, sequences that are capable of hybridizing to the nucleotide sequence under high stringency conditions (see, e.g., Sambrook et al. (1989). Among the equivalent sequences, there may also be mentioned the gene fragments conserving the immunogenicity of the complete sequence, e.g., an epitope of interest.

The homology of the whole genome between PCV types 1 and 2 is about 75%. For ORF1, it is about 86%, and for ORF2, about 66%. On the contrary, homologies between genomes and between ORFs within type 2 are generally above 95%.

Also, equivalent sequences useful in the practice of this present invention, for ORF1, are those sequences having an homology equal or greater than 88%, advantageously 90% or greater, preferably 92% or 95% or greater with ORF1 of strain Imp1010, and for ORF2, are those sequences having an homology equal or greater than 80%, advantageously 85% or greater, preferably 90% or 95% or greater with ORF2 of strain Imp1010.

ORF1 and ORF2 according to Meehan 1998 has the potential to encode proteins with predicted molecular weights of 37.7 kD and 27.8 kD respectively. ORF3 and ORF4 (according to Meehan et al. 1998, correspond to ORF7 and ORF10 respectively in WO-A-9918214) has the potential to encode proteins with predicted molecular weights of 11.9 and 6.5 kD respectively. The sequence of these ORFs is also available in Genbank AF 055392. They can also be incorporated in plasmids and be used in accordance with the invention alone or in combination, e.g. with ORF1 and/or ORF2.

The other ORFs 1–3 and 5, 6, 8–9, 11–12 disclosed in U.S. application Ser. No. 09/161,092 of Sep. 25 1998 (COLs 1–3 and 5, 6, 8–9, 11–12 in WO-A-9918214), or region(s) thereof encoding an antigen or epitope of interest, may be used in the practice of this invention, e.g., alone or in combination or otherwise with each other or with the ORFs 1 and 2 or region(s) thereof encoding antigen(s) or epitope (s).

This invention also encompasses the use of equivalent sequences; for instance, from ORFs of various PCV-2 strains cited herein. For homology, one can determine that there are equivalent sequences which come from a PCV strain having

an ORF2 and/or an ORF1 which have an homology as defined above with the corresponding ORF of strain 1010.

For ORF3 according to Meehan, an equivalent sequence has homology thereto that is advantageously, for instance, equal or greater than 80%, for example 85% or greater, preferably 90% or 95% or greater with ORF3 of strain Impl010. For ORF4 according to Meehan 1998, advantageously an equivalent sequence has homology that is equal or greater than 86%, advantageously 90% or greater, preferably than 95% or greater with ORF4 of strain Imp1010.

From the genomic nucleotide sequence, e.g. those disclosed in WO-A-99 18214, it is routine art to determine the ORFs using a standard software, such as MacVector®. Also, alignment of genomes with that of strain 1010 and comparison with strain 1010 ORFs allows the one skilled in the art to readily determine the ORFs of the genome of another strain (e.g. other strains disclosed in WO-A-99 18214 or in other herein cited documents).

Using software or making sequence alignment is not undue experimentation and provides direct access to equivalent ORFs or nucleic acid molecules.

Nucleotide sequence homology can be determined using the "Align" program of Myers and Miller, ("Optimal Alignments in Linear Space", CABIOS 4, 11–17, 1988, incorporated herein by reference) and available at NCBI. Alternatively or additionally, the term "homology" or "identity", for instance, with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref}-N_{dif})^* 100N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence similarity of 75% with the sequence AATCAATC (N_{ref}=8; N_{dif}=2).

Alternatively or additionally, "homology" or "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™Suite, Intelligenetics Inc. CA).. When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

RNA sequences within the scope of the invention can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

Additionally or alternatively, amino acid sequence similarity or identity or homology can be determined using the BlastP program (Altschul et al., Nucl. Acids Res. 25, 3389–3402, incorporated herein by reference) and available at NCBI. The following references (each incorporated herein by reference) provide algorithms for comparing the relative identity or homology of amino acid residues of two proteins, and additionally or alternatively with respect to the foregoing, the teachings in these references can be used for determining percent homology or identity: Needleman S B and Wunsch C D, "A general method applicable to the search for similarities in the amino acid sequences of two proteins," *J. Mol. Biol.* 48:444–453 (1970); Smith T F and Waterman M S, "Comparison of Bio-sequences," *Advances in Applied Mathematics* 2:482–489 (1981); Smith T F, Waterman M S and Sadler J R, "Statistical characterization of nucleic acid sequence functional domains," *Nucleic Acids Res.*, 11:2205–2220 (1983); Feng D F and Dolittle R F, "Progressive sequence alignment as a prerequisite to correct phylogenetic trees," *J. of Molec. Evol.*, 25:351–360 (1987); Higgins D G and Sharp P M, "Fast and sensitive multiple sequence alignment on a microcomputer," *CABIOS*, 5: 151–153 (1989); Thompson J D, Higgins D G and Gibson T J, "ClusterW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice, *Nucleic Acid Res.*, 22:4673–480 (1994); and, Devereux J, Haeberlie P and Smithies O, "A comprehensive set of sequence analysis program for the VAX," *Nucl. Acids Res.*, 12: 387–395 (1984).

This invention not only allows for administration to adult pigs, but also to the young and to gestating females; in the latter case, this makes it possible, in particular, to confer passive immunity onto the newborns (maternal antibodies). Preferably, female pigs are inoculated prior to breeding; and/or prior to serving, and/or during gestation. Advantageously, at least one inoculation is done before serving and it is preferably followed by an inoculation to be performed during gestation, e.g., at about mid-gestation (at about 6–8 weeks of gestation) and/or at the end of gestation (at about 11–13 weeks of gestation). Thus, an advantageous regimen is an inoculation before mating and/o serving and a booster inoculation during gestation. Thereafter, there can be reinoculation before each serving and/or during gestation at about mid-gestation and/or at the end of gestation. Preferably, reinoculations are during gestation. Male pigs also can be inoculated, e.g., prior to mating.

Piglets, such as piglets from vaccinated females (e.g., inoculated as herein discussed), are inoculated within the first weeks of life, e.g., inoculation at one and/or two and/or three and/or four and/or five weeks of life. Preferably, piglets are first inoculated within the first week of life or within the third week of life (e.g., at the time of weaning). Advantageously, such piglets are then boosted two to four weeks later.

The present invention is additionally described by the following illustrative, non-limiting Examples.

EXAMPLES

The invention in a preferred embodiment is directed to recombinant poxviruses containing therein a DNA sequence from PCV2 in a nonessential region of the poxvirus genome. The recombinant poxviruses express gene products of the foreign PCV2 gene. In particular, ORF2 and ORF1 genes encoding PCV2 proteins were isolated, characterized and inserted into ALVAC (canarypox vector) recombinants. The molecular biology techniques used are the ones described by Sambrook et al. (1989).

Cell Lines and Virus Strains. The strain of PCV2 designated Imp.1010-Stoon has been previously described (Meehan et al., 1998). It was isolated from mesenteric lymph node tissues from a diseased pig originating from Canada. Cloning of the PCV2 genome was described by Meehan et al. (1998). Plasmid pGem7Z-Imp1010-Stoon-EcoRI No. 14 contains the PCV2 genome as an EcoRI fragment inserted into the EcoRI site of plasmid pGem-7Z (Promega, Madison, Wis.). The complete nucleotide sequence of the Imp.1010-Stoon PCV2 strain has been determined by Mee-

han et al. (1998) and is available under the GenBank accession number AF055392.

The parental canarypox virus (Rentschler strain) is a vaccinal strain for canaries. The vaccine strain was obtained from a wild type isolate and attenuated through more than 200 serial passages on chick embryo fibroblasts. A master viral seed was subjected to four successive plaque purifications under agar and one plaque clone was amplified through five additional passages after which the stock virus was used as the parental virus in in vitro recombination tests. The plaque purified canarypox isolate is designated ALVAC. ALVAC was deposited Nov. 14, 1996 under the terms of the Budapest Treaty at the American Type Culture Collection, ATCC accession number VR-2547.

The generation of poxvirus recombinants involves different steps: (1) construction of an insertion plasmid containing sequences ("arms") flanking the insertion locus within the poxvirus genome, and multiple cloning site (MCS) localized between the two flanking arms (e.g., see Example 1); (2) construction of donor plasmids consisting of an insertion plasmid into the MCS of which a foreign gene expression cassette has been inserted (e.g. see Examples 2 to 5); (3) in vitro recombination in cell culture between the arms of the donor plasmid and the genome of the parental poxvirus allowing the insertion of the foreign gene expression cassette into the appropriate locus of the poxvirus genome, and plaque purification of the recombinant virus (e.g. see Example 6).

PCV2 recombinant immunogens may be used in association with PCV1 immunogens, for immunization of animals against PMWS. In a least preferred approach, PCV1 immunogens may be used without PCV2 immunogens.

Example 1

Construction of Canarypox Insertion Plasmid at C6 Locus

FIG. **1** (SEQ ID NO:1) is the sequence of a 3.7 kb segment of canarypox DNA. Analysis of the sequence revealed an ORF designated C6L initiated at position 377 and terminated at position 2254. The following describes a C6 insertion plasmid constructed by deleting the C6 ORF and replacing it with a multiple cloning site (MCS) flanked by transcriptional and translational termination signals. A 380 bp PCR fragment was amplified from genomic canarypox DNA using oligonucleotide primers C6A1 (SEQ ID NO:2) and C6B1 (SEQ ID NO:3). A 1155 bp PCR fragment was amplified from genomic canarypox DNA using oligonucleotide primers C6C1 (SEQ ID NO:4) and C6D1 (SEQ ID NO:5). The 380 bp and 1155 bp fragments were fused together by adding them together as template and amplifying a 1613 bp PCR fragment using oligonucleotide primers C6A1 (SEQ ID NO:2) and C6D1 (SEQ ID NO:5). This fragment was digested with SacI and KpnI, and ligated into pBluescript SK+ (Stratagene, La Jolla, Calif., USA) digested with SacI/KpnI. The resulting plasmid, pC6L was confirmed by DNA sequence analysis. It consists of 370 bp of canarypox DNA upstream of C6 ("C6 left arm"), vaccinia early termination signal, translation stop codons in six reading frames, an MCS containing SmaI, PstI, XhoI and EcoRI sites, vaccinia early termination signal, translation stop codons in six reading frames and 1156 bp of downstream canary pox sequence ("C6 right arm").

Plasmid pJP099 was derived from pC6L by ligating a cassette containing the vaccinia H6 promoter (described in Taylor et al. (1988c), Guo et al. (1989), and Perkus et al. (1989)) coupled to a foreign gene into the SmaI/EcoRI sites of pC6L. This plasmid pJP099 contains a unique EcoRV site and a unique NruI site located at the 3' end of the H6 promoter, and a unique SalI site located between the STOP codon of the foreign gene and the C6 left arm. The ~4.5 kb EcoRV/SalI or NruI/SalI fragment from pJP099 contains therefore the plasmid sequence (pBluescript SK+; Stratagene, La Jolla, Calif., USA), the 2 C6 arms and the 5' end of the H6 promoter until the EcoRV or NruI site.

Sequences of the Primers

Primer C6A1 (SEQ ID NO:3)

ATCATCGAGCTCGCGGCCGCCTATCAAAAGTCTT-AATGAGTT

Primer C6B1 (SEQ ID NO:4)

GAATTCCTCGAGCTGCAGCCCGGGTTTTTATAG-CTAATTAGTCATTTTTTCGTAAGTA AGTATTTT-TATTTAA

Primer C6C1 (SEQ ID NO:5)

CCCGGGCTGCAGCTCGAGGAATTCTTTT-TATTGATTAACTAGTCAAATGAGTATATA TAAT-TGAAAAAGTAA

Primer C6D1 (SEQ ID NO:6)

GATGATGGTACCTTCATAAATACAAGTTTGATTAAA-CTTAAGTTG

Example 2

Construction of ALVACC Donor Plasmid for PCV2 0RF2

Plasmid pGem7Z-Imp1010-Stoon-EcoRI No. 14, containing the PCV2 genome as an EcoRI fragment in plasmid pGem-7Z, was digested with EcoRI, and a 1768 bp fragment was isolated and ligated.

In order to insert PCV2 ORF 2 into an appropriate ALVAC insertion vector: Primers JP760 (SEQ ID NO:6) and JP773 (SEQ ID NO:7) were used to amplify PCV2 ORF 2 from the 1768 bp ligated EcoRI fragment (see above) resulting in PCR J1304. Primer JP760 (SEQ ID NO:7) contains the 3' end of the H6 promoter from EcoRV and the 5' end of PCV2 ORF 2. Primer JP773 (SEQ ID NO:8) contains the 3' end of PCV2 ORF 2 followed by a SalI site. The product of PCR J1304 was then digested with EcoRV/SalI and cloned as a ~750 bp fragment into a ~4.5 kb EcoRVISalI fragment from pJP099 (see above in Example 1). The resulting plasmid was confirmed by sequence analysis and designated pJP102 (see the map of pJP102 in FIG. **2** and the sequence (SEQ ID NO:9) in FIG. **3**). The sequence of ORF 2 matches sequence available in GenBank, Accession Number AF055392. The donor plasmid pJP102 (linearized with NotI) was used in an in vitro recombination (IVR) test to generate ALVAC recombinant vCP1614 (see Example 6).

Sequence of the Primers

JP760 (SEQ ID NO:7)

CAT-CAT-CAT-GAT-ATC-CGT-TAA-GTT-TGT-ATC-GTA-ATG-ACG-TAT-CCA-AGG-AGG-CG

JP773 (SEQ ID NO:8)

TAC-TAC-TAC-GTC-GAC-TTA-GGG-TTT-AAG-TGG-GGG-GTC

Example 3

Construction of an ALVACC Donor Plasmid for PCV2 0RF2 and ORF1

PCV2 ORF1 was amplified by PCR using primers JP774 (SEQ ID NO:9) and JP775 (SEQ ID NO:10) on plasmid pGem7Z-Imp1010-Stoon-EcoRI No. 14 resulting in PCR J1311. Primer JP774 (SEQ ID NO:10) contains the 3' end of the H6 promoter from NruI and the 5' end of PCV2 ORF1. Primer JP775 (SEQ ID NO:11) contains the 3' end of PCV2 ORF1 followed by a SalI site. The product of PCR J1311 (~1 Kb) was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.). The resulting plasmid was confirmed by sequence analysis and designated pJP104. The sequence of ORF1 matches

sequence available in GenBank, Accession Number AF055392. A ~970 bp NruIlSalI fragment was isolated from pJP104 and cloned into a ~4.5 kb NruI/SalI fragment from pJP099 (see Example 1), resulting in a plasmid which was confirmed by restriction analysis and designated pJP105 (see FIG. 4). The donor plasmid pJP105 could be used in an in vitro recombination test (described in Example 6) to generate ALVAC recombinant expressing the PCV2 ORF1.

A ~838 bp BamHI/SalI from pJP102 (see Example 2) was blunted using the Klenow fragment of DNA polymerase, and was cloned into the Klenow-blunted EcoRI site of pJP105. Clones were checked for orientation of insert by restriction analysis and a head-to-head orientation was chosen. This plasmid was confirmed by sequence analysis and designated pJP107 (see the map of pJP107 in FIG. 5 and the sequence (SEQ ID NO:11) in FIG. 6). The donor plasmid pJP107 (linearized with NotI) was used in an in vitro recombination 5 (IVR) test to generate the ALVAC recombinant vCP1615 (see Example 6).

Sequence of the Primers

JP774 (SEQ ID NO:7)

CAT-CAT-CAT-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GCC-CAG-CAA-GAA-GAA-TGG

JP775 (SEQ ID NO:12)

TAC-TAC-TAC-GTC-GAC-TCA-GTA-ATT-TAT-TTC-ATA-TGG

Example 4

Construction of ALVACC Donor Plasmid for PCV1 RF2

Plasmid pPCV1 (B. Meehan et al. J. Gen. Virol. 1997. 78. 221–227), containing the PCV1 genome as a PstI fragment in plasmid pGem-7Z, was used as a template to amplify the PCV1 ORF2.

In order to insert PCV2 ORF 2 into an appropriate ALVAC insertion vector: Primers JP787 (SEQ ID NO:16) and JP788 (SEQ ID NO:17) were used to amplify PCV1 ORF 2 from plasmid pPCV1 (see above) resulting in PCR J1315. Primer JP787 (SEQ ID NO:16) contains the 3' end of the H6 promoter from EcoRV and ORF 2 followed by a SalI site. The product of PCR J1315 was then digested with EcoRV/SalI and cloned as a ~750 bp fragment into a ~4.5 kb EcoRV/SalI fragment from pJP099 (see above in Example 1). The resulting plasmid was confirmed by sequence analysis and designated pJP113. The sequence of ORF 2 matches sequence available in GenBank, Accession Number U49186. The donor plasmid pJP113 (linearized with NotI) was used in an in vitro recombination (IVR) test to generate ALVAC recombinant vCP1621 (see Example 7).

Sequence of the Primers

JP787 (SEQ ID NO:17)

CAT-CAT-CAT-GAT-ATC-CGT-TAA-GTT-TGT-ATC-GTA-ATG-ACG-TGG-CCA-AGG-AGG-CG

JP788 (SEQ ID NO:13)

TAC-TAC-TAC-GTC-GAC-TTA-TTT-ATT-TAG-AGG-GTC-TTT-TAG-G

Example 5

Construction of an ALVACC Donor Plasmid for PCV1 RF2 and ORF1

Plasmid pPCV1 (see Example 4 above), containing the PCV1 genome as a PstI fragment in plasmid pGem-7Z, was digested with PstI, and a 1759 bp fragment was isolated and ligated.

Primers JP789 (SEQ ID NO:14) and JP790 (SEQ ID NO:15) were used to amplify PCV1 ORF1 from the 1759 bp ligated PstI fragment (see above), resulting in PCR J1316. Primer JP789 (SEQ ID NO:14) contains the 3' end of the H6 promoter from NruI and the 5' end of PCV1 ORF1. Primer JP790 (SEQ ID NO:15) contains the 3' end of PCV1 ORF1 followed by a SalI site. The product of PCR J1316 (~1 Kb) was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.). The resulting plasmid was confirmed by sequence analysis and designated pJP114. The sequence of ORF1 matches sequence available in GenBank, Accession Number U49186. A ~970 bp NruI/SalI fragment was isolated from pJP114 and cloned into a ~4.5 kb NruI/SalI fragment from pJP099 (see Example 1), resulting in a plasmid which was confirmed by restriction analysis and designated pJP115. The donor plasmid pJP115 could be used in an in vitro recombination test (described in Example 7) to generate ALVAC recombinant expressing the PCV1 ORF1.

A ~838 bp BamHI/SalI from pJP113 (see Example 4) was blunted using the Klenow fragment of DNA polymerase, and was cloned into the Klenow-blunted EcoRI site of pJP115. Clones were checked for orientation of insert by restriction analysis and a head-to-head orientation was chosen. This plasmid was confirmed by sequence analysis and designated pJP117. The donor plasmid pJP117 (linearized with NotI) was used in an in vitro recombination (IVR) test to generate the ALVAC recombinant vCP1622 (see Example 7).

Sequence of the Primers

JP789 (SEQ ID NO:18)

CAT-CAT-CAT-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GCC-AAG-CAA-GAA-AAG-CGG

JP790 (SEQ ID NO:19)

TAC-TAC-TAC-GTC-GAC-TCA-GTA-ATT-TAT-TTT-ATA-TGG

Example 6

Generation of ALVAC-PCV2 RECOMBINANTS

Plasmids pJP102 (see Example 2 and FIG. 2) and pJP107 (see Example 3 and FIG. 5) were linearized with NotI and transfected into ALVAC infected primary CEF cells by using the calcium phosphate precipitation method previously described (Panicali and Paoletti, 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific PCV2 radiolabeled probes and subjected to four sequential rounds of plaque purification until a pure population was achieved. One representative plaque from each IVR was then amplified and the resulting ALVAC recombinants were designated vCP1614 and vCP1615. The vCP1614 virus is the result of recombination events between ALVAC and the donor plasmid pJP102, and it contains the PCV2 ORF2 inserted into the ALVAC C6 locus. The vCP1615 virus is the result of recombination events between ALVAC and the donor plasmid pJP107, and it contains the PCV2 ORF2 and ORF1 inserted into the ALVAC C6 locus in a head-to-head orientation.

In a similar fashion, a recombinant ALVAC expressing only PCV2 ORF1 can be generated using the donor plasmid pJP105 described in Example 3.

Immunofluorescence. In order to determine if the PCV2 proteins were expressed in ALVAC recombinant infected Vero cells, immunofluorescence (IF) analysis was performed. Infected Vero cells were washed with PBS 24 hrs after infection (m.o.i. of approx. 10) and fixed with 95% cold aceton for 3 minutes at room temperature. Five monoclonal antibody (MAb) preparations (hybridoma supernatant) specific for PCV2 ORF1 (PCV2 199 1D3GA & PCV2 210 7G5GD) or ORF2 (PCV2 190 4C7CF, PCV2 190 2B1BC & PCV2 190 3A8BC) were used as the first antibody. These specific monoclonal antibodies were obtained from Merial-Lyon. Monoclonal antibodies can also be obtained following the teachings of documents cited herein, e.g. WO-A-99 18214, 1998, French applications Nos. 97/12382, 98/00873,

98/03707, filed Oct. 3, 1997, Jan. 22, 1998, and Mar. 20, 1998, and WO99/29717, incorporated herein by reference. The IF reaction was performed as described by Taylor et al. (1990).

PCV2 specific immunofluorescence with the three ORF2-specific antibodies could be detected in cells infected with vCP1614 and cells infected with vCP1615. PCV2 specific immunofluorescence with the two ORF1 -specific antibodies could be detected in cells infected with vCP1615 only. These results indicated that, as expected, vCP1614 expresses only ORF2, whereas vCP1615 expresses both ORF1 and ORF2. No fluorescence was detected in parental ALVAC infected Vero cells, nor in uninfected Vero cells.

Example 7

Generation of ALVAC-PCV1 Recombinants

Plasmids pJP113 (see Example 4) and pJP117 (see Example 5) were linearized with NotI and transfected into ALVAC infected primary CEF cells by using the calcium phosphate precipitation method previously described (Panicali and Paoletti, 1982; Piccini et al., 1987). Positive plaques were selected on the basis of hybridization to specific PCV1 radiolabeled probes and subjected to four sequential rounds of plaque purification until a pure population was achieved. One representative plaque from each IVR was then amplified and the resulting ALVAC recombinants were designated vCP1621 and vCP1622. The vCP1621 virus is the result of recombination events between ALVAC and the donor plasmid pJP113, and it contains the PCV1 ORF2 inserted into the ALVAC C6 locus. The vCP1622 virus is the result of recombination events between ALVAC and the donor plasmid pJP117, and it contains the PCV1 ORF2 and ORF1 inserted into the ALVAC C6 locus in a head-to-head orientation.

In a similar fashion, a recombinant ALVAC expressing only PCV1 ORF1 can be generated using the donor plasmid pJP115 described in Example 5.

Immunofluorescence. In order to determine if the PCV1 proteins were expressed in ALVAC recombinant infected Vero cells, immunofluorescence (IF) analysis was performed. Infected Vero cells were washed with PBS 24 hrs after infection (m.o.i. of approx. 10) and fixed with 95% cold aceton efor 3 minutes at room temperature. A specific anti-PCV1 pig polyclonal serum (Allan G. et al. Vet. Microbiol. 1999. 66: 115–123) was used as the first antibody. The IF reaction was performed as described by Taylor et al. (1990).

PCV1 specific immunofluorescence could be detected in cells infected with vCP1621 and cells infected with vCP1622. These results indicated that, as expected, vCP1621 and vCP1622 express PCV1-specific products. No fluorescence was detected with a PCV2-specific pig polyclonal serum in cells infected with vCP1621 and in cells infected with vCP1622. No fluorescence was detected in parental ALVAC infected Vero cells, nor in uninfected Vero cells.

Example 8

Formulation of Recombinant Canarypox Viruses with Carbopol™ 974P

For the preparation of vaccines, recombinant canarypox viruses vCP1614 and vCP1615 (Example 6) can be mixed with solutions of carbomer. In the same fashion, recombinant canarypox viruses vCP1621 and vCP1622 (Example 7) can be mixed with solutions of carbomer. The carbomer component used for vaccination of pigs according to the present invention is the Carbopol™ 974P manufactured by the company BF Goodrich (molecular weight of # 3,000,000). A 1.5 % Carbopol™ 974P stock solution is first prepared in distilled water containing 1 g/l of sodium chloride. This stock solution is then used for manufacturing a 4 mg/ml Carbopol™ 974P solution in physiological water. The stock solution is mixed with the required volume of physiological water, either in one step or in several successive steps, adjusting the pH value at each step with a 1N (or more concentrated) sodium hydroxide solution to get a final pH value of 7.3–7.4. This final Carbopol™ 974P solution is a ready-to-use solution for reconstituting a lyophilized recombinant virus or for diluting a concentrated recombinant virus stock. For example, to get a final viral suspension containing $10^{e}8$ pfu per dose of 2 ml, one can dilute 0,1 ml of a $10^{e}9$ pfU/ml stock solution into 1,9 ml of the above Carbopol™ 974P 4 mg/ml ready-to-use solution. In the same fashion, Carbopol™ 974P 2 mg/ml ready-to-use solutions can also be prepared.

Example 9

Immunization of Pigs and Subsequent Challenge

9.1. Immunization of 1 Day-Old Piglets

Groups of piglets, caesarian-derived at Day 0, are placed into isolators. The piglets are vaccinated by intramuscular route at Day 2 with various vaccine solutions. Vaccine viral suspensions are prepared by dilution of recombinant viruses stocks in sterile physiological water (NaCl 0.9 %). Suitable ranges for viral suspensions can be determined empiracally, but will generally range from 10^6 to 10^{10}, and preferably about 10^{10}, pfu/dose. Vaccine solutions can also be prepared by mixing the recombinant virus suspension with a solution of Carbopol™ 974P, as described in Example 8.

Piglets are vaccinated either with:

Recombinant virus vCP1614 (Example 2);

Recombinant virus vCP1615 (Example 3);

Recombinant virus vCP1614 mixed with Carbopol (4 mg/ml solution); or

Recombinant virus vCP1615 mixed with Carbopol (4 mg/ml solution).

The viral suspensions contain 10^8 plaque forming units (pfu) per dose. Each viral suspension is injected by intramuscular route under a volume of 1 ml. The intramuscular injection is administered into the muscles of the neck.

Two injections of viral suspensions are administered at Day 2 and Day 14 of the experiment. A challenge is done on Day 21 by an oronasal administration of a viral suspension prepared from a culture of PCV-2 virulent strain. After challenge, piglets are monitored during 3 weeks for clinical signs specific of the post-weaning multisystemic syndrome. The following signs are scored:

Rectal temperature: daily monitoring for 2 weeks post-challenge, then 2 measures of rectal temperature during the third week.

Weight: piglets are weighed right before the challenge, and then weekly during the first 3 weeks post-challenge.

Blood samples are taken at Day 2, day 14, Day 21, Day 28, Day 35 and Day 42 of the experiment in order to monitor viremia levels and anti-PCV-2 specific antibody titers.

Necropsies: at Day 42, all surviving piglets are humanely euthanized and necropsied to look for specific PWMS macroscopic lesions. Tissue samples are prepared from liver, lymph nodes, spleen, kidneys and thymus in order to look for specific histological lesions.

9.2. Immunization OF 5–7 Week-Old Piglets

5–7 week-old piglets, free of anti-PCV-2 specific maternal antibodies, are vaccinated by intramuscular route with various vaccine solutions. Vaccine viral suspensions are prepared by dilution of recombinant viruses stocks in sterile physiological water (NaCl 0.9%). Vaccine solutions can also

be prepared by mixing the recombinant virus suspension with a solution of Carbopol™ 974P, as described in Example 8.

Piglets are vaccinated either with:

Recombinant virus vCP1614 (Example 2);

Recombinant virus vCP1615 (Example 3);

Recombinant virus vCP1614 mixed with Carbopol (4 mg/ml solution); or

Recombinant virus vCP1615 mixed with Carbopol (4 mg/ml solution).

The viral suspensions contain 10^8 plaque forming units (pfu) per dose. Each viral suspension is injected by intramuscular route under a volume of 2 ml. The intramuscular injection is administered into the muscles of the neck. Two injections of the viral suspensions are administered at Day 0 and Day 21 of the experiment. A challenge is done at Day 35 by an oronasal administration of a viral suspension prepared from a culture of PCV-2 virulent strain. After challenge, piglets are monitored during 8 weeks for clinical signs specific of the post-weaning multisystemic syndrome. The clinical monitoring is identical to the one described in Example 9.1. except that total duration of monitoring is 8 weeks instead of 3 weeks.

Necropsies are done throughout the experiment for piglets dying from the challenge and at the end of the experiment (Day 97) for all surviving piglets. Tissue samples are the same as described in Example 9.1.

9.3. Immunization of Newborn Piglets

Groups of 3 or 4 piglets, caesarian-delivered day Oare placed into isolators. Day 2 the piglets are vaccinated with 10^8 pfu of vCP1614, vCP1615 or parental ALVAC vector in 1 ml of PBS by intramuscular route on the side of the neck. A second injection of vaccine or placebo is administered at day 14. Vaccination with ALVAC recombinant is well tolerated by piglets and no evidence of adverse reaction to vaccination is noted. The piglets are challenged day 21 by oronasal administration of a PCV-2 viral suspension, 1 ml in each nostril. Day 45 necropsies are performed and samples of tissues are collected for virus isolation. Necropsy results: PMWS is characterized generally by lymphadenopathy and more rarely by hepatitis or nephritis. So the gross findings in lymph nodes are scored for each piglet in the following manner: 0=no visible enlargement of lymph nodes; 1=mild lymph nodes enlargement, restricted to bronchial lymph nodes; 2=moderate lymph nodes enlargement, restricted to bronchial lymph nodes; 3=severe lymph nodes enlargement, extended to bronchial, submandibullar prescapular and inguinal lymph nodes.

Groups	Scores
vCP 1614	0.5
	0.0
	0.0
	1.0
mean	0.38
standard deviation	0.48
vCP 1615	0.0
	0.5
	0.5
	1.0
mean	0.5
standard deviation	0.41
Controls	2.0
	2.5
	2.5
	2.5
mean	2.38
standard deviation	0.25

Bronchial lymphadenopathy for PCV-2 is a prominent gross finding. A significant reduction of the lymph nodes lesion in relation to control group is observed after immunization with vCP 1614 and vCP1615 ($p \leqq 0.05$).

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

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Val Ile Phe Thr Thr Ser Asp Asp Asp Thr Val Val Val Ile Asn Glu
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 45                  50                  55                  60

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Phe Phe Asn Ser Phe Asn Asn Gly Leu Ser Lys Tyr Glu Thr Ile Ser
                 65                  70                  75

gat aca ata tta gat ata gat act cat aat tat tat ata cct agt tct      652
Asp Thr Ile Leu Asp Ile Asp Thr His Asn Tyr Tyr Ile Pro Ser Ser
             80                  85                  90

tct tct ttg tta gat att cta aaa aaa aga gcg tgt gat tta gaa tta      700
Ser Ser Leu Leu Asp Ile Leu Lys Lys Arg Ala Cys Asp Leu Glu Leu
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Glu Asp Leu Asn Tyr Ala Leu Ile Gly Asp Asn Ser Asn Leu Tyr Tyr
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aaa gat atg act tac atg aat aat tgg tta ttt act aaa gga tta tta      796
Lys Asp Met Thr Tyr Met Asn Asn Trp Leu Phe Thr Lys Gly Leu Leu
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gat tac aag ttt gta tta ttg cgc gat gta gat aaa tgt tac aaa cag      844
Asp Tyr Lys Phe Val Leu Leu Arg Asp Val Asp Lys Cys Tyr Lys Gln
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tat aat aaa aag aat act ata ata gat ata ata cat cgc gat aac aga      892
Tyr Asn Lys Lys Asn Thr Ile Ile Asp Ile Ile His Arg Asp Asn Arg
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Gln Tyr Asn Ile Trp Val Lys Asn Val Ile Glu Tyr Cys Ser Pro Gly
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tat ata tta tgg tta cat gat cta aaa gcc gct gct gaa gat gat tgg      988
Tyr Ile Leu Trp Leu His Asp Leu Lys Ala Ala Ala Glu Asp Asp Trp
    190                 195                 200

tta aga tac gat aac cgt ata aac gaa tta tct gcg gat aaa tta tac     1036
Leu Arg Tyr Asp Asn Arg Ile Asn Glu Leu Ser Ala Asp Lys Leu Tyr
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act ttc gag ttc ata gtt ata tta gaa aat aat ata aaa cat tta cga     1084
Thr Phe Glu Phe Ile Val Ile Leu Glu Asn Asn Ile Lys His Leu Arg
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gta ggt aca ata att gta cat cca aac aag ata ata gct aat ggt aca     1132
Val Gly Thr Ile Ile Val His Pro Asn Lys Ile Ile Ala Asn Gly Thr
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Ser Asn Asn Ile Leu Thr Asp Phe Leu Ser Tyr Val Glu Glu Leu Ile
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tat cat cat aat tca tct ata ata ttg gcc gga tat ttt tta gaa ttc     1228
Tyr His His Asn Ser Ser Ile Ile Leu Ala Gly Tyr Phe Leu Glu Phe
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Phe Glu Thr Thr Ile Leu Ser Glu Phe Ile Ser Ser Ser Ser Glu Trp
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tat gta aaa tat tgg aca aag aaa act ttg cag tat aag aac ttt ttt     1420
Tyr Val Lys Tyr Trp Thr Lys Lys Thr Leu Gln Tyr Lys Asn Phe Phe
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aaa gac ggt aaa cag tta gca aaa tat ata att aag aaa gat agt cag     1468
Lys Asp Gly Lys Gln Leu Ala Lys Tyr Ile Ile Lys Lys Asp Ser Gln
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gtg ata gat aga gta tgt tat tta cac gca gct gta tat aat cac gta     1516
Val Ile Asp Arg Val Cys Tyr Leu His Ala Ala Val Tyr Asn His Val
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Thr Tyr Leu Met Asp Thr Phe Lys Ile Pro Gly Phe Asp Phe Lys Phe
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tcc gga atg ata gat ata cta ctg ttt gga ata ttg cat aag gat aat     1612
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Glu Asn Ile Phe Tyr Pro Lys Arg Val Ser Val Thr Asn Ile Ile Ser
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gaa tct atc tat gca gat ttt tac ttt ata tca gat gtt aat aaa ttc     1708
Glu Ser Ile Tyr Ala Asp Phe Tyr Phe Ile Ser Asp Val Asn Lys Phe
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agt aaa aag ata gaa tat aaa act atg ttt cct ata ctc gca gaa aac     1756
Ser Lys Lys Ile Glu Tyr Lys Thr Met Phe Pro Ile Leu Ala Glu Asn
445                 450                 455                 460

tac tat cca aaa gga agg ccc tat ttt aca cat aca tct aac gaa gat     1804
Tyr Tyr Pro Lys Gly Arg Pro Tyr Phe Thr His Thr Ser Asn Glu Asp
                465                 470                 475

ctt ctg tct atc tgt tta tgc gaa gta aca gtt tgt aaa gat ata aaa     1852
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ggt tta ttt aca tct gtc gat ata aat acg gct gtt gag tta aga gga     1948
Gly Leu Phe Thr Ser Val Asp Ile Asn Thr Ala Val Glu Leu Arg Gly
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tat aaa ata aga gta ata gga tgt tta gaa tgg cct gaa aag ata aaa     1996
Tyr Lys Ile Arg Val Ile Gly Cys Leu Glu Trp Pro Glu Lys Ile Lys
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ata ttt aat tct aat cct aca tac att aga tta tta cta aca gaa aga     2044
Ile Phe Asn Ser Asn Pro Thr Tyr Ile Arg Leu Leu Leu Thr Glu Arg
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cgt tta gat att cta cat tcc tat ctg ctt aaa ttt aat ata aca gag     2092
Arg Leu Asp Ile Leu His Ser Tyr Leu Leu Lys Phe Asn Ile Thr Glu
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Asp Ile Ala Thr Arg Asp Gly Val Arg Asn Asn Leu Pro Ile Ile Ser
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Phe Ile Val Ser Tyr Cys Arg Ser Tyr Thr Tyr Lys Leu Leu Asn Cys
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His Met Tyr Asn Ser Cys Lys Ile Thr Lys Cys Lys Tyr Asn Gln Val
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Ile Tyr Asn Pro Ile
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Thr Ser Asp Asp Asp Thr Val Val Val Ile Asn Glu Asp Asn Val Leu
35 40 45

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

Phe Asn Asn Gly Leu Ser Lys Tyr Glu Thr Ile Ser Asp Thr Ile Leu
65 70 75 80

Asp Ile Asp Thr His Asn Tyr Tyr Ile Pro Ser Ser Ser Ser Leu Leu
85 90 95

Asp Ile Leu Lys Lys Arg Ala Cys Asp Leu Glu Leu Glu Asp Leu Asn
100 105 110

Tyr Ala Leu Ile Gly Asp Asn Ser Asn Leu Tyr Tyr Lys Asp Met Thr
115 120 125

Tyr Met Asn Asn Trp Leu Phe Thr Lys Gly Leu Leu Asp Tyr Lys Phe
130 135 140

Val Leu Leu Arg Asp Val Asp Lys Cys Tyr Lys Gln Tyr Asn Lys Lys
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Asn Thr Ile Ile Asp Ile Ile His Arg Asp Asn Arg Gln Tyr Asn Ile
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Trp Val Lys Asn Val Ile Glu Tyr Cys Ser Pro Gly Tyr Ile Leu Trp
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Leu His Asp Leu Lys Ala Ala Ala Glu Asp Asp Trp Leu Arg Tyr Asp
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Asn Arg Ile Asn Glu Leu Ser Ala Asp Lys Leu Tyr Thr Phe Glu Phe
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Ile Val His Pro Asn Lys Ile Ile Ala Asn Gly Thr Ser Asn Asn Ile
245 250 255

Leu Thr Asp Phe Leu Ser Tyr Val Glu Glu Leu Ile Tyr His His Asn
260 265 270

Ser Ser Ile Ile Leu Ala Gly Tyr Phe Leu Glu Phe Phe Glu Thr Thr
275 280 285

Ile Leu Ser Glu Phe Ile Ser Ser Ser Ser Glu Trp Val Met Asn Ser
290 295 300

Asn Cys Leu Val His Leu Lys Thr Gly Tyr Glu Ala Ile Leu Phe Asp
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Asp Thr Phe Lys Ile Pro Gly Phe Asp Phe Lys Phe Ser Gly Met Ile
385                 390                 395                 400

Asp Ile Leu Leu Phe Gly Ile Leu His Lys Asp Asn Glu Asn Ile Phe
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Tyr Pro Lys Arg Val Ser Val Thr Asn Ile Ile Ser Glu Ser Ile Tyr
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Ala Asp Phe Tyr Phe Ile Ser Asp Val Asn Lys Phe Ser Lys Lys Ile
        435                 440                 445

Glu Tyr Lys Thr Met Phe Pro Ile Leu Ala Glu Asn Tyr Tyr Pro Lys
    450                 455                 460

Gly Arg Pro Tyr Phe Thr His Thr Ser Asn Glu Asp Leu Leu Ser Ile
465                 470                 475                 480

Cys Leu Cys Glu Val Thr Val Cys Lys Asp Ile Lys Asn Pro Leu Leu
                485                 490                 495

Tyr Ser Lys Lys Asp Ile Ser Ala Lys Arg Phe Ile Gly Leu Phe Thr
            500                 505                 510

Ser Val Asp Ile Asn Thr Ala Val Glu Leu Arg Gly Tyr Lys Ile Arg
        515                 520                 525

Val Ile Gly Cys Leu Glu Trp Pro Glu Lys Ile Lys Ile Phe Asn Ser
    530                 535                 540

Asn Pro Thr Tyr Ile Arg Leu Leu Leu Thr Glu Arg Arg Leu Asp Ile
545                 550                 555                 560

Leu His Ser Tyr Leu Leu Lys Phe Asn Ile Thr Glu Asp Ile Ala Thr
                565                 570                 575

Arg Asp Gly Val Arg Asn Asn Leu Pro Ile Ile Ser Phe Ile Val Ser
            580                 585                 590

Tyr Cys Arg Ser Tyr Thr Tyr Lys Leu Leu Asn Cys His Met Tyr Asn
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625
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tatttttatt taa 73

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ttgaaaaagt aa 72

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cacgtttttg tatacatttc tttccatatt agtagctact actaaatgat tatcttcttt 420

catatcttgt agataagata gactatcttt atctttatta gtagaaaata cttctggcca 480

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gatacatata acagctgtaa atcctaaaaa attcctatca taattattaa tattaggtaa 720

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aga aga cac cgc ccc cgc agc cat ctt ggc cag atc ctc cgc cgc cgc      1479
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Pro Trp Leu Val His Pro Arg His Arg Tyr Arg Trp Arg Arg Lys Asn
            30                  35                  40

ggc atc ttc aac acc cgc ctc tcc cgc acc ttc gga tat act gtc aag      1575
Gly Ile Phe Asn Thr Arg Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys
        45                  50                  55

cgt acc aca gtc aca acg ccc tcc tgg gcg gtg gac atg atg aga ttt      1623
Arg Thr Thr Val Thr Thr Pro Ser Trp Ala Val Asp Met Met Arg Phe
    60                  65                  70

aaa att gac gac ttt gtt ccc ccg gga ggg ggg acc aac aaa atc tct      1671
Lys Ile Asp Asp Phe Val Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser
75                  80                  85                  90

ata ccc ttt gaa tac tac aga ata aga aag gtt aag gtt gaa ttc tgg      1719
Ile Pro Phe Glu Tyr Tyr Arg Ile Arg Lys Val Lys Val Glu Phe Trp
                95                  100                 105

ccc tgc tcc ccc atc acc cag ggt gat agg gga gtg ggc tcc act gct      1767
Pro Cys Ser Pro Ile Thr Gln Gly Asp Arg Gly Val Gly Ser Thr Ala
            110                 115                 120

gtt att cta gat gat aac ttt gta aca aag gcc aca gcc cta acc tat      1815
Val Ile Leu Asp Asp Asn Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr
        125                 130                 135

gac cca tat gta aac tac tcc tcc cgc cat aca atc ccc caa ccc ttc      1863
Asp Pro Tyr Val Asn Tyr Ser Ser Arg His Thr Ile Pro Gln Pro Phe
    140                 145                 150

tcc tac cac tcc cgt tac ttc aca ccc aaa cct gtt ctt gac tcc act      1911
Ser Tyr His Ser Arg Tyr Phe Thr Pro Lys Pro Val Leu Asp Ser Thr
155                 160                 165                 170

att gat tac ttc caa cca aat aac aaa agg aat cag ctt tgg ctg aga      1959
Ile Asp Tyr Phe Gln Pro Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg
                175                 180                 185

cta caa acc tct gga aat gtg gac cac gta ggc ctc ggc gct gcg ttc      2007
Leu Gln Thr Ser Gly Asn Val Asp His Val Gly Leu Gly Ala Ala Phe
            190                 195                 200

gaa aac agt aaa tac gac cag gac tac aat atc cgt gta acc atg tat      2055
Glu Asn Ser Lys Tyr Asp Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr
        205                 210                 215

gta caa ttc aga gaa ttt aat ctt aaa gac ccc cca ctt aaa ccc          2100
Val Gln Phe Arg Glu Phe Asn Leu Lys Asp Pro Pro Leu Lys Pro
    220                 225                 230
```

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taagtcgacc ccgggttttt atagctaatt agtcattttt tcgtaagtaa gtatttttat 2160

ttaatacttt ttattgtact tatgttaaat ataactgatg ataacaaaat ccattatgta 2220

ttatttataa ctgtaatttc tttagcgtag ttagatgtcc aatctctctc aaatacatcg 2280

gctatctttt tagtgagatt ttgatctatg cagttgaaac ttatgaacgc gtgatgatta 2340

aaatgtgaac cgtccaaatt tgcagtcatt atatgagcgt atctattatc tactatcatc 2400

atctttgagt tattaatatc atctacttta gaattgatag gaaatatgaa tacctttgta 2460

gtaatatcta tactatctac acctaactca ttaagacttt tgataggcgg ccgcgagctc 2520

<210> SEQ ID NO 10
<211> LENGTH: 233
<212> TYPE: PRT
<213> ORGANISM: ALVAC

<400> SEQUENCE: 10

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1 5 10 15

Ser His Leu Gly Gln Ile Leu Arg Arg Arg Pro Trp Leu Val His Pro
20 25 30

Arg His Arg Tyr Arg Trp Arg Arg Lys Asn Gly Ile Phe Asn Thr Arg
35 40 45

Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys Arg Thr Thr Val Thr Thr
50 55 60

Pro Ser Trp Ala Val Asp Met Met Arg Phe Lys Ile Asp Asp Phe Val
65 70 75 80

Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser Ile Pro Phe Glu Tyr Tyr
85 90 95

Arg Ile Arg Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr
100 105 110

Gln Gly Asp Arg Gly Val Gly Ser Thr Ala Val Ile Leu Asp Asp Asn
115 120 125

Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro Tyr Val Asn Tyr
130 135 140

Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
145 150 155 160

Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
165 170 175

Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg Leu Gln Thr Ser Gly Asn
180 185 190

Val Asp His Val Gly Leu Gly Ala Ala Phe Glu Asn Ser Lys Tyr Asp
195 200 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
210 215 220

Asn Leu Lys Asp Pro Pro Leu Lys Pro
225 230

<210> SEQ ID NO 11
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: ALVAC

<400> SEQUENCE: 11

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<210> SEQ ID NO 12

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<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: ALVAC

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<211> LENGTH: 3609
<212> TYPE: DNA
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atcacgtatc ctatttttag tattggtaga acgttttagt tctaaagtta aaatattaga 180

cataattggc atattgctta ttccttgcat agttgagtct gtagatcgtt tcagtatatc 240

actgattaat gtactactgt tatgatgaaa tatagaatcg atattggcat ttaactgttt 300

tgttatacta agtctagatt ttaaatcttc tagtaatatg ctatttaata taaaagcttc 360

cacgtttttg tatacatttc tttccatatt agtagctact actaaatgat tatcttcttt 420

catatcttgt agataagata gactatcttt atctttatta gtagaaaata cttctggcca 480

tacatcgtta aatttttttg ttgttgttag atataatatt aaatatctag aggatcctat 540

tatttgtggt aaaatgttta tagagtaaaa tgatctggct attaaacata ggccagttac 600

catagaatgc tgcttcccgt tacagtgttt taccataacc atagatctgc ctgtattgtt 660

gatacatata acagctgtaa atcctaaaaa attcctatca taattattaa tattaggtaa 720

ttcatttcca tgtgaaagat agactaattt tatatccttt acctccaaat aattatttac 780

atctcttaaa caatctattt taatatcatt aactggtatt ttataatatc cagaaaggtt 840

tgaaggggtt gatggaataa gtctattaac atcgttaagt aaattattaa tatcatgaat 900

ctttattata ttatacccat aagttaaatt tatatttact ttctcatcat ctgacttagt 960

tagtttgtaa taaggtgtgt ctgaaaaaat taaaaggtaa ttcgttgaat gaagctgtat 1020

ttgctgtatc atttttatct aattttggag atttagcagt acttacttca ttagaagaag 1080

aatctgccag ttcctgtcta ttactgatat ttcgtttcat tattatatga tttatatttt 1140

acttttcaa ttatatatac tcatttgact agttaatcaa taaaaagaat ttcgacttag 1200

ggtttaagtg gggggtcttt aagattaaat tctctgaatt gtacatacat ggttacacgg 1260

atattgtagt cctggtcgta tttactgttt tcgaacgcag cgccgaggcc tacgtggtcc 1320

acatttccag aggtttgtag tctcagccaa agctgattcc ttttgttatt tggttggaag 1380

taatcaatag tggagtcaag aacaggtttg ggtgtgaagt aacgggagtg gtaggagaag 1440

ggttgggggа ttgtatggcg ggaggagtag tttacatatg ggtcataggt tagggctgtg 1500

gcctttgtta caaagttatc atctagaata acagcagtgg agcccactcc cctatcaccc 1560

tgggtgatgg gggagcaggg ccagaattca accttaacct ttcttattct gtagtattca 1620

aagggtatag agattttgtt ggtccccct cccgggggaa caaagtcgtc aattttaaat 1680

ctcatcatgt ccaccgccca ggagggcgtt gtgactgtgg tacgcttgac agtatatccg 1740

aaggtgcggg agaggcgggt gttgaagatg ccatttttcc ttctccaacg gtagcggtgg 1800

cgggggtgga cgagccaggg gcggcggcgg aggatctggc caagatggct gcgggggcgg 1860

tgtcttcttc tgcggtaacg cctccttgga tacgtcatta cgatacaaac ttaacggata 1920

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tcgcgataat gaaataattt atgattattt ctcgctttca atttaacaca accctcaaga 1980

acctttgtat ttattttcac tttttaagta tagaataaag aagctggggg atcaattcct 2040

gcagccctgc agctaattaa ttaagctaca aatagtttcg ttttcacctt gtctaataac 2100

taattaatta aggatccccc agcttcttta ttctatactt aaaaagtgaa aataaataca 2160

aaggttcttg agggttgtgt taaattgaaa gcgagaaata atcataaatt atttcattat 2220

cgcgatatcc gttaagtttg tatcgtaatg cccagcaaga agaatggaag aagcggaccc 2280

caaccacata aaaggtgggt gttcacgctg aataatcctt ccgaagacga gcgcaagaaa 2340

atacgggagc tcccaatctc cctatttgat tattttattg ttggcgagga gggtaatgag 2400

gaaggacgaa cacctcacct ccaggggttc gctaattttg tgaagaagca aacttttaat 2460

aaagtgaagt ggtatttggg tgcccgctgc cacatcgaga aagccaaagg aactgatcag 2520

cagaataaag aatattgcag taaagaaggc aacttactta ttgaatgtgg agctcctcga 2580

tctcaaggac aacggagtga cctgtctact gctgtgagta ccttgttgga gagcgggagt 2640

ctggtgaccg ttgcagagca gcaccctgta acgtttgtca gaaatttccg cgggctggct 2700

gaacttttga aagtgagcgg gaaaatgcag aagcgtgatt ggaagaccaa tgtacacgtc 2760

attgtggggc cacctgggtg tggtaaaagc aaatgggctg ctaattttgc agacccggaa 2820

accacatact ggaaaccacc tagaaacaag tggtgggatg gttaccatgg tgaagaagtg 2880

gttgttattg atgactttta tggctggctg ccgtgggatg atctactgag actgtgtgat 2940

cgatatccat tgactgtaga gactaaaggt ggaactgtac ctttttggc ccgcagtatt 3000

ctgattacca gcaatcagac cccgttggaa tggtactcct caactgctgt cccagctgta 3060

gaagctctct atcggaggat tacttccttg gtattttgga agaatgctac agaacaatcc 3120

acggaggaag ggggccagtt cgtcaccctt tccccccat gccctgaatt tccatatgaa 3180

ataaattact gagtcgaccc cgggttttta tagctaatta gtcatttttt cgtaagtaag 3240

tatttttatt taatactttt tattgtactt atgttaaata taactgatga taacaaaatc 3300

cattatgtat tatttataac tgtaatttct ttagcgtagt tagatgtcca atctctctca 3360

aatacatcgg ctatcttttt agtgagattt tgatctatgc agttgaaact tatgaacgcg 3420

tgatgattaa aatgtgaacc gtccaaattt gcagtcatta tatgagcgta tctattatct 3480

actatcatca tctttgagtt attaatatca tctactttag aattgatagg aaatatgaat 3540

acctttgtag taatatctat actatctaca cctaactcat taagactttt gataggcggc 3600

cgcgagctc 3609

<210> SEQ ID NO 14
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<212> TYPE: PRT
<213> ORGANISM: ALVAC

<400> SEQUENCE: 14

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Met Thr Val Arg Ile Asn Tyr Asp Gln Asp Tyr Lys Ser Asn Glu Phe
20 25 30

Ala Ala Tyr Leu Tyr Val His Asp Val His Asp Val Asn Tyr Ser Thr
35 40 45

Gln Leu Arg Leu Trp Leu Gln Asn Arg Lys Asn Asn Pro Gln Phe Tyr
50 55 60

-continued

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Asp Ile Thr Ser Asp Leu Val Pro Lys Pro Thr Phe Tyr Arg Ser His
 65                  70                  75                  80

Tyr Ser Phe Pro Gln Pro Ile Thr His Arg Ser Ser Tyr Gln Val Tyr
                85                  90                  95

Pro Asp Tyr Thr Leu Ala Thr Ala Lys Thr Val Phe Gln Asp Asp Leu
            100                 105                 110

Ile Val Ala Thr Ser Gly Val Gly Arg Asp Gly Gln Thr Ile Pro Ser
        115                 120                 125

Cys Pro Trp Phe Glu Val Lys Val Lys Arg Ile Arg Tyr Tyr Glu Phe
    130                 135                 140

Pro Ile Ser Ile Lys Gln Thr Gly Gly Gly Pro Pro Val Phe Asp Asp
145                 150                 155                 160

Ile Lys Phe Arg Met Met Asp Val Ala Trp Ser Pro Thr Thr Val Thr
                165                 170                 175

Thr Arg Lys Val Thr Tyr Gly Phe Thr Arg Ser Leu Arg Thr Asn Phe
            180                 185                 190

Ile Gly Asn Lys Arg Arg Trp Arg Tyr Arg His Arg Pro His Val Leu
        195                 200                 205

Trp Pro Arg Arg Arg Leu Ile Gln Gly Leu His Ser Arg Pro Arg His
    210                 215                 220

Arg Arg Arg Arg Tyr Arg Arg Arg Pro Tyr Thr Met
225                 230                 235


<210> SEQ ID NO 15
<211> LENGTH: 314
<212> TYPE: PRT
<213> ORGANISM: ALVAC

<400> SEQUENCE: 15

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  1               5                  10                  15

Trp Val Phe Thr Leu Asn Asn Pro Ser Glu Asp Glu Arg Lys Lys Ile
             20                  25                  30

Arg Glu Leu Pro Ile Ser Leu Phe Asp Tyr Phe Ile Val Gly Glu Glu
         35                  40                  45

Gly Asn Glu Glu Gly Arg Thr Pro His Leu Gln Gly Phe Ala Asn Phe
     50                  55                  60

Val Lys Lys Gln Thr Phe Asn Lys Val Lys Arg Tyr Leu Gly Ala Arg
 65                  70                  75                  80

Cys His Ile Glu Lys Ala Lys Gly Thr Asp Gln Gln Asn Lys Glu Tyr
                85                  90                  95

Cys Ser Lys Glu Gly Asn Leu Leu Ile Glu Cys Gly Ala Pro Arg Ser
            100                 105                 110

Gln Gly Gln Arg Ser Asp Leu Ser Thr Ala Val Ser Thr Leu Leu Glu
        115                 120                 125

Ser Gly Ser Leu Val Thr Val Ala Glu Gln His Pro Val Thr Phe Val
    130                 135                 140

Arg Asn Phe Arg Gly Leu Ala Glu Leu Leu Lys Val Ser Gly Lys Met
145                 150                 155                 160

Gln Lys Arg Asp Trp Lys Thr Asn Val His Val Ile Val Gly Pro Pro
                165                 170                 175

Gly Cys Gly Lys Ser Lys Trp Ala Ala Asn Phe Ala Asp Pro Glu Thr
            180                 185                 190

Thr Tyr Trp Lys Pro Pro Arg Asn Lys Trp Trp Asp Gly Tyr His Gly
        195                 200                 205
```

-continued

```
Glu Glu Val Val Val Ile Asp Asp Phe Tyr Gly Trp Leu Pro Trp Asn
    210                 215                 220

Asp Leu Leu Arg Leu Cys Asp Arg Tyr Pro Leu Thr Val Glu Thr Lys
225                 230                 235                 240

Gly Gly Thr Val Pro Phe Leu Ala Arg Ser Ile Leu Ile Thr Ser Asn
                245                 250                 255

Gln Thr Pro Leu Glu Trp Tyr Ser Ser Thr Ala Val Pro Ala Val Glu
            260                 265                 270

Ala Leu Tyr Arg Arg Ile Thr Ser Leu Val Phe Trp Lys Asn Ala Thr
        275                 280                 285

Glu Gln Ser Thr Glu Glu Gly Gly Gln Phe Val Thr Leu Ser Pro Pro
    290                 295                 300

Cys Pro Glu Phe Pro Tyr Glu Ile Asn Tyr
305                 310


<210> SEQ ID NO 16
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: ALVAC

<400> SEQUENCE: 16

catcatcatg atatccgtta agtttgtatc gtaatgacgt ggccaaggag gcg           53


<210> SEQ ID NO 17
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: ALVAC

<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: ALVAC

<400> SEQUENCE: 18

catcatcatt cgcgatatcc gttaagtttg tatcgtaatg ccaagcaaga aaagcgg       57


<210> SEQ ID NO 19
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: ALVAC

<400> SEQUENCE: 19

tactactacg tcgactcagt aatttatttt atatgg                             36
```

What is claimed is:

1. A recombinant poxvirus comprising an isolated DNA molecule selected from the group consisting of ORFs 1 to 13 of porcine circovirus type II, wherein the poxvirus is:

ALVA C, or

a poxvirus having all the identifying characteristics of ALVAC.

2. The recombinant poxvirus of claim **1** wherein the recombinant poxvirus is a recombinant ALVAC canarypox virus.

3. The recombinant poxvirus of claim **2** wherein the isolated DNA molecule is in the C6 locus of the ALVAC canarypox virus genome.

4. The recombinant poxvirus of claim **1** wherein the isolated DNA molecule comprises PCV2 ORF1, PCV2 ORF2, or PCV2 ORF1 and PCV2 ORF2.

5. The recombinant poxvirus of claim **4** wherein the isolated DNA molecule is in the C6 locus of the ALVAC canarypox virus genome.

6. An immunological composition comprising the recombinant poxvirus of any one of claims **1** to **3**, **4** or **5**, and a carrier.

7. The immunological composition of claim **6**, which contains an adjuvant.

8. The immunological composition of claim **7**, wherein the adjuvant is a carbomer.

9. A method of expressing a porcine circovirus ORF in a cell cultured in vitro comprising introducing into the cell the recombinant poxvirus as claimed in any one of claims **1** to **3**, **4** or **5** under conditions which allow expression of the isolated DNA molecule by the recombinant poxvirus.

10. A method for inducing an immunological response against porcine circovirus in a host capable of an immunological response against porcine circovirus comprising administering to the host the recombinant poxvirus of any one of claims **1** to **3**, **4** or **5**.

11. The method of claim **10** wherein the recombinant poxvirus is administered with an adjuvant.

12. The method of claim **11** wherein the adjuvant is a carbomer.

13. An immunological composition comprising a recombinant avipox virus comprising an isolated DNA molecule selected from the group consisting of ORFs 1 to 13 of porcine circovirus type II, and a carbomer adjuvant.

14. The immunological composition of claim **13** wherein the isolated DNA molecule encodes PCV2 ORF1, PCV2 ORF2, or PCV2 ORF1 and PCV2 ORF2.

15. The immunological composition of any one of claim **13** or **14** wherein the avipox virus is a canarypox virus.

16. A recombinant avipox virus comprising an isolated DNA molecule comprising porcine circovirus type II (PCV2) open reading frames 1 and 2 (ORFs 1 and 2).

17. The recombinant avipox virus of claim **16** which is a recombinant canarypox virus.

18. An immunological composition comprising the recombinant avipox virus of any one of claim **16** or **17** and a carrier.

19. A method of expressing a gene product in a cell cultured in vitro comprising introducing into the cell the recombinant avipox virus as claimed in any one of claim **16** or **17** under conditions which allow expression of the isolated DNA molecule by the recombinant avipox virus.

20. A method for inducing an immunological response against porcine circovirus in a host capable of an immunological response against porcine circovirus comprising administering to the host the recombinant avipox virus of any of claim **16** or **17**.

21. A method for inducing an immunological response against porcine circovirus in a host capable of an immunological response against porcine circovirus comprising administering to the host the composition of any one of claim **13** or **14**.

22. A method for inducing an immunological response against porcine circovirus in a host capable of an immunological response against porcine circovirus comprising administering to the host the composition of claim **15**.

23. The method of claim **10** wherein the host is a porcine.

24. The method of claim **20** wherein the host is a porcine.

25. The method of claim **21** wherein the host is a porcine.

26. The method of claim **22** wherein the host is a porcine.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,497,883 B1
DATED : December 24, 2002
INVENTOR(S) : Michel Bublot et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.
Item [73], Assignee, change "Lyons" to -- Lyon --.

Signed and Sealed this

Fourth Day of March, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Journal of
Clinical Microbiology

Genetic Characterization of Type 2 Porcine Circovirus (PCV-2) from Pigs with Postweaning Multisystemic Wasting Syndrome in Different Geographic Regions of North America and Development of a Differential PCR-Restriction Fragment Length Polymorphism Assay To Detect and Differentiate between Infections with PCV-1 and PCV-2

Martijn Fenaux, Patrick G. Halbur, Mike Gill, Thomas E. Toth and Xiang-Jin Meng
***J. Clin. Microbiol.* 2000, 38(7):2494.**

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0095-1137/00/$04.00+0

Genetic Characterization of Type 2 Porcine Circovirus (PCV-2) from Pigs with Postweaning Multisystemic Wasting Syndrome in Different Geographic Regions of North America and Development of a Differential PCR-Restriction Fragment Length Polymorphism Assay To Detect and Differentiate between Infections with PCV-1 and PCV-2

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Received 23 March 2000/Accepted 24 April 2000

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in swine. Increasing evidence indicates that a variant strain of porcine circovirus (PCV), designated type 2 PCV (PCV-2), is responsible for PMWS. To determine the extent of genetic heterogeneity of PCV-2 isolates, the complete genomes of six PCV-2 isolates from different regions of North America were amplified by PCR and sequenced. Sequence and phylogenetic analyses confirmed that two distinct genotypes of PCV exist: nonpathogenic genotype PCV-1 and PMWS-associated genotype PCV-2. However, within the PCV-2 genotype, several minor branches that have been identified appear to be associated with geographic origins. The genomic sequences of two French PCV-2 isolates diverge the most from those of other PCV-2 isolates and form a distinct branch. Other minor but distinguishable branches have also been identified for a Taiwan PCV-2 isolate and two of the Canadian PCV-2 isolates. All the U.S. PCV-2 isolates are closely related, but the Canadian isolates vary, to some extent, in their genomic sequences. The data from this study indicate that although the genome of PCV-2 is generally stable among different isolates, PCV-2 isolates from different geographic regions vary in their genomic sequences. This variation may have important implications for PCV-2 diagnosis and research. On the basis of genetic analyses of available PCV strains, a universal PCR-restriction fragment length polymorphism (PCR-RFLP) assay was developed to detect and differentiate between infections with PCV-1 and PCV-2. This PCR-RFLP assay should be useful for studying the pathogenesis of PCV-2, for detecting PCV-2 infection in pigs from different geographic regions, and for screening donor pigs for use in xenotransplantation.

Porcine circovirus (PCV) was originally isolated as a noncytopathic contaminant of the porcine kidney cell line PK15 (50). PCV is a small nonenveloped virus that contains a single-stranded circular DNA genome of about 1.76 kb (50). On the basis of its morphology and genomic organization, PCV was classified as a member of *Circoviridae* family (30, 36), which consists of two other animal circoviruses, chicken anemia virus (CAV) and psittacine beak-and-feather disease virus, and three plant circoviruses, banana bunchy top virus, coconut foliar decay virus, and subterranean clover stunt virus. Members of the three recognized animal circoviruses, PCV, CAV, and psittacine beak-and-feather disease virus, do not share nucleotide sequence homology or antigenic determinants with each other (8, 54). More recently, a human circovirus, designated TT virus (TTV), was identified from individuals with posttransfusion hepatitis (38, 43). The human TTV is similar to the circovirus CAV in its genomic organization (38). Although antibodies to PCV were found in various animal species including humans, mice, cattle, and pigs (11, 12, 22, 23, 41, 52, 53), little is known regarding the pathogenesis of PCV in these animal species. Experimental infection of pigs with the PK15-derived PCV did not produce clinical disease, and thus, this virus is not considered pathogenic for pigs (1, 51).

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in pigs first described in 1991 (10, 20). The disease occurs in swine herds that are usually in good health and has a low rate of morbidity but causes a relatively high case fatality rate among 5- to 12-week-old pigs (6, 10). Clinically, PMWS is characterized by progressive weight loss, dyspnea, tachypnea, anemia, diarrhea, and jaundice. In an acute outbreak, the mortality rate associated with PMWS may peak at about 10% and can reach up to 50% in some cases (6, 20, 21). The microscopic lesions of PMWS include those associated with granulomatous interstitial pneumonia, lymphadenopathy, hepatitis, nephritis, and pancreatitis (20, 21). PMWS has now been recognized in pigs in Canada and most of the United States (2, 3, 6, 13, 18, 26, 27, 37, 39, 40), many European countries (4, 6, 24, 32, 48, 49), and some countries in Asia (6, 44) and has the potential to have a serious economic impact on the swine industry worldwide.

The etiology of PMWS is rather complicated, but it is believed that a variant strain of PCV, designated type 2 PCV (PCV-2), is responsible for PMWS in pigs (2, 3, 4, 6, 13, 18, 37, 39). The nonpathogenic PK15-derived PCV has been desig-

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nated PCV-1 to distinguish it from the PMWS-associated PCV, PCV-2 (2, 3). PCV-2 was isolated from pigs with clinical and pathological findings consistent with PMWS (2, 3, 4, 6, 13, 19, 27, 39, 40). PCV-2 DNA and antigen were detected in various tissues and organs from pigs with natural cases of PMWS (19, 27, 32, 37, 39, 40, 46). The complete genome of PCV-2 has been determined, and surprisingly, nonpathogenic PCV-1 and PMWS-associated PCV-2 are found to share only about 75% nucleotide sequence identity (18, 37, 39). Seven open reading frames (ORFs) have been identified for PCV-1 (31, 33, 36), whereas 6 or 11 ORFs have been identified for PCV-2 (18, 37, 39). Although PMWS has been reported in most of the United States, only a few PCV-2 isolates from the United States have been genetically characterized (37, 39). On the basis of the nucleotide sequences of the U.S. and other PCV-2 isolates sequenced thus far, it appears that there exists only one genotype of PCV-2 worldwide (19, 37, 39). Nevertheless, the value of diagnosing PCV-2 infections and studying the pathogenesis of PCV-2 by PCR and other molecular approaches will depend on knowledge of the extent of genetic variation among PCV-2 isolates from different geographic regions. In addition, the development of an effective vaccine against PMWS also requires a better understanding of the extent of genetic variation among PCV-2 isolates. In this study, we genetically characterized six PCV-2 isolates from pigs with confirmed PMWS in different geographic regions of North America. The extent of genetic variation among these six PCV-2 isolates and all other known PCV isolates (both PCV-1 and PCV-2) was analyzed. On the basis of the data generated from this study, we further developed a universal PCR-restriction fragment length polymorphism (PCR-RFLP) assay to detect and differentiate between infections with PCV-1 and PCV-2 in pigs from different geographic regions.

MATERIALS AND METHODS

Sample sources. Various tissue samples (liver, spleen, tonsil, lymph nodes, etc.) were collected from pigs with PMWS as confirmed by immunohistochemistry (IHC) (data not shown). The tissues were stored at −80°C until use. The complete PCV-2 genome was amplified, sequenced, and characterized from tissue samples from six selected pigs with PMWS that originated from different geographic regions of North America: two from Utah, one from Missouri, one from Iowa, one from Illinois, and one from Canada (Table 1). The isolates from these six pigs with PMWS, along with those from four more pigs with PMWS (Table 1) from Iowa, were also characterized by PCR-RFLP analyses.

Isolation of DNA from tissues. DNA was extracted from various tissue samples with the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, Calif.) according to the protocol supplied by the manufacturer. For each DNA extraction, 25 mg of tissue sample was used. The resulting DNA was eluted in DNase-, RNase-, and proteinase-free water (Eppendorf 5 Primer, Inc., Boulder, Colo.).

PCR amplification of the complete genome of PCV-2. Two sets of PCR primers were designed on the basis of the published PCV-2 sequence. These primers amplify two overlapping fragments that represent the entire genome of PCV-2 (Fig. 1). The first set of primers, CV1 and CV2 (Table 2), amplifies a 989-bp fragment, and the second set of primers, CV3 and CV4 (Table 2), amplifies a 1,092-bp fragment. The extracted DNA was amplified by PCR with AmpliTaq Gold polymerase (Perkin-Elmer, Norwalk, Conn.). The PCR consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min, followed by a terminal extension at 72°C for 7 min.

Nucleotide sequencing and sequence and phylogenetic analyses. The PCR products of the expected sizes were purified by electrophoresis on a 1% agarose gel, followed by extraction with a Geneclean Kit (Bio 101, Inc., La Jolla, Calif.). Both strands were sequenced with a variety of sequencing primers (Table 2) with an ABI automated DNA sequencer at the Virginia Polytechnic Institute and State University DNA Sequencing Facility. The sequences of the primers used to sequence the complete genome of PCV-2 are listed in Table 2, and their relative positions in the circular genome are indicated in Fig. 1. The sequences were compiled and analyzed with the MacVector program (Oxford Molecular Ltd., Beaverton, Oreg.). The percentages of sequence identity among different PCV isolates were determined with the Clustal alignment program in the MacVector package. Sequence alignments were performed with the ALIGN program in the MacVector package. Phylogenetic analyses were conducted with the aid of the PAUP program (from David L. Swofford, Smithsonian Institution, Washington, D.C., and distributed by Sinauer Associates, Inc., Sunderland, Mass.). The branch-and-bound searching and midpoint rooting options were used to produce a consensus tree.

Development of a PCR-RFLP assay. A PCR-RFLP assay was developed to differentiate between strains of PCV-1 and PCV-2 infecting the pigs. Briefly, the complete sequences of the six PCV-2 isolates from this study and the complete sequences of all other PCV sequences available in GenBank (those of both PCV-1 and PCV-2) were aligned with the Clustal program (data not shown). On the basis of this alignment, a set of conserved PCR primers (primers MCV1 and MCV2; Table 2) was designed to amplify a fragment of 243 bp from samples that contained either PCV-1 or PCV-2, or both. The sequences of the two PCR primers chosen from the sequences of PCV-1 and PCV-2 isolates are identical for all known PCV-1 and PCV-2 isolates including the six PCV-2 isolates sequenced in this study (Fig. 2). The PCR consisted of 37 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1.5 min. The amplified PCR products were subsequently digested with a unique restriction enzyme, *Nco*I, which is present in all PCV-2 isolates but not in PCV-1 isolates (Fig. 2). The digested PCR products are separated on a 2% agarose gel for RFLP analysis.

Nucleotide sequence accession numbers. The complete genomic sequences of the six PCV-2 isolates reported in this paper have been deposited with the GenBank database under accession numbers AF264038, AF264039, AF264040, AF264041, AF264042, and AF264043.

RESULTS

Genetic characterization of PCV-2 isolates from pigs with PMWS in different geographic regions. To determine the extent of genetic heterogeneity among PCV-2 isolates, the complete genome of PCV-2 was amplified and sequenced from one pig with PMWS in Canada (isolate 34464) and five pigs with PMWS in the United States: two from Utah (isolates 26606 and 26607), one from Missouri (isolate 40856), one from Iowa (isolate 40895), and one from Illinois (isolate 10489). The pigs with PMWS included in this study possessed clinical signs consistent with PMWS (Table 1) and were confirmed to be positive for PCV-2 antigen by IHC (data not shown). All six pigs with PMWS included in the study were negative for swine influenza virus, but four of the six pigs were found to be positive for porcine reproductive and respiratory syndrome virus (PRRSV) antigen (Table 1).

The lengths of the genomic DNAs of the PCV-1 isolates ranged from 1,758 to 1,760 bp. Sequence analyses of the complete genomes of six PCV-2 isolates from this study showed that, like all other PCV-2 isolates, the complete genome of each of the six PCV-2 isolates is 1,768 bp in length. All the PCV-2 isolates sequenced are closely related to each other, displaying 95 to 99% nucleotide sequence identities (Table 3). Two French PCV-2 isolates, isolates AF055393 and AF055394, displayed the most sequence divergence from the other PCV-2 isolates, with the identities ranging from 95 to 96%. Similarly, the four PCV-1 isolates sequenced thus far (isolates AF071879, Y09921, U49186, and AF012107) are closely related to each other, and their entire genomes share 98 to 99% nucleotide sequence identity (Table 3). Moreover, the nucleotide sequence identity between the entire genomes of PCV-1 and PCV-2 is only about 75 to 77%.

ORF2 of PCV is believed to code for the putative capsid protein (31, 33, 42). Sequence analysis indicated that the ORF2 genes of PCV-1 isolates encode a protein of 230 to 231 amino acid residues, whereas the ORF2 genes of PCV-2 isolates encodes a protein of 233 amino acid residues (Fig. 3). Pairwise sequence comparisons revealed that the ORF2 genes of all PCV-2 isolates shared 91 to 100% nucleotide sequence identity and 90 to 100% amino acid sequence identity (Table 3). The two French isolates, isolates AF055393 and AF055394, have only about 90 to 93% nucleotide sequence identity with the other PCV-2 isolates (Table 3). The ORF2 genes of the four PCV-1 isolates share 97 to 99% nucleotide sequence identity and 94 to 98% amino acid sequence identity. Between the ORF2 genes of PCV-1 and PCV-2 isolates, there exists

TABLE 1. PCV isolates used in this study as well as those reported previously

Type	Isolate no.	Geographic location	IHC		ISH[a] of PCV	Clinical sign(s)[b]	Histopathological lesions[c]	Reference or source
			PRRSV	PCV				
PCV-2	26606[d]	Utah	+	ND[e]	+	Resp	Pneumonia, lymphoid depletion, enteritis	This study
	26607[d]	Utah	+	ND	+	Resp, diarrhea	Pneumonia, enteritis, hepatitis, nephritis	This study
	40856[d]	Missouri	+	+	ND	Resp, wasting	Pneumonia, lymphoid depletion, hepatitis, nephritis	This study
	40895[d]	Iowa	−	+	ND	Resp, wasting	Pneumonia, lymphoid depletion	This study
	34464[d]	Canada	+	+	ND	Resp	Pneumonia	This study
	10489[d]	Illinois	−	+	ND	Resp, wasting	Pneumonia, lymphoid depletion	This study
	38835	Iowa	+	+	ND	Resp	Pneumonia, lymphoid depletion	This study
	36688	Iowa	+	+	ND	Resp, wasting, dermatitis	Pneumonia, lymphoid depletion, nephritis, dermatitis	This study
	40860	Iowa	+	+	ND	Resp	Pneumonia	This study
	40887	Iowa	+	+	ND	Resp	Pneumonia	This study
	AF055391	California						37
	AF027217	California						19
	AF109397	France[f]						29, GenBank
	AJ223185	Iowa						39
	AF055394	France						37
	AF085695	Canada						GenBank
	AF086836	Canada						GenBank
	AF086835	Canada						GenBank
	AF086834	Canada						GenBank
	AF112862	Canada						GenBank
	AF166528	Taiwan						GenBank
	AF109399	Canada						GenBank
	AF109398	Canada						GenBank
	AF117753	Canada						GenBank
	AF055393	France						37
	AF055392	Canada						37
PCV-1	AF071879	PK15 cell						42
	Y09921	Germany						31
	U49186	Ireland						36
	AF012107	France						31

[a] ISH, in situ hybridization.
[b] Clinical signs: Resp, respiratory disease; wasting, anorexia and weight loss.
[c] Histopathological lesions: pneumonia, interstitial pneumonia.
[d] PCV-2 isolates sequenced in this study.
[e] ND, not determined.
[f] Bovine isolate.

only 65 to 67% nucleotide sequence identity and 63 to 68% amino acid sequence identity (Table 3). However, sequence analysis revealed that the N-terminal region of ORF2 is very rich in basic amino acid residues (arginine and lysine) and is highly conserved among PCV isolates, both PCV-1 and PCV-2 isolates (Fig. 3).

Phylogenetic analysis of PCV-1 and PCV-2 isolates from different geographic regions worldwide. To gain a better understanding of the genetic relationship and evolution of PCV, phylogenetic analyses were performed on the basis of the complete genomic sequences of 26 PCV isolates (both PCV-1 and PCV-2) worldwide, including the six North American PCV-2 isolates sequenced in this study (Fig. 4). These sequences either were published (18, 19, 31, 32, 33, 36, 37, 39, 41, 42, 54) or are available in GenBank (Table 1). Phylogenetic analysis confirmed that two distinct genotypes of PCV exist: PCV-1 and PCV-2 (Fig. 4). All 22 PCV-2 isolates are clustered together and form one distinct branch. Similarly, all four PCV-1 isolates are closely related and form another branch. Within the major genotype of PCV-2, a few minor branches were identified, and some of these minor branches appear to be associated with the geographic origins of the isolates. All the PCV-2 isolates that were from different geographic regions of the United States and that were sequenced in this study are grouped closely with other U.S. and most of the Canadian PCV-2 isolates (Fig. 4). Canadian isolate 34464, sequenced in this study, is closely related to another Canadian isolate, 109399, but is less related to the U.S. and other Canadian isolates. Two other Canadian isolates, AF109398 and AF117753, form a distinguishable branch and are distantly related to other Canadian and U.S. isolates. An isolate of PCV-2 from Taiwan, AF166526, is clustered with the North American PCV-2 isolates but forms a single minor branch. The two French isolates of PCV-2, AF055393 and AF055394, are closely related to each other but diverge the most from North American PCV-2 isolates. Interestingly, a bovine isolate of circovirus is most closely related to the U.S. isolates of PCV-2.

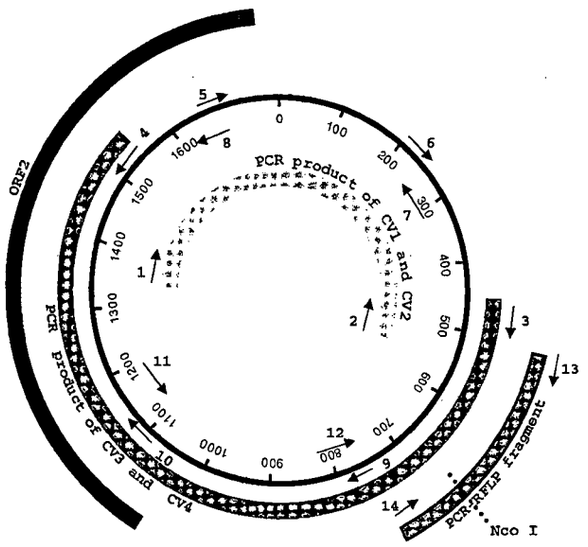

FIG. 1. Genome organization of PCV-2. The origin of replication (0), the putative capsid gene (ORF2), the PCR-RFLP fragment, and the two overlapping PCR fragments used to determine the complete genome of PCV-2 are indicated in the circular map. The relative positions of the oligonucleotide primers used in this study are indicated by arrows with respective numbers: 1, CV1; 2, CV2; 3, CV3; 4, CV4; 5, CV1-1; 6, CV1-2; 7, CV2-1; 8, CV2-2; 9, CV3-1; 10, CV3-2; 11, CV4-1; 12, CV4-2; 13, MCV1; 14, MCV2. The sequences and designations of these primers are listed in Table 2.

Development of a PCR-RFLP assay to diagnose PCV-2 infection and to differentiate between PCV-1 and PCV-2 infections. On the basis of the sequence alignments of all PCV-1 and PCV-2 isolates sequenced thus far, a set of consensus PCR primers was selected from two conserved regions of the PCV genome to amplify a fragment of 243 bp for both PCV-1 and PCV-2 isolates (Fig. 5A). To test the feasibility of using these primers for the detection of both PCV-1 and PCV-2 isolates in clinical samples, DNA was extracted from tissue samples from the six pigs with PMWS. The PCV-2 genomic sequences from these samples have been determined. DNA was also extracted from tissue samples from four additional pigs with PMWS from Iowa (Table 1), but the PCV-2 sequences in samples from these four pigs with PMWS have not been determined. DNA extracted from the PK15 cell line (ATCC CCL-33) was used as the source of PCV-1 DNA. DNA extracted from a sample of liver tissue collected from a specific-pathogen-free pig was used as a negative control. We were able to amplify an expected fragment of the PCV genome from tissue samples from all 10 pigs with PMWS as well as from the PCV-1-contaminated PK15 cells. By using an unique restriction enzyme site (*Nco*I) that is present only in the sequences of PCV-2 isolates (Fig. 2), a PCR-RFLP assay was developed to differentiate between infections with PCV-1 and PCV-2. After digestion of the PCR products with *Nco*I, the resulting RFLP patterns revealed that all products amplified from PCV-2 isolates produced two fragments of 168 and 75 bp, whereas the PCR product amplified from PCV-1 produced only the undigested fragment of 243 bp (Fig. 5). DNA extracted from a sample that contained both PCV-2 (from pig 40860) and PCV-1 (PK15 cells) was also subjected to PCR amplification. After digestion with the *Nco*I restriction enzyme, the PCR product amplified from the sample with both PCV-1 and PCV-2 produced three fragments of 243, 168, and 75 bp, respectively (Fig. 5B). Thus, this PCR-RFLP assay is able to detect PCV-1 and PCV-2 in clinical samples with potential dual infection with PCV-1 and PCV-2.

DISCUSSION

PMWS is a new and unique disease of swine. Since the first recognition of the disease in 1991 (10, 20), PMWS has emerged to be an economically important global disease of swine (2, 3, 4, 6, 13, 18, 24, 26, 27, 32, 37, 39, 40, 44, 48, 49). The clinical and pathological presentations and etiology of PMWS are very complicated (6, 10, 16, 20, 21, 22, 46, 47). Many known swine pathogens such as PRRSV, swine influenza virus, hemagglutinating encephalomyocarditis virus, the bacterium that causes porcine proliferative enteropathy, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, the bacterium that causes postweaning colibacillosis, etc., could cause postweaning wasting of pigs (21). However, increasingly, data indicate that PCV-2 is the causative agent of PMWS (2, 3, 4, 6, 13, 18, 37, 39). Experimental inoculation of conventional pigs with homogenates of tissue from pigs with clinical PMWS produced PMWS-like lesions, and PCV-2 DNA and antibody to PCV-2 were detected in the inoculated pigs (7). Ellis et al. (14) experimentally inoculated neonatal gnotobiotic piglets with filtered tissue culture materials and homogenates of tissue from PMWS-affected pigs. The inoculated gnotobiotic piglets devel-

TABLE 2. Oligonucleotide primers used in the study

Primer	Primer sequence	Application	Position[a]
CV1	5′-AGGGCTGTGGCCTTTGTTAC-3′	PCR, Seq[b]	1336–1355
CV2	5′-TCTTCCAATCACGCTTCTGC-3′	PCR, Seq	536–556
CV3	5′-TGGTGACCGTTGCAGAGCAG-3′	PCR, Seq	452–471
CV4	5′-TGGGCGGTGGACATGATGAG-3′	PCR, Seq	1525–1544
CV1-1	5′-GAGGATCTGGCCAAGATGGCTG-3′	Seq	1674–1695
CV1-2	5′-AGGACGAACACCTCACCTCCAG-3′	Seq	213–234
CV2-1	5′-GCAGCGGGCACCCAAATACCAC-3′	Seq	279–300
CV2-2	5′-ACGTATCCAAGGAGGCGTTACC-3′	Seq	1718–1739
CV3-1	5′-AGACTAAAGGTGGAACTGTACC-3′	Seq	770–791
CV3-2	5′-TTGTACATACATGGTTACACGG-3′	Seq	1083–1104
CV4-1	5′-TGTGGACCACGTAGGCCTCGGC-3′	Seq	1146–1167
CV4-2	5′-TGGTAATCAGAATACTGCGGGC-3′	Seq	799–820
MCV1	5′-GCTGAACTTTTGAAAGTGAGCGGG-3′	PCR-RFLP	508–531
MCV2	5′-TCACACAGTCTCAGTAGATCATCCCA-3′	PCR-RFLP	724–749

[a] The relative positions of these oligonucleotide primers are indicated in Fig. 1.
[b] Seq, primers used for DNA sequencing to determine the complete genome of PCV-2.

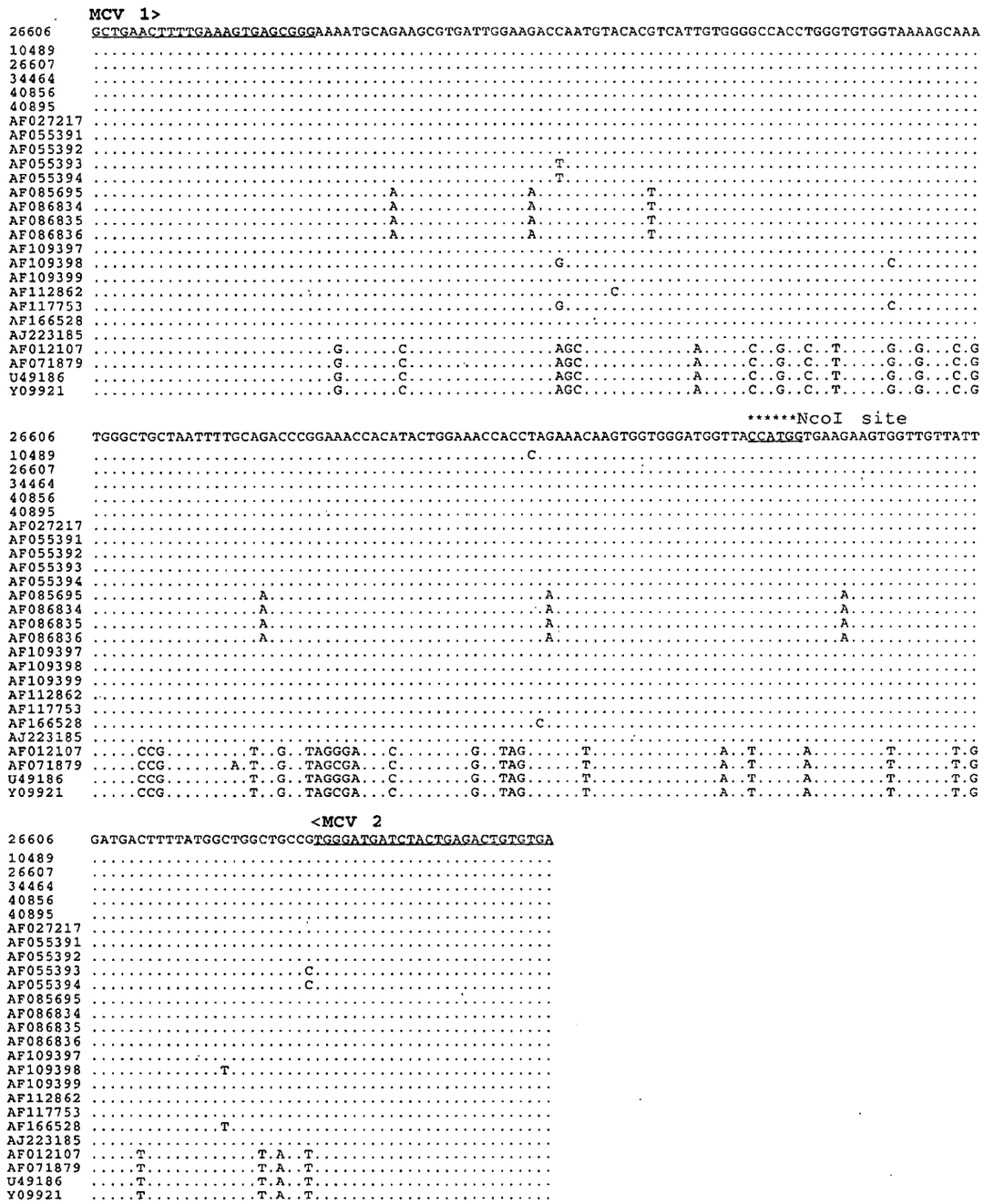

FIG. 2. Nucleotide sequence alignment of the region amplified in the PCR-RFLP assay. The regions from which the consensus PCR primers (MCV1 and MCV2) were chosen are underlined. The unique *Nco*I restriction enzyme site that is present in all PCV-2 isolates is indicated by asterisks. The sequence of PCV-2 isolate 26606 from this study is shown on top, and only differences from that sequence are indicated for the other isolates. The sequences used in the alignment are cited in the text.

oped lesions typical of PMWS, but the study was complicated by the detection of porcine parvovirus (PPV) in inoculated piglets. In fact, coinfection with PPV and PCV in pigs with naturally acquired PMWS has been reported (16). It has also been shown that PCV-2 alone induced PMWS lesions in colostrum-deprived conventional pigs but that concurrent infection with PPV increased the severity of the lesions (5, 25), suggesting that PMWS is a complex disease syndrome and that multiple factors may be involved in the pathogenesis of PMWS. It has been suspected that some of the clinical signs and pathological lesions attributable to PRRSV may actually be induced by PCV-2 as a result of PCV-2 infection or coinfection (15, 27). Synergism between a circovirus (CAV) and a reovirus was observed following dual infection of chickens by a natural route (34). In the present study, PCV-2 was readily detectable from pigs with PMWS in different regions of the

TABLE 3. Pairwise comparison of the complete genomic and putative capsid gene (ORF2) sequences of PCV-1 and PCV-2

Isolate	% Sequence identity[a]																								
	26606	10489	26607	40895	34464	40856	AF085695	AF086834	AF086835	AF086836	AF109398	AF109399	AF112862	AF117753	AF027217	AF055391	AF055392	AF055393	AF055394	AF109397	AF166528	AJ223185	**AF012107**	**AF071879**	**U49186**	**YO9921**
26606		98/96	99/99	98/95	96/95	99/98	98/97	98/96	98/97	98/97	93/93	94/93	97/95	93/93	98/95	98/95	98/96	92/92	92/92	97/95	95/95	98/96	66/66	65/64	65/65	65/65
10489	99		98/97	99/98	97/97	98/96	98/98	99/100	99/98	98/98	94/94	95/95	98/98	93/93	99/98	99/98	98/98	92/93	92/93	99/98	96/98	99/99	67/68	66/66	66/66	66/66
26607	99	99		98/96	96/95	99/98	98/97	98/97	99/97	98/97	93/93	94/93	97/95	93/93	98/96	98/96	98/97	92/92	92/92	98/96	95/95	98/96	66/66	65/64	66/65	66/65
40895	99	99	99		97/97	98/95	98/96	99/98	98/97	98/96	94/95	95/95	98/96	93/93	99/99	99/99	98/96	92/93	92/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
34464	98	98	98	98		96/95	96/95	97/97	97/96	96/95	94/93	97/97	96/95	93/92	97/96	97/96	96/95	92/91	92/91	97/96	96/96	97/97	66/67	65/65	65/66	65/66
40856	99	99	99	98	98		98/97	98/96	98/97	98/97	93/93	94/93	97/95	92/92	98/95	98/95	98/96	92/92	92/92	97/95	95/95	98/96	66/66	65/63	65/64	65/64
AF085695	98	98	98	98	97	98		99/98	99/99	100/100	94/94	94/93	97/96	93/93	98/96	98/96	99/99	92/93	92/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF086834	98	98	98	98	97	98	99		99/98	99/98	94/94	95/95	98/98	93/93	99/98	99/98	99/98	92/93	93/93	99/98	96/98	99/99	67/68	66/66	66/66	66/66
AF086835	98	98	98	98	97	98	99	99		99/99	94/95	94/93	98/96	93/93	98/97	98/97	99/99	93/93	93/93	98/97	96/97	98/98	67/67	66/65	66/66	66/66
AF086836	98	98	98	98	97	98	99	98	99		94/94	94/93	97/96	93/93	98/96	98/96	99/99	92/93	92/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF109398	96	96	96	96	96	96	95	95	95	95		93/92	94/94	97/96	94/95	94/95	94/94	93/93	93/93	95/96	93/94	94/95	67/67	66/65	66/66	66/66
AF109399	97	97	97	97	98	97	96	96	96	96	96		94/93	93/91	95/94	95/94	94/93	91/90	91/90	95/94	94/94	95/95	66/65	65/63	65/63	65/63
AF112862	98	99	98	99	98	98	98	98	98	97	96	97		93/92	98/96	98/96	98/96	92/92	92/92	98/97	95/96	98/97	67/67	66/65	66/66	66/66
AF117753	96	96	96	96	96	95	95	95	95	95	97	96	96		93/93	93/93	93/93	91/91	92/91	93/93	93/93	93/93	66/66	65/64	65/65	65/65
AF027217	99	99	99	99	98	99	98	98	98	98	96	97	99	96		99/99	98/96	93/93	93/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
AF055391	99	99	99	99	98	99	98	98	98	98	96	97	99	96	99		98/96	93/93	93/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
AF055392	99	99	99	99	98	99	99	98	98	98	96	97	99	96	99	99		93/93	93/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF055393	95	95	95	95	95	95	95	95	95	95	95	95	95	95	96	96	96		99/99	92/93	93/93	92/93	67/68	66/66	66/66	66/66
AF055394	95	96	95	96	95	95	95	95	95	95	95	95	95	95	96	96	96	99		92/92	93/93	92/93	67/68	66/66	66/66	66/66
AF109397	99	99	99	99	98	99	98	98	98	98	97	97	99	96	99	99	99	95	95		96/97	99/99	67/68	66/66	66/66	66/66
AF166528	97	97	97	97	97	97	96	97	96	97	96	96	97	95	97	97	97	96	96	97		96/98	66/67	65/65	65/66	65/66
AJ223185	99	99	99	99	98	98	98	98	98	98	96	97	99	96	99	99	99	95	95	99	97		66/68	66/66	66/66	66/66
AF012107	76	77	76	76	76	76	76	76	76	76	76	76	76	76	76	76	76	77	77	77	76	76		97/95	97/96	97/94
AF071879	76	76	76	76	76	76	76	75	76	76	76	76	76	75	76	76	76	76	76	76	76	76	98		99/98	98/95
U49186	76	76	76	76	76	76	76	76	76	76	76	76	76	75	76	76	76	76	77	76	76	76	98	99		98/96
YO9921	76	76	76	76	76	76	76	76	76	76	76	76	76	75	76	76	76	76	76	76	76	76	98	99	99	

[a] The values in the table are percent identity of amino acid or nucleotide sequences. The percent nucleotide sequence identities of the complete genomes are presented in the lower left half. The four PCV-1 isolates (AF012107, AF071879, U49186, YO9921) are highlighted in boldface type. The nucleotide/amino acid sequence identities of the putative capsid (ORF2) gene are shown at the upper right.

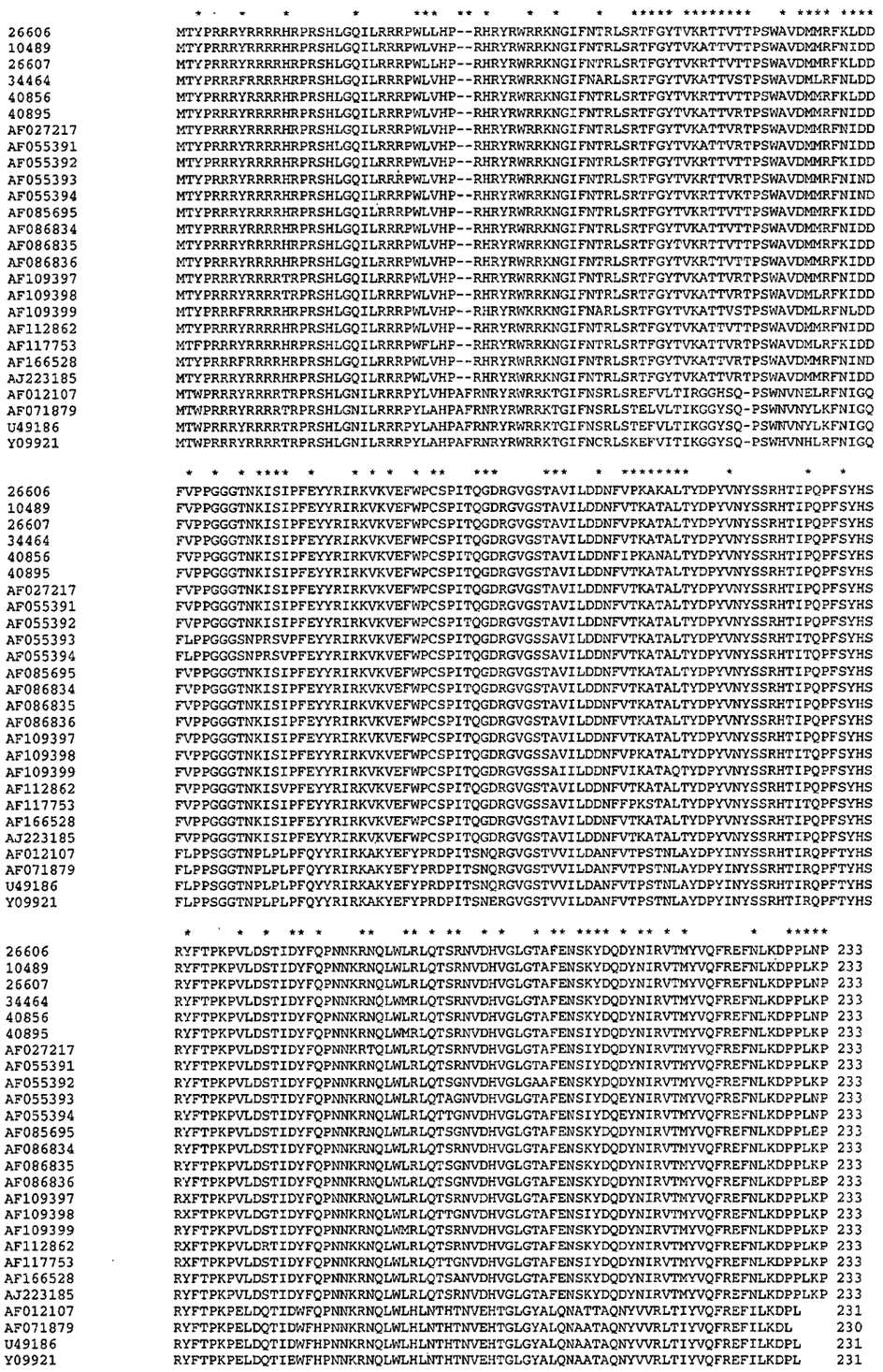

FIG. 3. Amino acid sequence alignment of the putative capsid protein (ORF2) of PCV-1 and PCV-2 isolates sequenced thus far. Deletions are indicated by hyphens. Amino acid sequence differences are indicated with asterisks above the alignment. The sequences used in the alignment are cited in the text.

North America, but PRRSV antigen was also detected in most of the pigs with PMWS (Table 1). The etiological significance of PCV-2 in PMWS and its interrelationship with PRRSV, PPV, or other agents need to be further studied.

The extent of genetic variation of PCV-2 isolated from different geographic regions of the United States is not known since only a few PCV-2 isolates from the United States have been genetically characterized (37, 39). In this study, we ge-

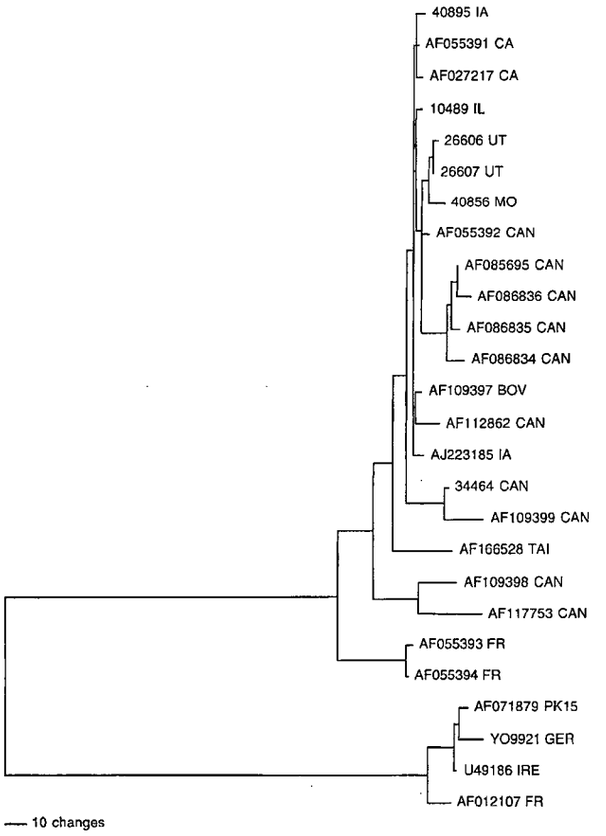

FIG. 4. Phylogenetic tree based on the complete genomic nucleotide sequences of all PCV isolates. The tree was constructed with the aid of the PAUP program. Branch-and-bound searching and midpoint rooting options were used to produce a consensus tree. A scale bar that represents the numbers of character-state changes is shown. Branch lengths are proportional to the numbers of character-state changes. The geographic locations of the isolates are also indicated with the usual state abbreviations and the following country abbreviations: CAN, Canada; TAI, Taiwan; FR, France; GER, Germany; IRE, Ireland. BOV, bovine.

netically characterized six North American isolates of PCV-2 (one Canadian isolate and five U.S. isolates) from pigs with PMWS from different geographic regions. Sequence analysis of the complete genome and of the putative capsid gene ORF2 indicated that these six North American isolates of PCV-2 are closely related to other known PCV-2 isolates worldwide. The putative capsid gene (ORF2) of PCV is highly variable, and the ORF2 genes of certain PCV-2 isolates share as little as 90% sequence identity. Despite the overall heterogeneic nature of ORF2, the N-terminal region of ORF2 among all PCVs is highly conserved and possesses a high percentage of basic amino acids, suggesting that the amino-terminal region of the putative capsid protein may have DNA binding activity and may be in contact with the PCV DNA in the native virion (42). Phylogenetic analysis revealed that all PCV-2 isolates sequenced thus far form a major genotype, whereas all PCV-1 isolates are closely related and form another genotype. On the basis of phylogenetic analysis, it is evident that both PCV-1 and PCV-2 evolved from the same ancestor, but they may have undergone divergent evolution. Gibbs and Weiller (17) analyzed the genomes of circoviruses and plant nanoviruses and showed that circoviruses most likely evolved from a plant nanovirus. It is believed that the plant nanovirus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus (17). Within the major genotype of PCV-2, several minor branches were also identified. The two French PCV-2 isolates (isolates AF055393 and AF055394) diverge the most from all other PCV-2 isolates. The clinical significance of this divergence is not known. LeCann et al. (29) reported that a PCV-1-like virus was isolated from pigs with wasting disease in France, but they failed to experimentally reproduce the disease with this isolate. Phylogenetically, all the U.S. PCV-2 isolates sequenced thus far are closely related. However, genetic variation was observed among the Canadian PCV-2 isolates. Two of the Canadian isolates, isolates AF109398 and AF117753, form a minor branch that is separate from other Canadian or U.S. isolates. Two other Canadian isolates (isolate 34464, sequenced in this study, and isolate AF109399) also differ phylogenetically from the U.S. and other Canadian PCV-2 isolates. A PCV-2 isolate from Taiwan (isolate AF116528) also forms a distinguishable minor branch. The origin of the bovine circovirus isolate is not known, but its close genetic relatedness to PCV-2 suggested that the bovine circovirus may be of swine origin and that cross-species infection of PCV between bovines and swine is possible. These data suggest that although the genome of PCV-2 is relatively stable in general, minor genetic differences do exist among PCV-2 isolates from different geographic regions. This observed difference might have important implications for the diagnosis of PCV-2 infection by nucleic acid-based assays such as PCR. Genetic characterization of additional PCV-2 isolates from other geographic regions worldwide is warranted.

Since PCV-1 is nonpathogenic and widespread in pig populations (6, 11, 12, 22, 23, 51, 52, 53), a test is needed to differentiate between infections with PCV-1 and PCV-2. In addition, since PCV antibody has been detected in humans (53), a major and growing concern is the inadvertent transmission of PCV from pig organs to human recipients during xenotransplantation. In xenotransplantation, there is zero tolerance for circovirus infection, regardless of its pathogenic potential, since nonpathogenic PCV-1 may become pathogenic in immunocomprised xenograft recipients. Therefore, sensitive and easy-to-perform assays are needed to screen for both PCV-1 and PCV-2 infection in xenograft donor pigs. Several techniques such as PCR (19, 27, 28, 39, 40, 45), IHC (35, 39, 46), and in situ hybridization (9, 35, 39, 46) are available for the detection of PCV-2 infection; however, the ability of these tests to detect PCV-2 isolates from different geographic regions is not known. The data from this study suggest that the genomic sequences of PCV-2 isolates from different geographic regions vary to some extent. Thus, a universal and more sensitive PCR assay that can detect PCV isolates from various geographic regions is needed. On the basis of genetic analyses of all PCV isolates, we developed a universal PCR-RFLP assay for the diagnosis of PCV-2 infection and differentiation between infections with PCV-1 and PCV-2 in pigs. This assay uses a pair of PCR primers selected from two conserved regions of the PCV-1 and PCV-2 genomes and a unique *Nco*I restriction enzyme site that exists only in PCV-2 isolates. The feasibility of this PCR-RFLP assay to detect PCV-2 and to differentiate between PCV-2 and PCV-1 was validated by using clinical samples from pigs from different geographic regions with confirmed cases of PMWS and a sample that intentionally contained both PCV-1 and PCV-2. Our results indicate that this PCR-RFLP assay is accurate and fast in diagnosing PCV-2 infection in pigs with PMWS from different geographic regions, in differentiating PCV-1 and PCV-2 infections, and in detecting dual infection with PCV-1 and PCV-2. This universal PCR-RFLP assay should help clinicians diagnose cases of

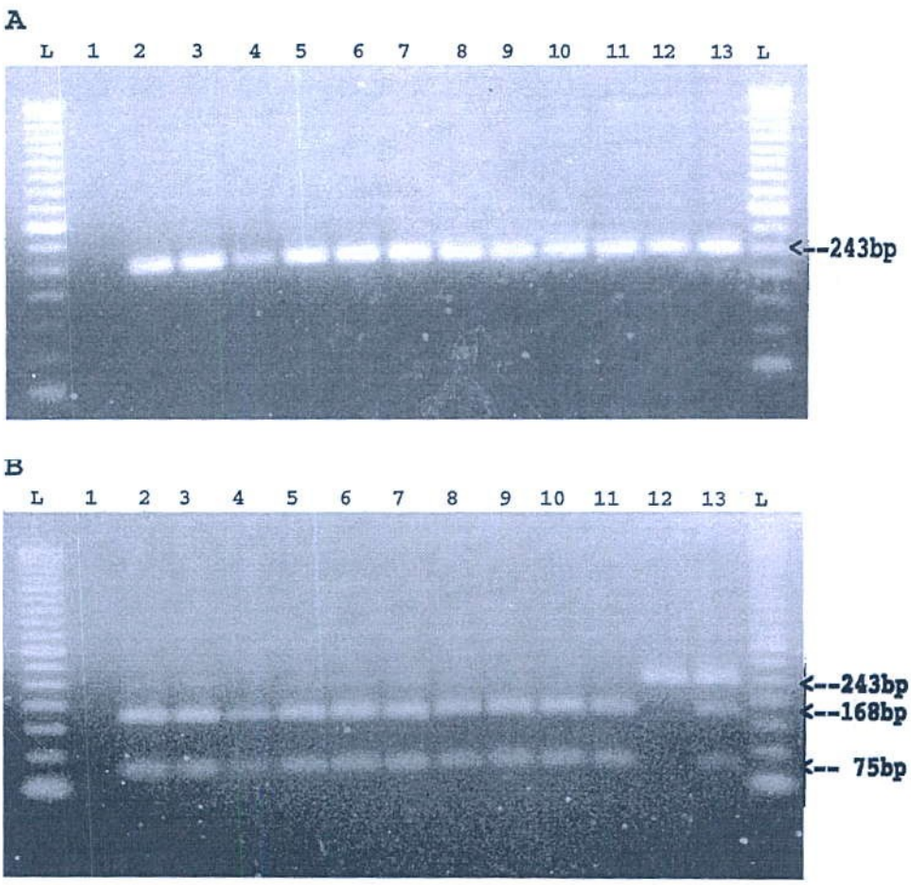

FIG. 5. Detection and differentiation of PCV infections by a PCR-RFLP assay. (A) Results of PCR amplification of a 243-bp fragment from tissue samples that contained PCV isolates (both PCV-1 and PCV-2) but not from a negative control liver tissue sample (lane 1). (B) Results of RFLP analysis of the PCR products. Lane L, 50-bp DNA ladder; lane 1, a sample of liver tissue from a control specific-pathogen-free pig; lanes 2 to 11, tissue samples from 10 pigs with PMWS, respectively; lane 12, PK15 cells containing PCV-1; lane 13, a sample containing both PCV-1 and PCV-2. The expected PCR fragment (A) and three RFLP fragments of 243, 168, and 75 bp, respectively (B), are indicated with arrows.

PMWS associated with PCV-2 infection in different geographic regions of the world and should also be useful for the screening of xenograft donor pigs to ascertain that they are free of circovirus infection.

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Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
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Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
                165                 170                 175

Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg Leu Gln Thr Ser Arg Asn
            180                 185                 190

Val Asp His Val Gly Leu Gly Thr Ala Phe Glu Asn Ser Lys Tyr Asp
        195                 200                 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
    210                 215                 220

Asn Leu Lys Asp Pro Pro Leu Glu Pro
225                 230
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<210> 7
<211> 756
<212> DNA
<213> Artificial

<220>
<223> This sequence is from porcine circovirus type 2, open reading frame 2, together with a portion from the pGEM T-easy vector.

<400> 7
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caccgccccc gcagccatct tggccagatc ctccgccgcc gcccctggct cgtccacccc 120

cgccaccgct accgttggag aaggaaaaat ggcatcttca acacccgcct ctcccgcacc 180

ttcggatata ctgtcaaggc taccacagtc acaacgccct cctgggcggt ggacatgatg 240

agatttaata ttgacgactt tgttcccccg ggaggggggga ccaacaaaat ctctataccc 300

tttgaatact acagaataag aaaggttaag gttgaattct ggccctgctc ccccatcacc 360

cagggtgata ggggagtggg ctccactgct gttattctag atgataactt tgtaacaaag 420

gccacagccc taacctatga cccatatgta aactactcct cccgccatac aatcccccaa 480

cccttctcct accactcccg ttacttcaca cccaaacctg ttcttgactc cactattgat 540

tacttccaac caaataacaa aaggaatcag ctttggctga ggctacaaac ctctagaaat 600

gtggaccacg taggcctcgg cactgcgttc gaaaacagta aatacgacca ggactacaat 660

atccgtgtaa ccatgtatgt acaattcaga gaatttaatc ttaaagaccc cccacttgaa 720

ccctaagaat tctatcacta gtgaattcgc ggccgc 756

<210> 8
<211> 10387
<212> DNA
<213> Artificial

<220>
<223> This is the porcine circovirus type 2, ORF2 construct, which includes baculovirus and pGEM T-easy coding sequences.

<400> 8
aagctttact cgtaaagcga gttgaaggat catatttagt tgcgtttatg agataagatt 60

gaaagcacgt gtaaaatgtt tcccgcgcgt tggcacaact atttacaatg cggccaagtt 120

ataaaagatt ctaatctgat atgttttaaa acacctttgc ggcccgagtt gtttgcgtac 180

gtgactagcg aagaagatgt gtggaccgca gaacagatag taaaacaaaa ccctagtatt 240

ggagcaataa tcgatttaac caacacgtct aaatattatg atggtgtgca ttttttgcgg 300

gcgggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc tgaaagcata 360

gttcaagaat ttattgacac ggtaaaagaa tttacagaaa agtgtcccgg catgttggtg 420

ggcgtgcact gcacacacgg tattaatcgc accggttaca tggtgtgcag atatttaatg 480

cacaccctgg gtattgcgcc gcaggaagcc atagatagat tcgaaaaagc cagaggtcac 540

aaaattgaaa gacaaaatta cgttcaagat ttattaattt aattaatatt atttgcattc 600

tttaacaaat actttatcct attttcaaat tgttgcgctt cttccagcga accaaaacta 660

tgcttcgctt gctccgttta gcttgtagcc gatcagtggc gttgttccaa tcgacggtag 720

gattaggccg gatattctcc accacaatgt tggcaacgtt gatgttacgt ttatgctttt 780

ggttttccac gtacgtcttt tggccggtaa tagccgtaaa cgtagtgccg tcgcgcgtca 840

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<210> 9
<211> 20
<212> PRT
<213> Porcine circovirus

<400> 9

Ser Tyr Pro Arg Arg Arg Tyr Arg Arg Arg Arg His His Pro Pro Ser
1 5 10 15

His Leu Gly Gln
20

<210> 10
<211> 19
<212> PRT
<213> Porcine circovirus

<400> 10

Pro Arg His His Tyr Arg Pro Arg Arg Lys Asn Gly Ile Phe Asn Thr
1 5 10 15

Thr Leu Ser

<210> 11
<211> 233
<212> PRT
<213> Artificial

<220>
<223> This is an amino acid sequence for porcine circovirus type 2, open reading frame 2.

<400> 11

Met Thr Tyr Pro Arg Arg Arg Tyr Arg Arg Arg Arg His Arg Pro Arg
1 5 10 15

Ser His Leu Gly Gln Ile Leu Arg Arg Arg Pro Trp Leu Val His Pro
20 25 30

Arg His Arg Tyr Arg Trp Arg Arg Lys Asn Gly Ile Phe Asn Thr Arg
35 40 45

Leu Ser Arg Thr Phe Gly Tyr Thr Val Lys Ala Thr Thr Val Arg Thr

50 55 60

Pro Ser Trp Ala Val Asp Met Met Arg Phe Asn Ile Asp Asp Phe Val
65 70 75 80

Pro Pro Gly Gly Gly Thr Asn Lys Ile Ser Ile Pro Phe Glu Tyr Tyr
85 90 95

Arg Ile Lys Lys Val Lys Val Glu Phe Trp Pro Cys Ser Pro Ile Thr
100 105 110

Gln Gly Asp Arg Gly Val Gly Ser Thr Ala Val Ile Leu Asp Asp Asn
115 120 125

Phe Val Thr Lys Ala Thr Ala Leu Thr Tyr Asp Pro Tyr Val Asn Tyr
130 135 140

Ser Ser Arg His Thr Ile Pro Gln Pro Phe Ser Tyr His Ser Arg Tyr
145 150 155 160

Phe Thr Pro Lys Pro Val Leu Asp Ser Thr Ile Asp Tyr Phe Gln Pro
165 170 175

Asn Asn Lys Arg Asn Gln Leu Trp Leu Arg Leu Gln Thr Ser Arg Asn
180 185 190

Val Asp His Val Gly Leu Gly Thr Ala Phe Glu Asn Ser Ile Tyr Asp
195 200 205

Gln Asp Tyr Asn Ile Arg Val Thr Met Tyr Val Gln Phe Arg Glu Phe
210 215 220

Asn Leu Lys Asp Pro Pro Leu Lys Pro
225 230