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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: Expediente N°. 13-019.546

Solicitud de Patente de Invención titulada: "VACUNAS DE VIRUS
VARICELA ZÓSTER INACTIVADO Y MÉTODOS DE
PRODUCCIÓN DE LAS MISMAS"

Solicitante: MERCK SHARP & DOHME CORP.

Nuestra Ref.: P2013/000022

RESPUESTA AL OFICIO No. 15825, NOTIFICADO POR FIJACIÓN EN LISTA No. 001
DE FECHA 06 DE ENERO DE 2015
(SEGUNDO EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal de prórroga previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. En primer lugar, con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad el Capítulo Reivindicatorio Modificado presentado el 11 de julio de 2014.

Así entonces, en el Capítulo Reivindicatorio Modificado adjunto contiene los siguientes cambios:

- se incluyen las Reivindicaciones 2-4, las cuales encuentran soporte en las antiguas Reivindicaciones 1 y 7;
- se eliminan las Reivindicaciones 4, 10 y 13 a 18;

- se limita la Reivindicación 1, que ahora reclama un método de preparación de un virus de varicela zóster inactivado, además, especificando un rango de radiación gamma entre 10 a 25 kGy;
- las Reivindicaciones 5-7, 8-9, 10-11 y 12 corresponden a las antiguas Reivindicaciones 2-4, 6-7, 9-10 y 12.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial.

2. a- La Examinadora presenta las siguientes objeciones respecto a la claridad de las solicitud:

i) la Reivindicación 1 menciona un virus de varicela zóster que pertenece a una cepa Oka o una cepa derivada de Oka, sin especificar su número de certificado de depósito biológico. En este mismo sentido, la Examinadora también argumenta que el Capítulo Descriptivo no es lo suficientemente completo para que la persona medianamente versada en la materia pueda reproducir o ejecutar la materia aquí reclamada, ya que corresponde a material biológico sin incluir el Certificado de Depósito de la cepa Oka;

ii) la Reivindicación 1 menciona que el virus VVZ inactivado induce una respuesta inmune contra VVZ, lo que corresponde a un resultado a alcanzar y no define el virus.

b- En respuesta a las objeciones de la Examinadora, me permito señalar lo siguiente:

i) En primer lugar, la nueva Reivindicación 1, ahora reclama:

“Un **método de preparación de un virus de varicela zóster (VVZ) inactivado** que comprende **irradiar con radiación gamma** una muestra que comprende un VVZ, usando de **10 a 25kGy de radiación gamma**, en donde la radiación gamma se proporciona a la muestra mediante la exposición de la muestra a rayos de ⁶⁰Co, en donde la infectividad del VVZ inactivado es <0,040 unidades formadoras de placas (UFP)/mL y en donde el VVZ inactivado induce una respuesta inmune contra VVZ.”

ii) Ahora bien, teniendo en cuenta la drástica modificación realizada al Capítulo Reivindicatorio, que ahora se dirige en la reivindicación independiente a un método de preparación de un VVZ, en cuanto al requerimiento del certificado del depósito de la cepa Oka, éste resulta

innecesario, ya que el método reivindicado es aplicable a cualquier virus VVZ. Por lo tanto, la presente invención no reclama una cepa Oka particular.

Por todo lo anterior, para la persona medianamente versada en la materia es claro que la materia reclamada es el proceso y por lo tanto, el alcance de las nuevas reivindicaciones es absolutamente claro y conciso.

ii) En cuanto a las propiedades de infectividad y inmunogenicidad especificadas en la Reivindicación 1 para el virus VVZ obtenido a través del método reclamado, me permito respetuosamente llamar la atención de la Examinadora sobre las directrices del Manual Andino de Patentes (MAP), que indica que los parámetros y los resultados a alcanzar son admitidos en las reivindicaciones siempre y cuando las características técnicas de la invención (en este caso, el proceso de la invención) hayan sido previamente definidas.¹ En el mismo sentido, la SIC señala respecto a los resultados a alcanzar que: *"El resultado a alcanzar no es una característica técnica de la invención. Puede aparecer en la reivindicación, pero siempre acompañando las características técnicas que definen la invención"*.² Así las cosas, la obtención de un virus de varicela zóster está definido en la Reivindicación 1 por el procedimiento de obtención, correspondiendo a sus características técnicas que son el procedimiento en sí.

En cuanto a la nueva Reivindicación 4, dirigida al virus obtenido por el método de la Reivindicación 1, me permito resaltar respetuosamente que según la Superintendencia de Industria y Comercio, en su Instructivo para el Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad, respecto a los productos definidos por su proceso de fabricación, estos son perfectamente aceptables en la reivindicaciones, siempre y cuando *"los productos como tales cumplen los requisitos de patentabilidad, es decir, cuando son nuevos e inventivos, y cuando no se pueden definir por sus características estructurales"*³, situación completamente aplicable a la Reivindicación 4 de este caso.

Teniendo en cuenta lo anterior, considero superadas las anteriores objeciones, en tanto que el Capítulo Reivindicatorio Modificado adjunto cumple con los requisitos de claridad establecidos en la Decisión 486, y a la luz del Manual Andino de Patentes (MAP) y el Instructivo de la SIC, de tal forma que le permite a cualquier persona medianamente versada en la materia determinar inequívocamente el alcance de la materia reclamada.

¹Manual Andino de Patentes. (2004). Secciones 4.6.6 y 4.6.7. (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

²Instructivo Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 2.6.5.8. Página 41. (disponible electrónicamente en http://www.sic.gov.co/recursos_user/documentos/propiedad_industrial/Formatos/PI02-I06.pdf)

³Instructivo Examen de Solicitudes de Patentes de Invención y Modelo de utilidad. Superintendencia de Industria y Comercio. 2012. Sección 2.6.5.10. Página 81. (disponible electrónicamente en http://www.sic.gov.co/recursos_user/documentos/propiedad_industrial/Formatos/PI02-I06.pdf)

3. a- La Examinadora argumenta que la presente invención carece de nivel inventivo a la luz de la patente US6214354 (D1) y el documento "Virus inactivation studies using ion beams, electron and gamma irradiation"⁴ (D2).

D1 se considera como el documento más cercano a la presente invención, ya que esté divulga un método para inactivar un virus VZV que consiste en exponer a radiación gamma el virus VZV (D1, col. 9, línea 60), donde el virus corresponde a la cepa Oka (D1, col 4, línea 19). D1 también divulga una composición que contiene el virus en forma de vacuna (D1, col. 6, línea 15-65, Ejemplo 2). En particular, D2 divulga la inactivación del virus de la varicela zóster por exposición a radiación gamma (20-30 kGy) (D2, Fig. 1, col 60, pág. 250). La Examinadora entiende que la diferencia entre D1 y la presente solicitud consiste en que el virus se expone a una radiación gamma con dosis de entre 5 a 25kGy.

De hecho, en las Figuras 1 y 2 de esta solicitud se describe que la exposición de la muestra viral a radiación gamma de entre 5 a 25 kGy, mostró una pérdida de ½ unidad logarítmica, sin llegar al 100% de la pérdida de infectividad. Lo cual no evidencia un efecto técnico sorprendente para un método para inactivar un virus de la varicela zóster.

La Examinadora define que el problema técnico que pretende resolver la presente invención es ¿cómo proveer un virus de la varicela zóster inactivo alternativo?. Sin embargo, D2 ya describe un virus de varicela zóster inactivado a partir de la exposición a radiación gamma de Co 60 (20-30 kGy) con un 100% de inactivación total (D1, Fig. 1). La Examinadora concluye que la persona medianamente versada en la materia usaría diferentes dosis de rayos gamma (como se enseña en D2) en el método divulgado en el D1, para llegar así al objeto de las Reivindicaciones 1 y 13.

b- i) En primer lugar, llamo nuevamente la atención de la Examinadora sobre el Capítulo Reivindicatorio Modificado adjunto, en particular sobre la Reivindicación 1, la cual reclama específicamente un método como se transcribió en el punto anterior 2.b.i.

ii) Ahora bien, D1 divulga vacunas de virus varicela zóster (VVZ) para la aliviar la neuralgia post-herpética aprovechando la estimulación antigénica y enfocándose exclusivamente en vacunas con VVZ completo, vivo y atenuado. Tal y como lo ilustra el Ejemplo 2 de D1, esta referencia muestra la preparación de una vacuna de virus varicela zóster inactivado solamente por calor (véanse las columnas 6 y 7) y solamente menciona la posibilidad de preparar una vacuna de VVZ inactivado mediante la irradiación con rayos gamma, con una única referencia general en la

⁴Eduardo E. Smolko, Nuclear Instruments and Methods in Physics Research Section B: Beam interactions with Materials & Atoms, 2005, vol. 236, issues 1-4, páginas 249-253.

columna 4 a radiación gamma “en cantidades conocidas de tiempo e intensidad”, sin detalle adicional particular alguno.

Por lo tanto, la única enseñanza concreta de D1 corresponde al Ejemplo 2, en relación el tratamiento de virus VVZ para inactivarlo con calor, alcanzando una infectividad de 2,4 unidades formadoras de placas/mL, lo cual es bastante diferente de lo que se reclama específicamente en la presente solicitud.

En otras palabras, D1 es insuficiente en cuanto a enseñanzas relacionadas con virus de VVZ inactivado por radiación gamma. Aunque D1 mencione la radiación gamma, es evidente que dicho documento no proporciona datos o información relevantes. Por el contrario, D1 solamente proporciona información esencial para producir vacunas mediante la inactivación por calor, dejando la producción de vacunas por radiación como una simple especulación. En conclusión D1 no sugiere las condiciones de radiación que específicamente aplica la presente invención, en donde se reclama un rango de radiación gamma de 10 a 25kGy.

iii) Por otro lado D2 divulga un método para la inactivación de virus, particularmente enseñando pruebas con radiación gamma en Virus de Fiebre Aftosa (VFA), virus Rauscher de leucemia (RLV) y virus del herpes simple (VHS). Evidentemente, D2 no contiene enseñanza alguna sobre la aplicación y utilidad de la inactivación de VVZ usando radiación gamma.

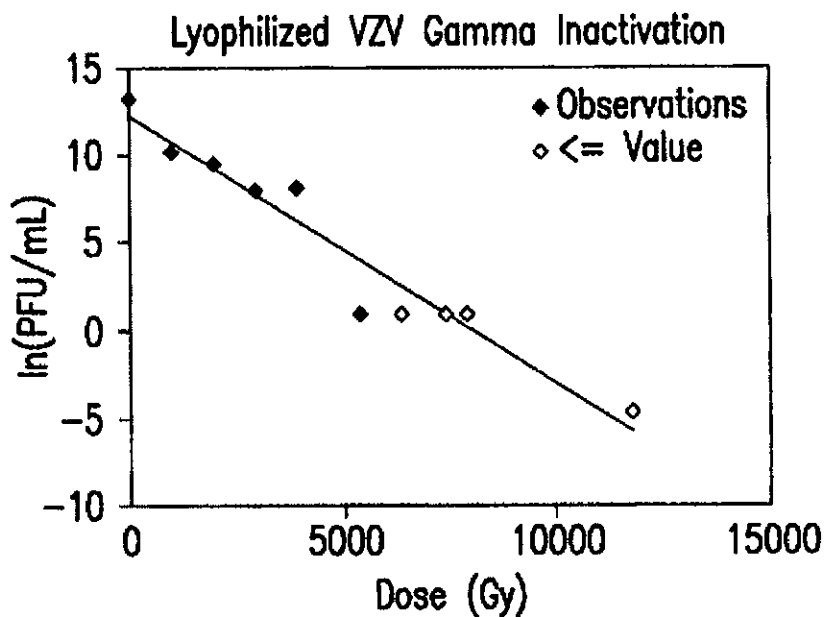
D2 tan solo muestra resultados de inactivación en la Figura 1 para cada uno de los virus citados, en donde se logra un 0.0001% de actividad de los virus alrededor de una radiación gamma de 20kGy. Sin embargo, la presente invención proporciona un virus activo alrededor de 0.000015% de virus activo, lo cual corresponde al menos a **10 veces** más virus inactivados que lo descrito en D2. Claramente, D2 no conduce a la persona medianamente versada en la materia a usar la radiación gamma específica sobre VZV para llegar a la presente invención, pues ni siquiera se refiere al mismo tipo de virus, ni brinda enseñanzas sobre los resultados obtenidos.

En conclusión, D2 se refiere a virus inactivos pero no al virus de varicela zóster, si no que muestra la inactivación de virus diferentes a los de la presente invención, sin sugerir un protocolo de inactivación específico. D2 sólo describe diferentes estructuras de virus que no llevan a la persona medianamente versada en la materia a llegar al objeto aquí reclamado. Es más, D2 enseña que la inactivación de diferentes virus no es alcanzable con un protocolo de inactivación general (que pueda predecirse, funcionaría para todos (ver Figuras 1 u 2 de D2)).

De otra parte, D2 no proporciona ningún dato biológico en su publicación en relación con muestras de virus que fueron irradiados con radiación gamma. D2 solamente muestra la inactivación de un

virus a la mitad del volumen. Es imposible entender a partir de D2 que la inactivación de VVZ usando una alta cantidad de radiación sea útil para inactivar completamente la muestra, pues el daño que puede causarse a las muestras (ADN y proteínas) puede resultar en virus incapaces de inducir una respuesta inmune efectiva. Así, inactivar un virus corresponde sólo a la mitad del trabajo.

iv) De otra parte, considero respetuosamente que la Examinadora malinterpreta los datos que se muestran en la Figura 1 de esta solicitud, en donde se muestra la cinética de inactivación de VVZ liofilizado, sometido a irradiación γ (log UFP/ml v. dosis de radiación, véase el Ejemplo 4). Se irradiaron viales de VVZ con diferentes niveles de rayos ^{60}Co , usando un irradiador ^{60}Co Gammacell®. Las UFP residuales/ml de cada vial por dosis de radiación (Gy) se determinaron usando el ensayo en placas de varicela en células MRC-5. Los puntos de datos observados se muestran como rombos negros ("observaciones") y los datos máximos medibles por el ensayo, con valores mayores de 5kGy, los valores se muestran por rombos grises (" $\leq$ Valor"), para los que no se observaron placas. Es decir, se muestran resultados para radiación de 0 a 5 kGy, en donde la ineffectividad está marcada con rombos negros y las plaquetas no observadas son reportadas como " $\leq$ " (dicho valor indica que el título pudo haber tenido 1 plaqueta detectada en el volumen medido) como se puede observar a continuación:



En la solicitud también se ensayó un volumen de material grande de 100mL, el cual fue medido en la Figura 1 (Ejemplo 4, pág. 18). Los 100mL de volumen de material fueron irradiados con 12 kGy y no se detectó ineffectividad residual. En este caso, el mínimo de ineffectividad detectable pudo ser 1 PFU en 100mL o 0.01 PFU/mL. El valor medido indica que la ineffectividad del materia irradiado a 12 kGy es entonces ≤ 0.01 PFU/mL. En la escala $\ln(\text{PFU/mL})$ eje-y de la Figura 1, esto correspondería -4.6.

El argumento de la Examinadora afirmando que la muestra del virus pierde la mitad de una unidad logarítmica a 5-25 kGy de radiación gamma sin lograr un 100% de desactivación, es incorrecto. Para las pruebas del volumen en la Figura 1, se obtuvo la desactivación completa (por ejemplo, cero plaquetas fueron detectadas en ensayos con varicela), con la cantidad de radiación probada mayor de 5kGy. Los diamante blancos en la gráfica indican el límite de detección del ensayo. Aunque no se muestran resultados a 25 kGy en la Figura 1, el $\ln(\text{PFU/mL})$ puede extrapolarse basado en la linealidad de la curva, que sería bastante bajo. Esto resulta completamente inesperado, ya que el rango de radiación reclamado resulta en tales niveles menores de ineffectividad residual, mientras se mantienen el requerimiento de inmunogenicidad, antigénicidad y características estructurales requeridas para la vacuna propuesta.

v) Para terminar, y como la Examinadora debe saberlo, el análisis del nivel inventivo no puede basarse en si la invención era posible de alcanzar. Aplicar un estándar de "posibilidad de alcanzar" en el análisis de solicitudes de patente haría que ninguna invención fuera susceptible de patentarse, haciendo inviable el sistema de patentes. En otras palabras, para la fecha de prioridad aquí reclamada, la persona medianamente versada en la materia habría podido llegar a los compuestos de la presente invención. Sin embargo, la cuestión a determinar es si la persona medianamente versada en la materia, a la fecha de prioridad aquí reclamada, lo habría realizado, motivado por el conocimiento general y estimulado por el arte previo.

Finalmente, me permito llamar la atención a la Examinadora en cuanto el Manual Andino de Patentes (MAP), señala que, las invenciones en el campo de la química [biología] tienen nivel inventivo cuando la materia reclamada tiene una estructura inesperada (no derivable por la persona medianamente versada en la materia) o cuando presentan un efecto técnico inesperado o mejorado impredecible del arte previo⁵. En el presente caso, es claro que el método propuesto y el producto VVZ inactivado obtenido, provee efectos inesperados no derivables del arte previos, además de que dicho arte previo no proporciona las enseñanzas específicas para realizar tal método en particular.

Además, la Guía de la SIC establece claramente que "si el estado de la técnica provee las enseñanzas para derivar la solución, revisar si el conjunto de enseñanzas del estado de la técnica lo llevarían de forma inmediata a la solución propuesta por la solicitud. Si se requiere de pasos adicionales o de corroboración o de superar obstáculos revelados por las propias enseñanzas entonces el estado de la técnica no revela la solución al problema"⁶ (subrayado y negrilla fuera de texto). Evidentemente, la dicha persona medianamente versada en la materia necesitaría

⁵ Manual Andino de Patentes. (2004). Sección 10.8.1 Ejemplo 1 (<http://www.comunidadandina.org/public/patentes.pdf>).

⁶ Superintendencia de Industria y Comercio, Guía para Examen de solicitudes de Patente de Invención y Modelo de Utilidad, 2014, sección 2.6.5.4, p. 76.

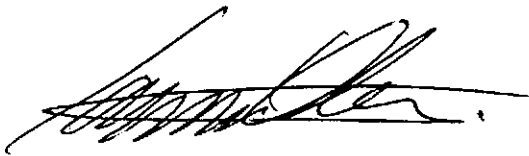
investigación y experimentación adicional con el fin de establecer el método reclamado y los virus VVZ obtenidos.

4. Finalmente, me permito informar que para el concepto inventivo comprendido en la presente solicitud, han sido otorgadas hasta el momento patentes en Australia, Nueva Zelanda y Sur África.

Si bien es cierto que las concesiones de patentes en oficinas de otros países no son vinculantes, en el sentido que obliguen a la SIC a tomar una decisión en el mismo sentido, considero que dichas concesiones sugieren el cumplimiento de los requisitos de patentabilidad de la materia reivindicada, ya que los criterios de evaluación y estudio aplicados para concluir la concesión de dichas patentes resultan equivalentes a los que la SIC debe emplear.

5. Por todo lo anterior, considero que las observaciones presentadas por la Examinadora han sido resueltas y por consiguiente solicito respetuosamente que el Despacho tenga por cumplidos los requisitos de patentabilidad, y se proceda con la concesión de la patente.

Atentamente,



CARLOS R. OLARTE

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T.P. 74.295

ANEXOS:

- Capítulo Reivindicatorio Modificado;
- ANEXO 1: "Comparison of the complete DNA sequences of the Oka varicella vaccine and its parental virus";
- ANEXO 2: "Complete DNA sequences of two Oka strain VZV genomes", J Virol 2008;
- ANEXO 3: "Correction to Comparison of the Complete DNA sequences of the Oka varicella vaccine and its parental virus", J Virol, 2003;
- ANEXO 4: "Population diversity in batches of the varicella Oka vaccine", Kanda et al. Vaccine, 2011;
- ANEXO 5: "Molecular Analysis of the Oka Vaccine Strain of Varicella-Zoster Virus", Yamanishi et al., 2008.

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CAPÍTULO REIVINDICATORIO MODIFICADO

(Segundo Examen de Fondo)

1. Un método de preparación de un virus de varicela zóster (VVZ) inactivado que comprende:
 - irradiar con radiación gamma una muestra que comprende un VVZ, usando de 10 a 25 kGy de radiación gamma,
 - en donde la radiación gamma se proporciona a la muestra mediante la exposición de la muestra a rayos de ^{60}Co ,
 - en donde la infectividad del VVZ inactivado es $<0,040$ unidades formadoras de placas (UFP)/mL y
 - en donde el VVZ inactivado induce una respuesta inmune contra VVZ.
2. El método de la Reivindicación 1, en donde la muestra de VVZ es una cepa Oka o una cepa derivada de la cepa Oka.
3. El método de la Reivindicación 1, en el que la muestra se liofiliza antes de irradiarse.
4. Un virus VVZ inactivo producido por el método de cualquiera de las Reivindicaciones 1 a 3.
5. Una vacuna de VVZ inactivado que comprende una cantidad terapéuticamente efectiva del VVZ inactivado de la Reivindicación 4 y un vehículo farmacéuticamente aceptable.
6. La vacuna de VVZ inactivado de la Reivindicación 5, en donde la vacuna es una formulación líquida a granel.
7. La vacuna de VVZ inactivado de cualquiera de las Reivindicaciones 5 o 6, en donde la infectividad del VVZ inactivado es $\leq 0,010$ unidades formadoras de placas (UFP)/mL.
8. La vacuna de VVZ inactivado de cualquiera de las Reivindicaciones 5 o 6, en donde la infectividad del VVZ inactivado es $\leq 0,002$ unidades formadoras de placas (UFP)/mL.
9. La vacuna de VVZ inactivado de las Reivindicaciones 7 u 8, en donde la respuesta inmune inducida por la el VVZ inactivado no es significativamente diferente de la respuesta inmune inducida por una muestra de control que comprende la misma cantidad del VVZ que no se ha inactivado.

10. La vacuna de VVZ inactivado de las Reivindicaciones 7 u 8, en donde la infectividad del VVZ se determina por un ensayo en placas de varicela.

11. La vacuna de VVZ inactivado de las Reivindicaciones 7 u 8, en donde la respuesta inmune se mide con un ensayo de ELISA.

12. La vacuna de VVZ inactivado de las Reivindicaciones 7 u 8, en donde la respuesta inmune se mide con un ensayo IFN γ ELISPOT.

Comparison of the Complete DNA Sequences of the Oka Varicella Vaccine and Its Parental Virus

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The DNA sequences of the Oka varicella vaccine virus (V-Oka) and its parental virus (P-Oka) were completed. Comparison of the sequences revealed 42 base substitutions, which led to 20 amino acid conversions and length differences in tandem repeat regions (R1, R3, and R4) and in an origin of DNA replication. Amino acid substitutions existed in open reading frames (ORFs) 6, 9A, 10, 21, 31, 39, 50, 52, 55, 59, 62, and 64. Of these, 15 base substitutions, leading to eight amino acid substitutions, were in the gene 62 region alone. Further DNA sequence analysis showed that these substitutions were specific for V-Oka and were not present in nine clinical isolates. The immediate-early gene 62 product (IE62) of P-Oka had stronger transactivational activity than the mutant IE62 contained in V-Oka in 293 and CV-1 cells. An infectious center assay of a plaque-purified clone (S7-01) from the V-Oka with 8 amino acid substitutions in ORF 62 showed smaller plaque formation and less-efficient virus-spreading activity than did P-Oka in human embryonic lung cells. Another clone (S-13) with only five substitutions in ORF 62 spread slightly faster than S7-01 but not as effectively as P-Oka. Moreover, transient luciferase assay in 293 cells showed that transactivational activities of IE62s of S7-01 and S7-13 were lower than that of P-Oka. Based on these results, it appears that amino acid substitutions in ORF 62 are responsible for virus growth and spreading from infected to uninfected cells. Furthermore, the Oka vaccine virus was completely distinguishable from P-Oka and 54 clinical isolates by seven restriction-enzyme fragment length polymorphisms that detected differences in the DNA sequence.

Varicella-zoster virus (VZV) is a human herpesvirus that causes chickenpox (varicella) and shingles (herpes zoster). A live attenuated varicella vaccine, the Oka strain was originally developed by Takahashi et al. in Japan (56) and is routinely used in children in Japan and other countries, including the United States. Clinical symptoms caused by this live vaccine are very rare in healthy children. Although the Oka vaccine virus (V-Oka) is an avirulent virus, its parental virus (P-Oka), isolated from a patient with typical varicella, is thought to be virulent in vivo. It has not been clarified which gene(s) is involved in the pathogenicity of VZV infection. Thus, comparison of the complete genomes of V-Oka and P-Oka should reveal correlations between DNA sequence and virulence.

The complete DNA sequence of the VZV Dumas strain was first determined by Davison and Scott (9). The genome is a linear double-stranded DNA of ca. 125,000 bp and consists of unique long regions (ULs) flanked by terminal repeat long (TRL) and internal repeat long (IRL) inverted repeat regions, and a unique short (US) region flanked by internal repeat short (IRS) and terminal repeat short (TRS) inverted repeat regions. The genome contains ca. 70 open reading frames (ORFs), three of which exist in both IRS and TRS regions; genes 62 through 64 correspond to genes 69 through 71.

We previously determined the sequences of several genes from V-Oka and P-Oka and found differences between them

(15, 16). As many as 15 base substitutions, representing 8 amino acid differences, were found in gene 62, located within the IRS region (or in gene 71 located within the TRS region) and its flanking regions, although no mutations were found in genes encoding other transactivators, such as genes 4, 10, 61, and 63. In addition to our report, another author reported that the DNA sequences of the P-Oka virus and a cosmid clone from the vaccine virus were different in gene 62 (2). The immediate-early gene 62 product (IE62) is an immediate-early (IE) protein consisting of 1,310 amino acids and is functionally conserved with herpes simplex virus type 1 (HSV-1) ICP4 (13). High levels of IE62 are associated with viral tegument (24) and a recombinant HSV-1 with both copies of ICP4 replaced by VZV IE62 has been constructed (11). IE62 can transactivate all three putative kinetic classes of VZV gene promoters—i.e., the IE, early, and late gene promoters (5, 10, 15, 16, 22, 38, 47, 52)—and can transrepress its own promoter (5, 12, 49). Production of infectious VZV generated by the transfection of purified viral DNA is strongly increased by the addition of an IE62-expressing plasmid, and it has been hypothesized that virion-associated IE62 may play a crucial role in stimulating the IE events upon infection (44). In our previous study, the transcriptional activity of the P-Oka IE62 was higher than that of V-Oka IE62, which possessed all eight amino acid substitutions, on all classes of VZV gene promoters, leading us to hypothesize that IE62 might play an important role in the VZV replicative cycle and, moreover, in the attenuation of VZV (15, 16). However, it is possible that other substitution(s) are responsible for the pathogenicity of VZV, since the DNA sequence comparisons between the Oka viruses have only been

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done for several genes, but not for the complete genome. To resolve this issue, the complete DNA sequence of both viruses was needed.

We determined here the complete genome sequences of the Oka viruses and found remarkable differences in some of the VZV genes. The present study provides the first step toward investigating the genes involved in VZV attenuation. Furthermore, new methods for distinguishing V-Oka from clinical isolates, including P-Oka, were established, based on the newly identified substitutions. These methods can be used for typing VZV strains and for quality control of the Oka varicella vaccine.

MATERIALS AND METHODS

Cells and viruses. P-Oka was isolated in Japan from a patient with varicella and was propagated only in human embryonic lung (HEL) cells. A live, attenuated varicella vaccine (BIKEN, lot V-65), manufactured by the Research Foundation for Microbial Diseases of Osaka University, was used as V-Oka. Virus strains used as clinical isolates in the present study were isolated in Japan from patients with varicella (Kawaguchi, Inoue, RT-283, RT-342, and OZ-4) or zoster (Z-4, Z-5, Z-8, or RT-345) who had never been vaccinated. Strain RT-342 was isolated from the vesicles of a patient 10 days after vaccination, and strain RT-345 was isolated from the vesicles of a patient with shingles 19 months after vaccination. Clinical samples were isolated by the inoculation of HEL cells with vesicular fluids from patients and were propagated by three to five passages in HEL cells or MRC-5 cells.

PCR. Genomic DNA was extracted from the infected cells as described previously (42). Approximately 500 primers (25 bp in length) were designed by referring to the nucleotide sequence of the Dumas strain (9). Almost all regions covering each genome were amplified as 43 overlapping pieces by PCR. The reaction mixtures for PCR contained 2.5 U of Ex Taq (Takara Shuzo Co., Kyoto, Japan), 200 μ M concentrations of each deoxynucleoside triphosphate, 0.3 mM concentrations of each primer, and an extremely low amount of template DNA in 50 μ l of Ex Taq buffer. Thirty cycles of amplification were performed, in which each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1.5 min, and an extension step at 72°C for an appropriate period (1 min/kbp) in a thermal cycler (Takara Shuzo). For only the regions containing the R3 reiteration region and gene 62, the PCR conditions were as described above except that the denaturation time and the annealing time were each changed to 30 s. For an origin of replication region only, the amplified DNA fragments were obtained by shuttle PCR by using 2.5 U of LA Taq polymerase (Takara Shuzo), 200 μ M concentrations of each deoxynucleoside triphosphate, 0.3 μ M concentrations of each primer (32 bp in length), and an extremely low amount of template DNA in 50 μ l of LA Taq buffer. Shuttle PCR conditions were as follows: an incubation at 98°C for 2 min for the hot start and 30 cycles of 98°C for 30 s and 68°C for 2.5 min. All amplified products were purified by using a QIA PCR purification kit (Qiagen) to remove the primers.

Cloning of PCR products. Determinations of the DNA sequences at both ends of the genomes, the flanking regions between the internal repeat long and IRS regions, and the R3 repeat were difficult to obtain by direct sequencing of the PCR products as described below, implying that various clones containing different sequences existed. Thus, PCR products containing the regions were individually inserted into a pCR2.1 vector and then transformed into competent *Escherichia coli* TOP10 5' cells by the TA cloning method (Invitrogen). The plasmid DNAs were purified from cultured cells by using the QIA Prep Spin Kit (Qiagen).

Sequencing. Direct sequencing of purified PCR products and plasmids was performed by using the BigDye-terminator cycle sequencing kit and a Genetic Analyzer 310 (both from Perkin-Elmer/Applied Biosystems, Chiba, Japan). The viral sequences were compiled by using the DNASIS computer programs (Hitachi Computer Engineering, Tokyo, Japan).

Plasmids. Two effector plasmids were constructed for a transient luciferase assay to analyze the transcriptional activity of ORF 10 protein containing an amino acid substitution, which was caused by a partial base substitution within the *HindIII* site. A DNA fragment containing ORF 10 from V-Oka was amplified by PCR under the conditions described above. The amplified product was inserted into the pCR2.1 vector by the TA cloning method and then checked for the direction of the inserted fragment and whether the *HindIII* site was present or not. Plasmids pCG10(-) and pCG10(+) were constructed by inserting the

KpnI-XhoI fragment from clones obtained by TA cloning into pCDNA3.1 (Invitrogen) containing the cytomegalovirus IE promoter. The *HindIII* site existed in pCG10(-), which bore the ORF 10 from P-Oka, but not in pCG10(+), which contained the ORF 10 present in V-Oka.

Five kinds of effector plasmids expressing IE62s were constructed. pPar-G62 and pVac-G62, which had eight amino acids that were different from each other, were selected from the clones and used for our previous study (15). After pPar-G62 and pVac-G62 were digested by *NcoI* and *PstI* (Takara Shuzo), pPV-G62*Nco* and pVP-G62*Nco* were constructed by connecting the small fragment from the digested pPar-G62 and pVac-G62 and the large fragment from the digested pVac-G62 and pPar-G62, respectively. pVmut4 was also selected from the clones from V-Oka; this clone had four amino acid differences in ORF 62 compared to pPar-G62. The reporter plasmid pPol-Luc contained ca. 750 bases upstream from the initiation codon of gene 29. This was constructed by transferring the *NheI-BglII* fragment from plasmid pPol-CAT as described previously (15) to the region upstream of the firefly luciferase gene in pGL3-Basic (Promega).

Transfection. To analyze the transactivational activity of the ORF 10 proteins, CV1 cells, which were cultured in 35-mm plastic dishes at 10^5 cells/dish, were transfected by lipofection with SuperFect (Qiagen). Each reaction mixture for transfection consisted of 0.5 μ g of the reporter plasmid, various amounts (0 to 2 μ g) of either effector plasmid pCG10(+) or pCG10(-), and 0.25 μ g of β -galactosidase-expressing plasmid pCHI10 (Amersham Pharmacia) for normalizing the transfection efficiency between different dishes. The total amount of plasmid DNA in each transfection experiment was kept constant at 2.75 μ g by adding the vector pCDNA3.1.

To analyze the transactivational activity of the different IE62s, 293 cells and guinea pig embryonic (GPE) cells (derived from the fourth week of pregnant guinea pigs), which were cultured in 35-mm plastic dishes at 10^5 cells/dish, were transfected with the SuperFect and Lipofectamine Plus reagents (Gibco-BRL), respectively. Each reaction mixture for transfection consisted of 0.25 mg of the reporter plasmid, the same amount of one of five effector plasmids that express IE62s, and 2 mg of the vector pUC19. All experiments were repeated at least three times independently.

Luciferase and β -galactosidase assays. At 24 h after transfection, the cells were washed twice with phosphate-buffered saline. The cells were then lysed by the addition of 100 μ l of reporter lysis buffer (Promega), followed by mixing for 15 min at room temperature. The lysate was collected in a new microcentrifuge tube, and the cell debris was removed by centrifugation at 12,000 rpm for 2 min. The luciferase activity of 10 μ l of supernatant was determined by using a Berthold luminometer, model LB9507 (Berthold Japan, Tokyo, Japan), after the addition of 50 μ l of substrate buffer (Promega). To normalize this transient assay for ORF 10 proteins to the transfection efficiency, the β -galactosidase activity of 10 μ l of supernatant was also determined by using the luminometer, after the addition of 100 μ l of the reaction buffer of the luminescent β -galactosidase detection kit II (Clontech) and incubation of the mixture at room temperature for 1 h. In another transient assay for various IE62s, the luciferase activity in each lysate was standardized to the protein concentration determined with the Bio-Rad protein reagent, because all of the internal plasmids used to normalize the transfection efficiency were stimulated by the IE62s.

Comparison of plaque sizes and infectious center assay. HEL cells were infected with cell-free V-Oka, and virus clones were plaque purified twice. Two clones, S7-01 and S7-13, were obtained after DNA sequencing for use in further experiments. These clones were then propagated in HEL cells, and cell-free viruses were harvested by sonication as described previously (15). HEL or GPE cells were infected with the cell-free cloned viruses, P-Oka, and V-Oka, and their plaque sizes were compared after fixation and staining with methylene blue. An infectious center assay was also performed to examine the spreading of the viruses. HEL or GPE cells in 35-mm dishes were infected with cell-free viruses (ca. 50 PFU/plate). At 0 to 5 days postinfection, they were washed with phosphate-buffered saline (37°C) and treated with trypsin. The trypsin-treated cells were diluted and transferred onto monolayers of HEL cells in 35-mm dishes, and the numbers of infected cells were assessed by counting the plaques 7 days after the inoculation.

Restriction fragment length polymorphism (RFLP) analysis. Six parts of the genome were amplified by PCR with six sets of primer pairs as shown in Table 1. The conditions for the PCR were as described above. Portions (3 μ l) of the amplified products with three primer pairs—01-N12 and 01-R13, 50-N15 and 50-R17, or 60-N11 and 60-R12—were digested with 4 U of the restriction endonucleases *AhaI* (Takara Shuzo), *BstXI* (Takara Shuzo), or *BstI* (New England Biolabs), respectively. The same volumes of the products obtained by using primer pair 60-N06 and 60-R06 and primer pair 60-N26 and 60-R28 were digested independently with 4 U of the enzymes *SfaNI* (New England Biolabs)-

TABLE 1. Primers used in the RFLP analysis in this study^a

Name	Sequence	Position (nucleotide range)
01-N12	5'-ATTGTATGCATGCGATTGCTATGCC-3'	5372-5396
01-R13	5'-GGTCTTCCACTTTAAAGGGGTTTGC-3'	6134-6110
50-N15	5'-CGATCACGTCGCTCACATCCAACCC-3'	93685-93709
50-R17	5'-ATGGCAGAAGAAACAGTATTGCCG-3'	94457-94433
60-N06	5'-GAGGACAACAGCTCCACCTTGACCG-3'	105277-105301
60-R06	5'-GAGTAATGTGGCCGCCCGTTTGG-3'	105613-105589
60-N26	5'-CCAAAACCGGGCGGCCACATTACTC-3'	105589-105613
60-R28	5'-ATTACTGTGACCCGAGACCTGGCC-3'	106380-106356
60-N11	5'-AAGGGCTTCGTCGGGCATCATGAG-3'	107729-107753
60-R12	5'-TCGGGTAAAAAGCCGGCGATGAGC-3'	108497-108473

^a These primers were designed by referring to the nucleotide sequence of the Dumas strain. Nucleotide positions refer to those in the sequence of the Dumas strain.

AclII (Takara Shuzo) and *SacII* (New England Biolabs)-*SmaI* (New England Biolabs), respectively. Samples were digested for 1 h at 25°C for *SmaI*; 37°C for *AclII*, *SfaNI*, and *SacII*; 45°C for *BstXI*; 60°C for *AclII*; and 65°C for *BstI*. The digested products were analyzed by electrophoresis in a 4% agarose gel (NuSieve 3.1; FMC BioProducts Co., Rockland, Maine) with ethidium bromide staining and then visualized with short-wave UV illumination of the gel.

RESULTS

Comparison of the complete DNA sequences of V-Oka and P-Oka. (i) **Substitutions of bases and amino acids.** The entire VZV DNA sequences of V-Oka and P-Oka were determined mainly by the direct sequencing of PCR products. Thus, the determined sequences reflect the VZV DNA population in infected cells; that is, if mutated viruses also exist within a sample, more than two kinds of nucleotides would be recognized at one base position by this method. The genome sizes of both the Oka viruses were ca. 125 kbp, and >95% of the sequences were determined by this method on both the sense and antisense strands of the DNAs. When the complete DNA sequences of the Oka viruses were compared, only 42 bases were found to be different (Fig. 1A and Table 2), not including the substitutions in the TRS region, which are duplications of the IRS region. Two base substitutions were found in genes 10 and 14 for the first time in the present study, although we had missed them in our earlier study (15, 16). Base substitutions at 20 nucleotide positions in V-Oka caused amino acid conversions, and the base substitutions at 17 nucleotide positions were silent mutations. The remaining five base substitutions were found to be in noncoding regions. Thirty-one base substitutions in V-Oka were mixtures of two kinds of base at each of these positions (Table 2). Every base substitution was a G-versus-A or a C-versus-T conversion except for a G-versus-T conversion only at position 39227 in gene 22. Fifteen amino acid substitutions in V-Oka were partial, resulting in mixtures of two kinds of amino acids at each of these positions. Surprisingly, more than one-third (15 of 42) of the substitutions were found in gene 62 (or gene 71) and its flanking regions, and about half of the amino-acid substitutions (8 of 20) were within ORF 62. Thus, the gene 62 region was obviously the most variable region in the V-Oka virus. The major transactivating domains of IE62 are divided into five regions (regions 1 to 5) based on sequence homology in comparison with HSV-1 ICP4

(9, 35). Regions 4 and 5 contained one and three amino acid substitutions each, respectively.

There were 12 amino acid substitutions between the Oka viruses in the entire genome outside of gene 62. These existed in genes 6, 9A, 10, 21, 31, 39, 50, 52, 55, 59, and 64 (Fig. 1A and Table 2). The HSV-1 helicase-primase complex consists of three proteins encoded by UL52, UL8, and UL5 (8); their homologs in VZV are ORFs 6, 52, and 55, respectively. Interestingly, four amino acid substitutions were found in these proteins. One amino acid substitution was located near the carboxyl terminus of gene 6, which was Ser in P-Oka and Gly in V-Oka. Another substitution occurred in gene 52. In this case, the amino acid residue in P-Oka was Ile, which was incompletely altered to Val in V-Oka; V-Oka contained two kinds of base at a single position, resulting in two kinds of ORF 52 protein. The remaining two amino acid substitutions were located in ORF 55. An Ala at position 97748 and a Cys at position 97796 in P-Oka were partially substituted by Thr and Arg in V-Oka, respectively.

There was only one amino acid substitution among the seven known VZV surface glycoproteins gK, gC, gB, gH, gL, gI, and gE, encoded in genes 5, 14, 31, 37, 60, 67, and 68, respectively. gB contained an amino acid substitution in which an Ile in P-Oka was substituted by Val in about half of the virus population of V-Oka. Another substitution was found in ORF 50, which is homologous to the gene encoding the HSV-1 glycoprotein gM, but it has not been established whether gM exists and is functional in VZV. Gene 9A is homologous to HSV-1 UL49.5, which encodes a hydrophobic protein with only 91 amino acids (4). The gene product in VZV, the ORF 9A protein, is present in the membrane fraction of infected MeWo cells, although it is absent in the cytosolic fraction, and cells infected with a recombinant virus that is unable to express the ORF 9A protein produce smaller plaques than cells infected with the parental virus (50). A partial amino acid substitution was located at the end of the carboxyl terminus of ORF 9A protein. It is possible that this substitution affects the stability of the protein. ORF 10 protein is a tegument protein (24) that regulates positively to the IE62 gene promoter, and V-Oka contained both Ala and Val at a single position (i.e., 12779) in the middle of the protein. In the gene 21 protein, whose function is unknown, Thr in P-Oka was incompletely substituted by Ile in V-Oka as a result of a base substitution at position 31732. Amino acid substitutions also existed in genes 39 and 64. The HSV-1 homologue to the gene 39 product is the UL20 product, which is known to be a membrane protein, and the homologue to gene 64 is the US10 product, which is known to be a tegument protein, but the function of either of these products in VZV is not clear. Finally, an amino acid substitution was found in the gene 59-encoded uracil-DNA glycosylase, which is a DNA repair enzyme responsible for the removal of uracil residues misincorporated into DNA.

The DNA sequence differences of the Oka viruses were then compared with the Dumas strain. The G+C content was 46.1% for both V-Oka and P-Oka; this value is very close to that of the Dumas strain (46.0%). When the complete DNA sequences of V-Oka and P-Oka were compared with the Dumas strain, 213 and 171 base substitutions leading to 75 and 55 amino acid conversions, respectively (Fig. 1B), were found. All 176 substitutions between the Dumas virus and P-Oka are

13

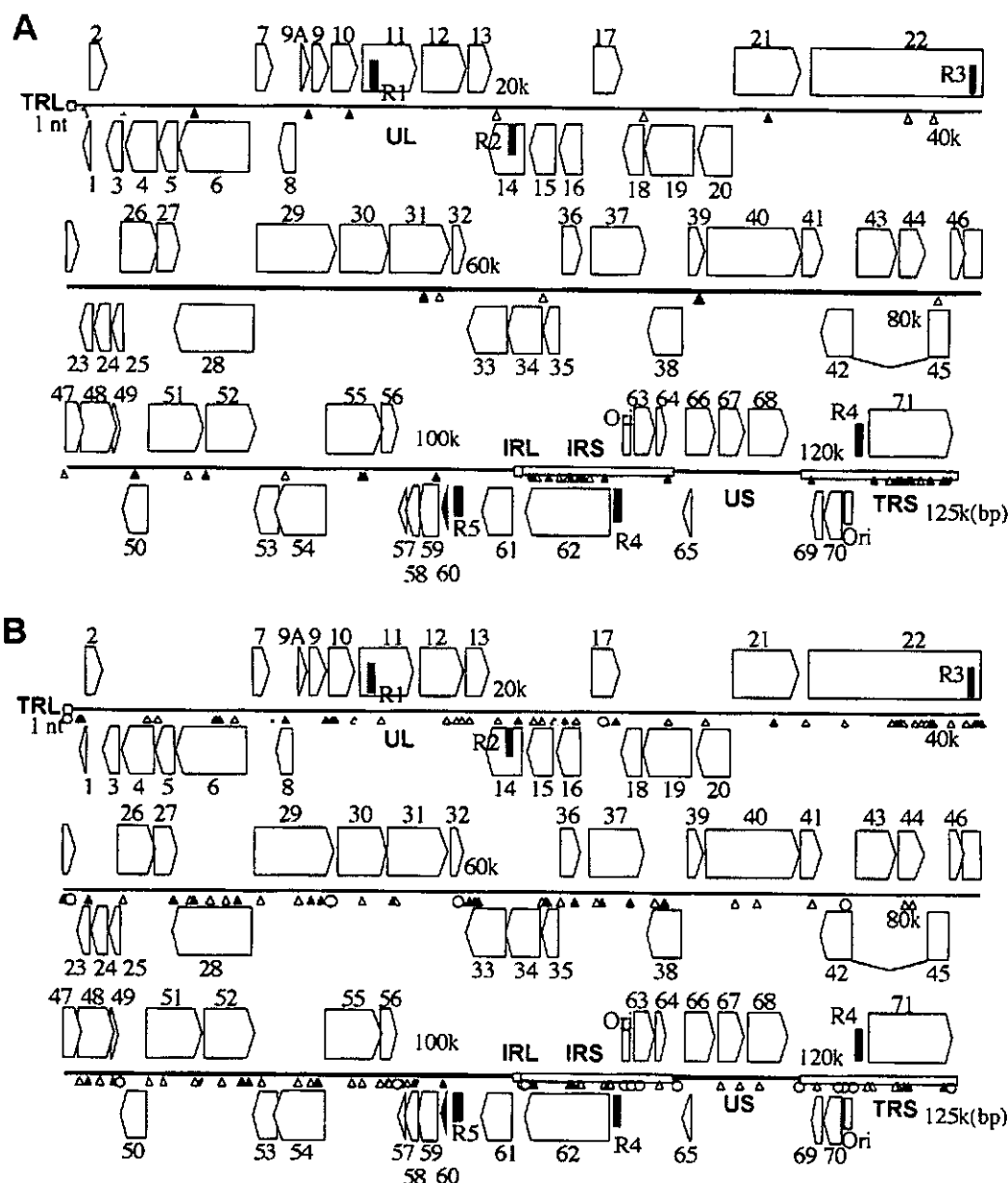


FIG. 1. Comparisons of complete DNA sequences. The genome is presented in sections; horizontal lines indicate unique regions, and open boxes indicate inverted repeat regions. ORFs 1 to 71 are illustrated as pentagons. ORFs 62 through 64 are duplications of ORFs 69 through 71. Solid, shaded, and open symbols show the base substitutions causing amino acid conversions, base substitutions in noncoding regions, and silent mutations, respectively. (A) Forty-two base substitutions between P-Oka and V-Oka. (B) One hundred seventy-six base substitutions between the Dumas virus and P-Oka. All 176 substitutions between the Dumas virus and P-Oka are contained within 218 substitutions between the Dumas virus and V-Oka; the 218 substitutions between the Dumas virus and V-Oka were the total of 176 plus 42 substitutions.

contained within 218 substitutions between the Dumas virus and V-Oka; 218 substitutions between the Dumas virus and V-Oka were the total of 176 plus 42 substitutions. In the entire DNA sequences, excluding the repeat regions, the mutation rates of P-Oka and V-Oka were 0.16 and 0.19% compared with the Dumas strain, respectively. Thus, P-Oka is genetically slightly closer to the Dumas strain than is V-Oka. Several regions in gene 22 and genes 55 through 61 were variable between the Dumas strain and P-Oka (Fig. 1B). The gene 62 region was an additional variable region when the Dumas

strain was compared with V-Oka; 24 base substitutions existed in gene 62 and its flanking regions between V-Oka and the Dumas strain. At the amino acid level, the mutation rate in all of the ORFs between the Dumas strain and P-Oka was 0.16%, whereas that between the Dumas strain and V-Oka was 0.21%. V-Oka ORF62 possessed the greatest mutation rate (0.84%) among all of the ORFs compared with the Dumas strain.

(ii) **Deletions and insertions in V-Oka.** V-Oka had several deletions and insertions compared to P-Oka in certain regions, including the tandem repeat regions and an origin of DNA

110087

Dumas :GTACGCCAATCGGATACACTCTTTTATCTGCATTTCGCACTTCCCGTTTTTCACTGTATGGGTTTCATT
 Parental:GTACGCCAATCGGATACACTCTTTTAATCTGCATTTCGCACTTCCCGTTTTTCACTGTATGGGTTTCATT
 Vaccine :GTACGCCAATCGGATACACTCTTTAATCTGCATTTCGCACTTCCCGTTTTTCACTGTATGGGTTTCATT

110350

FIG. 3. Comparisons of DNA sequences in the origin region. An almost palindromic sequence (underline) is located in the middle of this region. P-Oka and V-Oka have deletions, (TA)₄ and (TA)₇, in the palindromic sequence, respectively. The other deletion site is in the (GA) repeat just after the palindromic sequence.

base numbers of elements were multiples of 3. R4 is located in both the IRS and TRS regions and consists of a single element, a, which is 27 bp in length, and a partial copy of it, x, which is 11 bp in length. V-Oka had 9 repeat elements in the R4 region, whereas P-Oka had 10 elements (Fig. 2c).

Finally, the VZV genome contains origin regions of replication in both the IRS and the TRS regions, and each origin region is ca. 270 bp in length (Fig. 3). P-Oka contained 269 bp in this region and was 8 bp longer than V-Oka. The Dumas strain has an almost perfect palindrome of 45 bp in the middle of the origin region (Fig. 3, underline) (9, 54), and both Oka viruses had additional sequence following the palindrome. P-Oka had part of the palindromic sequence (TA)₁₂ and just following (GA)₁₀, whereas V-Oka had a different sequence (TA)₉ and (GA)₉. The genome size of V-Oka was slightly longer than that of P-Oka due to these insertions and deletions. The whole genomes of V-Oka and P-Oka consisted of 125,076 and 125,069 bp, respectively.

Transactivational activity of ORF 10 protein containing an amino acid substitution. ORF 10 protein, which is a homolog of HSV-1 VP16, is a virion-associated transactivator (24). It is reported that the ORF 10 protein can stimulate the IE62 gene promoter but has no regulatory effect on the gene 4 promoter (43). A base substitution that existed within the *Hind*III site in ORF 10 caused an amino acid conversion in which an Ala in the middle of the ORF 10 protein in P-Oka was altered to a mixture of Ala and Val in V-Oka. When the PCR product containing the site from V-Oka was digested by *Hind*III, approximately half of the product was cleaved similar to P-Oka. To compare the transcriptional activity of the ORF 10 protein from clones with the amino-acid substitution, a transient luciferase assay was performed. The effector plasmids pCG10(+) and pCG10(-) had Ala and Val at the substituted positions, respectively. ORF 10 proteins from the V-Oka and P-Oka viruses could both transactivate the gene 62 promoter in CV1 cells in a dose-dependent fashion. The transactivational activity of pCG10(-) on the gene 62 promoter was slightly higher than that of pCG10(+), but this difference was not significant.

Plaque sizes and infectious center assay of cloned viruses.

V-Oka was a mixture of different clones (15, 16). To obtain clones of V-Oka, plaque purification was carried out and 13 clones were obtained. Because we found 20 base replacements that led to amino acid differences between V-Oka and P-Oka as described above, these clones were sequenced. In gene 62, one of the clones, S7-01, had all eight amino acid substitutions compared to P-Oka. In contrast, another clone, S7-13, had the minimum number of substitutions compared to P-Oka, with only five amino acid differences, and the remaining three amino acids conserved with P-Oka (Table 3). Thus, these two clones were chosen to determine whether the mutations in gene 62 were correlated with viral growth. A comparison of the plaque sizes of S7-01, S7-13, V-Oka, and P-Oka showed that P-Oka gave rise to the largest plaques (Fig. 4). V-Oka and S7-13 showed relatively larger plaques than S7-01. In particu-

TABLE 3. Amino acid substitutions in clones S7-01 and S7-13 relative to V-Oka and P-Oka

Position	Gene	Amino acid substitution(s)			
		Oka parental	Oka vaccine	S7-01	S7-13
5745	6	A (Ser)	G (Pro)	G (Pro)	G (Pro)
10900	9A	T (Trp)	T/C (Trp/Arg)	T (Trp)	C (Arg)
12779	10	T (Ala)	T/C (Ala/Val)	C (Val)	C (Val)
31732	21	C (Thr)	C/T (Thr/Ile)	T (Ile)	C (Thr)
58595	31	A (Ile)	A/G (Ile/Val)	G (Val)	A (Ile)
71252	39	T (Met)	T/C (Met/Thr)	C (Thr)	C (Thr)
87306	50	T (Ser)	T/C (Ser/Gly)	T (Ser)	T (Ser)
90535	52	A (Ile)	A/G (Ile/Val)	A (Ile)	G (Val)
97748	55	G (Ala)	G/A (Ala/Thr)	G (Ala)	A (Thr)
97796	55	T (Cys)	T/C (Cys/Arg)	C (Arg)	T (Cys)
101089	59	A (Leu)	A/G (Leu/Pro)	A (Leu)	A (Leu)
105310	62	A (Leu)	A/G (Leu/Ser)	G (Ser)	G (Ser)
105356	62	T (Ile)	C (Val)	C (Val)	C (Val)
105544	62	A (Val)	G (Ala)	G (Ala)	G (Ala)
106262	62	A (Arg)	C (Gly)	C (Gly)	C (Gly)
107252	62	T (Ser)	C (Gly)	C (Gly)	C (Gly)
107599	62	A (Val)	A/G (Val/Ala)	G (Ala)	A (Val)
107797	62	A (Leu)	A/G (Leu/Pro)	G (Pro)	A (Leu)
108838	62	A (Met)	A/G (Met/Thr)	G (Thr)	A (Met)
111650	64	A (Gln)	A/G (Gln/Arg)	G (Arg)	A (Gln)

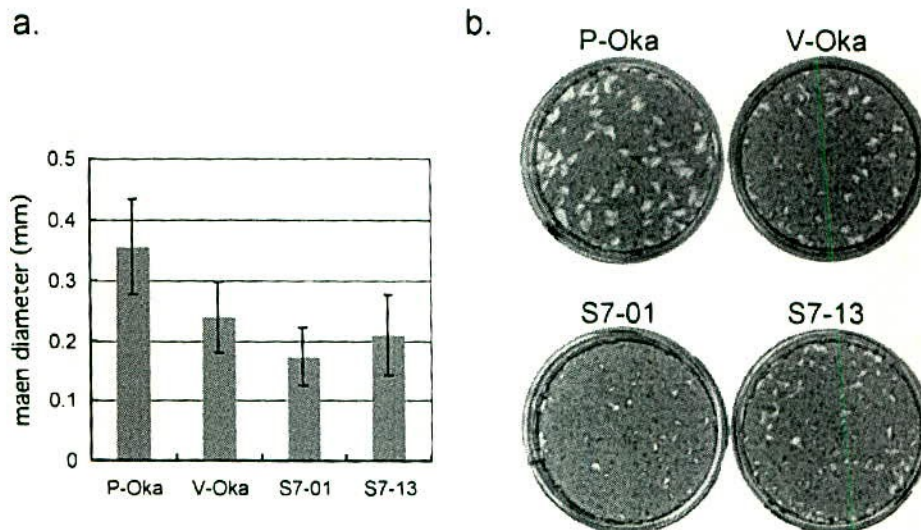


FIG. 4. (a) Plaque sizes after infection with cell-free P-Oka, V-Oka, S7-01, and S7-13. (b) HEL cells were infected with each cell-free virus and cultured for 10 days. The cells were stained with methylene blue.

lar, S7-01 showed significantly smaller plaques compared with P-Oka ($P < 0.01$); the mean diameter of the plaques resulting from S7-01 infection (1.73 mm) was about half the size of those resulting from P-Oka (3.55 mm). V-Oka and S7-13 had smaller plaque diameters (2.40 and 2.10 mm, respectively) than did P-Oka. In addition, the cytopathic effect (CPE) of P-Oka appeared 2 days after infection, whereas the CPE of the other viruses appeared in 3 to 4 days. Thus, an infectious center assay was performed to examine the difference in cell-to-cell spreading of progeny viruses. HEL cells were infected with P-Oka, V-Oka, S7-01, or S7-13, and the numbers of infected cells from 0 to 5 days were titrated. In this assay, P-Oka exhibited an obviously faster spread than the other viruses in HEL cells. After 5 days, the numbers of cells infected by P-Oka were 2.1-, 4.2-, and 2.6-fold higher than the numbers infected by V-Oka,

S7-01, and S7-13, respectively (Fig. 5a). These results suggested that the virus growth was slightly different among the V-Oka clones and that P-Oka grew best among these Oka viruses in HEL cells. On the other hand, in GPE cells, the plaque sizes of the viruses derived from Oka vaccine were not significantly different from those of its parental viruses (data not shown). Moreover, both Oka viruses showed similar propagation in cell-to-cell spreading by infectious center assay in GPE cells (Fig. 5b).

Transactivational activities of mutant IE62s. We next analyzed whether the transactivational activities of the IE62s of these clones differed from the parental activity. pLuc-Pol, containing the cloned VZV DNA polymerase gene promoter upstream of the luciferase ORF, was used as the reporter plasmid for transient luciferase assays, because the transactivation of

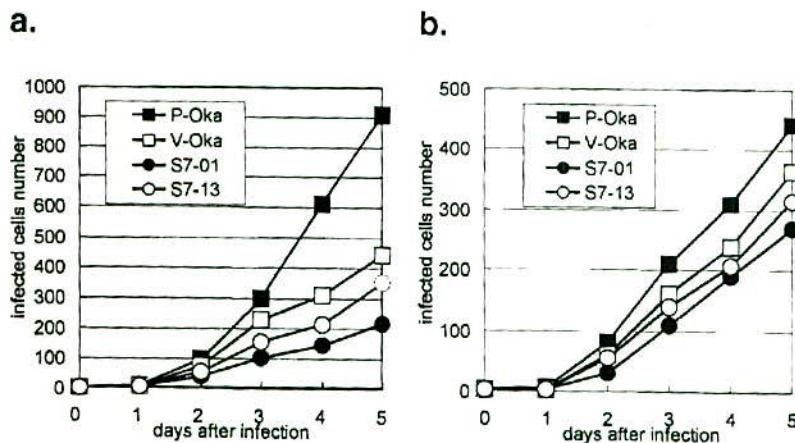


FIG. 5. Infectious center assay of P-Oka, V-Oka, S7-01, and S7-13. HEL cells (a) and GPE cells (b) in 35-mm dishes were infected independently with similar titers of these four kinds of cell-free viruses, and then the cells were washed and treated with trypsin from 1 to 5 days postinfection. The trypsin-treated cells were diluted and transferred onto monolayers of uninfected HEL cells in 35-mm dishes, and the numbers of infected cells were assessed by counting the number of VZV plaques appearing after 7 days. The number of infected cells was normalized to the initial viral titer per dish; the fold increase indicates the number of infected cells spread from one initial infected cell at day 0.

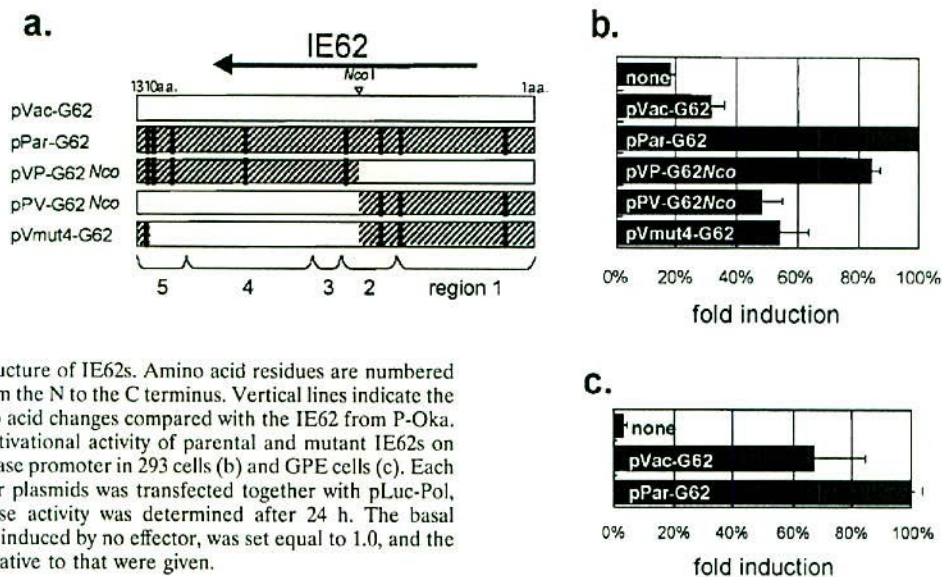


FIG. 6. (a) Structure of IE62s. Amino acid residues are numbered from 1 to 1310 from the N to the C terminus. Vertical lines indicate the positions of amino acid changes compared with the IE62 from P-Oka. (b and c) Transactivational activity of parental and mutant IE62s on the DNA polymerase promoter in 293 cells (b) and GPE cells (c). Each of the five effector plasmids was transfected together with pLuc-Pol, and each luciferase activity was determined after 24 h. The basal luciferase activity, induced by no effector, was set equal to 1.0, and the other activities relative to that were given.

this promoter by IE62 is well known. Five kinds of effector plasmids expressing IE62s were constructed (Fig. 6a). Two of them, pPar-G62 derived from P-Oka and pVac-G62 derived from V-Oka, were used in our previous study (15, 16). One clone containing an IE62 gene was selected from V-Oka and used as pVac-G62 because this clone had all eight amino acid mutations compared to pPar-G62. In addition, another clone was selected from V-Oka, called pVmut4-G62, which had the minimum four mutations; all of the other clones contained in V-Oka had these four mutations and one to four of the other mutations. pPV-G62 *Nco* and pVP-G62 *Nco* were chimeric IE62-expressing plasmids, which were bounded by an *NcoI* site. pPV-G62 *Nco* expressed the amino terminus of parental IE62 and the carboxyl terminus of pVac-G62. pVP-G62 *Nco* was constructed to contrast with pPV-G62 *Nco* and expressed the amino terminus of pVac-G62 and the carboxyl terminus of pPar-G62. The IE62 genes of P-Oka, S7-01, and S7-13 corresponded with those of pPar-G62, pVac-G62, and pPV-G62 *Nco*, respectively. V-Oka contained from four (like pVmut4-G62) to eight (like pVac-G62) amino acid mutations. The relative luciferase activity was set equal to 100% in cells, where the reporter plasmid pLuc-Pol was cotransfected with pPar-G62. The basal activity of the DNA polymerase promoter was 18% of the activity of parental IE62 (Fig. 6b). pVac-G62 and pVmut4-G62 showed 32 and 54% activities. Therefore, the mutant IE62s contained in the clones of V-Oka were likely to have lower activity than the parental IE62. The chimeric IE62 expressed by pVP-G62 *Nco* transfection possessed an activity that was still high (84%), whereas the other chimera expressed by pPV-G62 *Nco* showed 48% activity, suggesting that the first five amino acid mutations from the carboxyl terminus, rather than the other three mutations, were more important for transactivating the promoter. The mutation closest to the carboxyl terminus changed IE62's activity moderately but significantly. pPar-G62 and pVac-G62 exhibited the highest and lowest activities among the forms we analyzed, respectively, and pPV-G62 *Nco* led to more effective luciferase expression than pVac-G62 but was not as effective as pPar-G62. These transient assay

results roughly showed trends that were consistent with both the mean plaque sizes and the square roots of the number of infected cells determined in the infectious center assay. These findings support the possibility that viral growth and spreading may be regulated by the transactivational activity of IE62.

Transient luciferase assay with GPE cells were also performed to analyze the transactivational activities of the Oka vaccine IE62 and its parental IE62 in these cells. The assay showed that pVac-G62, the most heavily substituted, had 67% of the activity of pPar-G62 (Fig. 6c), suggesting that the transactivational activity of the IE62 of V-Oka was relatively higher in GEF cells compared to that found in other cells as described above.

Differentiation of the DNA sequence in gene 62 among clinical isolates. Comparison of the DNA sequences of the Oka viruses showed that gene 62 was the most variable region in the VZV genes, as described above. Furthermore, V-Oka consisted of at least eight different virus clones (15). Thus, we determined the nucleotide sequences of gene 62 from clinical isolates and compared the sequences among different VZV strains (Fig. 7b). Four clinical isolates (RT-283, OZ-4, Inoue, and Kawaguchi) were derived from patients with varicella. The first two strains were recently isolated but the latter two were isolated more than 30 years ago. Three isolates (Z-4, Z-5, and Z-8) were obtained from patients with zoster. Only the RT-342 and RT-345 strains were obtained from vaccinated patients, as described in Materials and Methods. Seven bases (at positions 105356, 105544, 105705, 106262, 107136, 107252, and 108111) were conserved in the clinical isolates and were only different in V-Oka, and four of these led to amino acid substitutions that were specific for V-Oka. Some of the clones contained in V-Oka showed different bases at eight positions (i.e., 105169, 105310, 106710, 107599, 107797, 108838, 109137, and 109200) that were not seen in the clinical isolates, and four of them led to partial amino acid substitutions. In summary, all of the clinical isolates differed from V-Oka in 15 bases that were found to be different between V-Oka and P-Oka. Only one base substitution (at position 105451) causing an amino acid

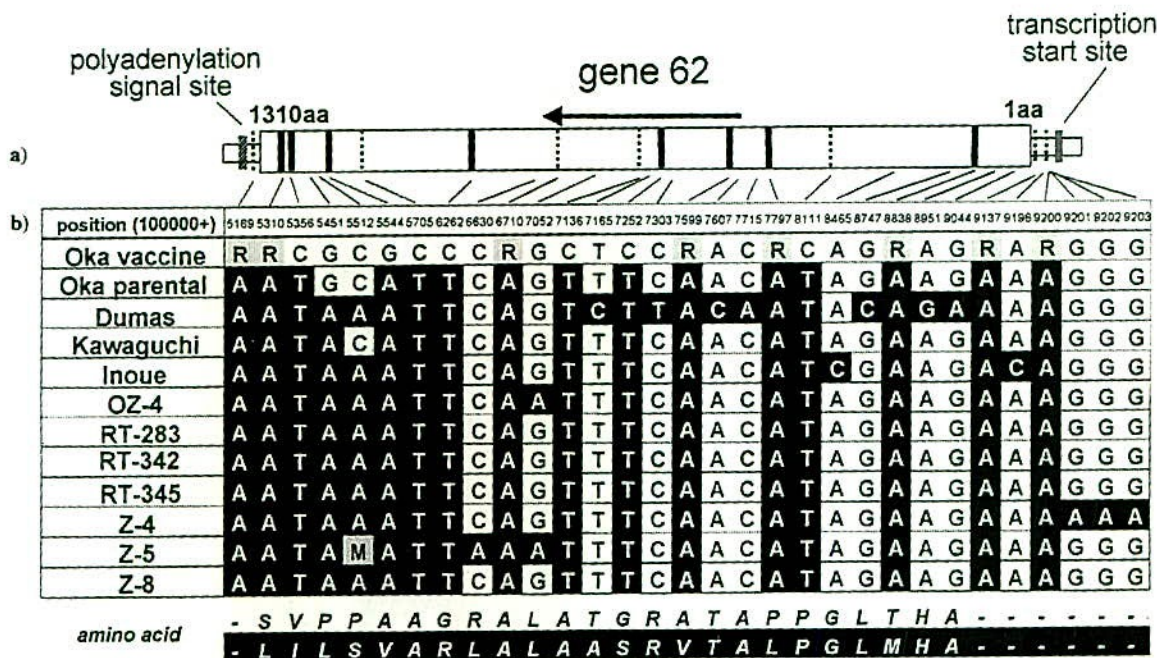


FIG. 7. (a) Structure of gene 62. Amino acid residues are numbered from 1 to 1310 from the N to the C terminus. Fifteen vertical lines indicate the positions of base changes between Oka vaccine and its parental virus. Bold or broken lines show base mutations with or without amino acid conversions, respectively. (b) Sequence analysis of gene 62 from the V-Oka vaccine, Oka parental, Dumas strains, and nine clonal isolates. Filled cells indicate base differences compared with V-Oka. All nine viruses have identical bases at these 15 positions, but V-Oka has different bases. R, mixture of G and A; M, mixture of A and C.

conversion was specific for the Oka viruses. Interestingly, an identical sequence was seen in RT-283, RT-342, and RT-345. These strains were isolated from patients in the same clinic; thus, it was likely that the same virus strain was circulating there.

Genetic differentiation of the Oka vaccine virus from other viruses by RFLP analysis. Comparison of the complete DNA sequences of the Oka viruses revealed 11 bases that were completely substituted by other bases. These bases (at positions 560, 5745, 26125, 94167, 105356, 105544, 105705, 106262, 107136, 107252, and 108111) were located within sites recognized by the restriction enzymes *Nla*III, *Alu*I, *Mae*II, *Bst*XI, *Sfa*NI, *Acc*II, *Sac*II, *Sma*I, *Bss*HII, *Nae*I, and *Bsr*I, respectively. A method for distinguishing V-Oka from other viruses, including P-Oka, was established in our previous study, which demonstrated that the cleavage sites for *Bss*HII and *Nae*I were useful for identifying V-Oka (15). Here we sought to establish new methods for distinguishing the viruses by using the eight other restriction enzyme sites, and V-Oka was successfully identified by using all of these enzymes except for *Nla*III and *Mae*II. First, the base substitution at position 5745 in gene 6, which encodes a putative subunit of the helicase-primase complex of V-Oka, resulted in the loss of a restriction site that is recognized by *Alu*I (Fig. 8a). Immediately adjacent to this position, another site recognized by this enzyme was present in every virus and was predicted to be cleaved. The PCR product containing these sites in gene 6 of V-Oka was cleaved at only one site with the enzyme, resulting in two fragments, whereas that of P-Oka was cleaved at two sites by the enzyme, resulting in three fragments. The RFLP study was further performed with 54 clinical isolates. The results of this PCR-RFLP analysis

demonstrated that the clinical isolates tested did not show a pattern identical to that of V-Oka. Similar results were obtained in six other experiments. P-Oka and all 54 clinical isolates contained cleavage sites for *Sfa*NI and *Bsr*I, whereas only V-Oka lost those sites. In contrast, four other sites, which were recognized by *Bst*XI, *Acc*II, *Sac*II, and *Sma*I, were specific only for V-Oka (Fig. 8b to g) but not for the clinical isolates. Moreover, V-Oka could be distinguished from five clinical isolates such as RT-342 and RT-348, which were from vaccinated patients with clinical symptoms, by using some of these restriction enzyme sites in gene 62 (data not shown). Therefore, these findings suggested that these clinical symptoms were caused by wild-type viruses and not by V-Oka.

DISCUSSION

We found here that the VZV DNA sequences were quite highly conserved (>99.8%) among the Dumas strain, P-Oka, and V-Oka. In particular, there was only a 0.034% sequence difference between the two Oka viruses. However, clinical reactions after vaccination with the Oka varicella vaccine are very rare in healthy children. It was reported that V-Oka grows better than wild-type viruses, including the Oka parental virus, in GPE cells (34) and that the immune response in guinea pigs could be induced by V-Oka but not by wild-type strains (61). These results indicate that a sequence difference of ca. 0.034% of can make a biological difference in such properties as the pathogenicity and growth of VZV. In this and previous studies, gene 62 has emerged as having the potential to be involved in the attenuation of the Oka virus, based on the following remarkable features of V-Oka. First, gene 62 shows the highest

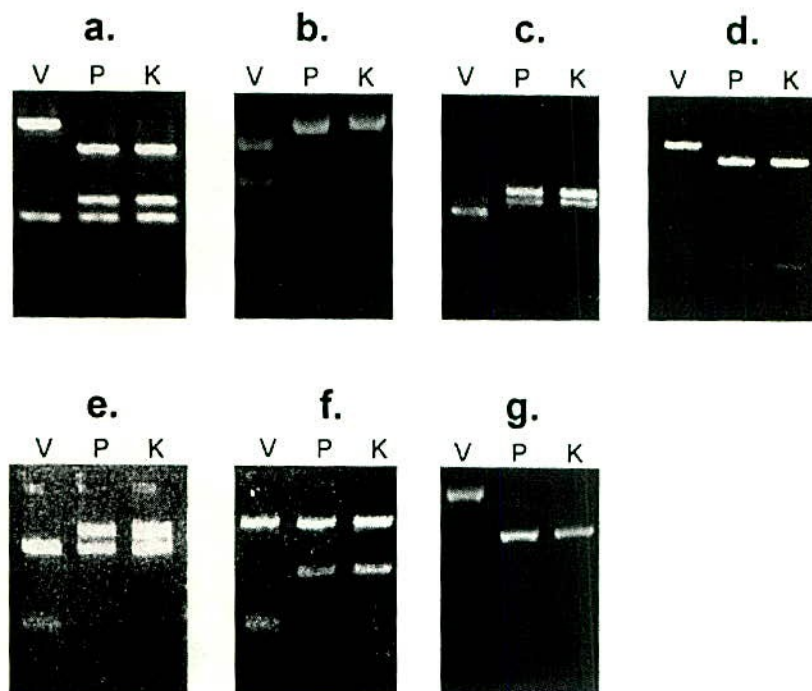


FIG. 8. RFLP analysis of the PCR products from V-Oka, P-Oka, and 54 wild-type viruses. V-Oka (V) can be distinguished not only from P-Oka (P) but also from all wild-type viruses. The Kawaguchi strain (K) was used to represent the wild-type viruses by using *AluI* (a), *BsrXI* (b), *AccII* (c), *SfaNI* (d), *SacII* (e), *SmaI* (f), and *BsrI* (g).

divergence in both base and amino acid sequences when V-Oka is compared to P-Oka. In particular, comparison of the Oka viruses revealed that gene 62 and its duplicate gene (gene 71), which cover only 7% of the whole genome, contained more than one-third of the base substitutions, although the other three IE genes (genes 4, 61, and 63) had no substitutions. Second, comparisons of gene 62 from nine clinical isolates showed that all 15 base substitutions leading to eight amino acid conversions were specific for V-Oka. Third, the sequence analysis of a fragment including the gene 62 region from nine clones revealed that V-Oka is a mixture of at least eight different viruses that had a variety of mutations (15). Fourth, our previous studies had demonstrated that the vaccine IE62, which contained all eight amino acid substitutions, had less transactivational activity than parental IE62 in activating all three kinetic classes of VZV promoters in CV1 cells (15, 16). Fifth, S7-01 virus, which had mutations in all eight amino acids in IE62, showed slower virus spreading in HEL cells. Thus, the substitutions that have accumulated in gene 62 are most likely to be important for the differences in the replication and the attenuation of VZV. Because V-Oka had been passaged in guinea pig cells and also in human fibroblast cells at a low temperature (56), mutant viruses must have been selected and grown under selective pressure. The reason why so many amino acid substitutions were accumulated in gene 62 of V-Oka is still unclear, but it is possible that the mutant IE62s contained in V-Oka may have a competitive advantage over parental IE62 in interacting with some cellular transcription factors in guinea pig cells. The five amino acid substitutions closest to the carboxyl terminus in IE62 directly reduced its transactivational activity, and these locate in regions 4 and 5.

Region 2 contains the DNA-binding domain (5), which is located between amino acids 472 and 646 (59, 60) and is essential for the protein's regulatory functions (58). A substitution of Lys residue 548, which lies within a highly conserved quartet of residues, WLQN, in region 2 drastically reduces the DNA-binding activity (58). Although the DNA-binding region of V-Oka had three amino acid mutations, no substitutions were found in the WLQN region. There were also no amino acid substitutions in other domains of IE62 that are important for its transcriptional activity, such as the proline-rich domain between regions 4 and 5 (5), the nuclear localization signal (5, 26), the potent activation domain in the N terminus (48), the kinase catalytic domain, and the ATP-binding domain (23). Thus, further studies are needed to determine the mechanism by which the expression of VZV genes is affected by the amino acid substitutions in IE62.

We detected insertions and a deletion in three repeat regions—R1, R3, and R4—that could potentially lead to different biological behaviors in V-Oka. R1 and R3 lie in ORF 11 and ORF 22, respectively, but both of these reiteration elements are multiples of 3 bp, so there were no frameshifts. The function of the ORF 11 protein is unclear, although it is homologous to HSV-1 UL47, a nucleotidylated tegument phosphoprotein reported to modulate the activity of VP16 (6, 63, 64). It is difficult to differentiate V-Oka from wild-type viruses in Japan by analyzing R1 because a predominant R1 structure is contained in both the wild-type viruses and V-Oka (62). The R1 sequence in V-Oka determined by us is also identical to that in some viruses from patients with acute retinal necrosis syndrome (1). Thus, the deletion in the R1 region is not likely to be important for viral pathogenesis. R3 is unstable, as de-

scribed above, and lies within the longest ORF in VZV, ORF 22. The ORF 22 product is homologous to the UL36 virion tegument phosphoprotein (36, 37). It is not clear whether the variable R3 exerts a function in ORF 22, because little is known about this gene. R4 lies in a noncoding region situated ca. 550 bp upstream from the transcription start site, ca. 620 bp upstream from the initiator codon, of gene 62. It is possible that the deletion of R4 in V-Oka affects the expression level of IE62 despite its far distance from the transcription start site. In contrast, Perera et al. (46) reported that IE62 could autoactivate a truncated IE62 promoter that contained only 45 bp upstream of the transcription start site and suggested that IE62 self-activation might be mediated by a TATA sequence-dependent mechanism. Thus, at least the autoactivating activity of IE62 is independent of R4. Finally, a comparison of the Oka viruses showed that V-Oka had a deletion of (TA)₃ and GA in the origin regions in IRS and TRS. Plasmid replication experiments showed that the yields of deletion mutants that lost (TA)₆ and (TA)₁₀ in the corresponding region from the Dumas virus were 45 and 18%, respectively (54). Thus, the deletion in V-Oka may cause a reduced replication of VZV. Further studies are required to clarify the correlation between these candidates, including IE62, and pathogenicity. Recombinant virus could be prepared from viral DNA clones as previously reported (7, 32). Viral pathogenesis and proteins that are important for virus replication could be analyzed by using two systems: the SCID-hu mouse implanted with human fetal tissue (39, 40) and human mononuclear cells in umbilical cord blood infected with VZV (53). Studies are in progress to examine the pathogenesis of the IE62 mutant virus. The sequence differences presented here and the application of these techniques may provide new insights into mechanisms for the molecular pathogenesis of VZV infection.

Interestingly, differences in virus spreading to HEL cells were found among P-Oka, V-Oka, S7-01, and S7-13. P-Oka is known to be as virulent *in vivo* as other wild-type viruses. Naturally infected children who develop varicella have viremia that is detectable by virus isolation, whereas no viruses are isolated from healthy children immunized by the vaccine (3). Despite the different infection routes in these situations, the findings suggest that the vaccine virus cannot propagate *in vivo* to the same level as wild-type viruses. The behavior of V-Oka and its constituents both *in vitro* and *in vivo* differ from that of P-Oka and the wild-type viruses. Our data showed that viral pathogenicity was affected in association with 20 amino acid substitutions and slow spreading was associated with 15 of them, in ORFs 6, 10, 21, 31, 39, 55, 62, and 64, in the S7-01 virus. IE62 may be the most attractive candidate for causing the different biological properties of the vaccine. S7-01 had 14 amino acid substitutions, including all 8 amino acid substitutions in IE62. The IE62 of S7-01 is identical to a mutant IE62 that exhibited less transcriptional activity than the IE62 of P-Oka in CV1 cells in our previous study (15, 16), and similar results were obtained with the human 293 cell line (data not shown). It is possible that the lower activity of the mutant IE62 may be involved in the ineffective spreading or, moreover, the avirulence of the vaccine. On the other hand, S7-01 did not have mutations in genes 9A, 50, 52, 55, or 59. P-Oka not only is known to be virulent *in vivo* but also was more efficient in spreading *in vitro* than was S7-01 in the present study. Thus,

the amino acid mutations in these gene products, which were the ORF 9A protein, two helicase-primase components, gM, and uracil-DNA glycosylase, did not seem to be important for efficient spreading. On the other hand, S7-13 exhibited moderately better growth than did S7-1, based on the analysis of plaque sizes and spreading. Seven amino acids, in genes 21, 31, 55, 62, and 64, of S7-13 were found to be identical with P-Oka but not with S7-01. Three of these amino acids were in IE62, and when we expressed this mutant IE62 from pPV-G62Nco, it was shown to have <50% of the activity of parental IE62. These data indicate that the difference in viral spreading was likely to have been determined by the three mutations in IE62 rather than the four mutations in other gene products, ORF21 protein, gB glycoprotein, helicase-primase component, or ORF64 protein. Studies with recombinant VZVs will be needed to confirm the amino acid substitution(s) responsible for the differences in spreading activity.

Several candidates other than IE62 are potentially responsible for the biological differences in different forms of the virus. First, ORF 10 protein transactivates the IE62 gene promoter, although it does not transactivate the IE ORF 4 and ORF 61 promoters (43, 44). In the present study, both the parent and mutant ORF 10 proteins stimulated the IE62 promoter, and the amino acid substitution in ORF 10 failed to affect its transactivation activity. Thus, the mutant ORF 10 protein is unlikely to cause attenuation by repressing the expression level of IE62. Second, there were four amino acid substitutions in ORFs 6, 52, and 55, which are homologous to HSV-1 UL52, UL8, and UL5, respectively. ORFs 52 and 55 encode two kinds of protein each, as a result of partial amino acid substitutions, whereas ORF 6 is a single clone, as shown by RFLP analysis, indicating that eight (i.e., 2³) isomeric complexes may exist by combining these three proteins. It is possible that different helicase-primase activities might exist among different isomeric complexes. Third, a base substitution led to an amino acid conversion in the uracil-DNA glycosylase. If this amino acid substitution were responsible for making the base substitutions in gene 62 in V-Oka, then uracil residues should misincorporate randomly in all of the genes, and substitutions should accumulate in dispensable genes rather than in essential genes. Nonetheless, more than one-third of the substitutions accumulated in gene 62, the homologue of HSV-1 ICP-4, which is essential *in vitro* (31). Therefore, the amino acid substitution in uracil-DNA glycosylase is not involved in promoting mutations during replication.

VZV glycoproteins are known to induce a strong humoral response both in natural infection and in vaccination with the Oka varicella vaccine. VZV produces at least seven glycoproteins, as described above. The expression level of gC, the gene 14 product, was dependent on the strain of VZV, and the gC level of V-Oka was much lower than that of wild-type viruses (25, 30). SCID-hu mouse experiments showed that a decrease in gC played a critical role in attenuation (39). However, we found no differences in its ORF or flanking regions except for a silent substitution in a termination codon of the gC gene: an ochre (TAA) incompletely altered to an amber (TAG). In our earlier study we found no evidence that the decrease in gC in V-Oka was caused by amino acid substitutions in IE62 because the gC promoter was unresponsive to IE62. The transactivational activity of IE62 is negatively regulated at later stages of

the infectious cycle by its phosphorylation by the ORF66 protein kinase, which inhibits its nuclear import (23, 26). Therefore, vaccine IE62 is not directly involved with the decrease in gC by transactivating the gene to a lesser extent. We found amino acid substitution in only gB of V-Oka; i.e., an Ile in the gB of P-Oka was Ile plus Val in V-Oka. gB is the second most abundant and immunogenic glycoprotein in VZV. It appears to play a role in the attachment and penetration of particles, along with gH and gC, and can elicit a monoclonal antibody that neutralizes viral infectivity in vitro. VZV gB is associated with cell-to-cell infection, as is HSV-1 gB (41, 45). gB was also shown to have great fusogenic properties in the presence of gE (33). Nonetheless, it is thought that the amino acid substitution in gB is not crucial for the antigenicity of the vaccine because about half of the clones in V-Oka contain identical sequences to P-Oka in this gene; moreover, Ile and Val have similar properties, and this substitution would be unlikely to change the protein's properties in the remaining half of the clones. These findings indicate that the cell-mediated and humoral immunities led by the glycoproteins of V-Oka are identical to those of P-Oka without adapting to the heterogeneous host, guinea pig cells. V-Oka is likely to have few functional differences in viral attachment and penetration from P-Oka. Although it was reported that V-Oka shows a higher adsorption rate than P-Oka in GPE cells as determined by the infectious center assay (34), the higher adsorption of V-Oka appeared to be caused by the virus's capacity to replicate in guinea pig cells but not by the substitution in gB.

Some cases of chickenpox or shingles in immunocompromised individuals after vaccination have been reported. In rare cases, even healthy vaccine recipients develop rashes within a few weeks after vaccination. However, it has not been clear whether these cases were caused by the vaccine virus or by a wild-type virus. In addition, some reports concerning transmission of the vaccine virus have been reported (21, 29, 51). Therefore, it is epidemiologically important to differentiate the Oka vaccine virus from circulating wild-type viruses. At present, the Oka viruses, which include V-Oka and P-Oka, are identified by using restriction endonuclease digestion of extracted purified viral DNA (14, 19), analysis of repeat regions (17, 55), and RFLP assays after PCR (17, 20, 27, 28, 55, 57). We also have reported an identifying method that combines the single-strand conformational polymorphism of repeat region 2 (R2) with *Pst*I cleavage of the *Pst*I-site-less region (42). Although the Oka viruses can be distinguished from other clinical isolates by these methods, it is difficult to distinguish V-Oka from P-Oka. Therefore, we attempted to identify only V-Oka by another method that uses the simplified RFLP analysis by *Nae*I and *Bss*HIII (15, 16). This method was established based on the first known sequence differences we found in gene 62, and V-Oka could be distinguished not only from clinical isolates but also from P-Oka. Therefore, comparisons of the gene 62 sequence may enable the epidemiological typing of VZV strains. Thus, the comparison of the DNAs is important for quality control of varicella vaccine, molecular epidemiology and, in particular, the development and improvement of new vaccines.

The mechanism of attenuation is still unclear because the mutations were found not only in gene 62 but also in other genes. Therefore, further study to analyze more in detail by

using recombinant viruses and suitable animals are needed to resolve the mechanism of attenuation of VZV.

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Complete DNA Sequences of Two Oka Strain Varicella-Zoster Virus Genomes[▽]

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Varicella-zoster virus (VZV) is a herpesvirus and is the causative agent of chicken pox (varicella) and shingles (herpes zoster). Active immunization against varicella became possible with the development of live attenuated varicella vaccine. The Oka vaccine strain was isolated in Japan from a child who had typical varicella, and it was then attenuated by serial passages in cell culture. Several manufacturers have obtained this attenuated Oka strain and, following additional passages, have developed their own vaccine strains. Notably, the vaccines Varilrix and Varivax are produced by GlaxoSmithKline Biologicals and Merck & Co., Inc., respectively. Both vaccines have been well studied in terms of safety and immunogenicity. In this study, we report the complete nucleotide sequence of the Varilrix (Oka-V_{GSK}) and Varivax (Oka-V_{Merck}) vaccine strain genomes. Their genomes are composed of 124,821 and 124,815 bp, respectively. Full genome annotations covering the features of Oka-derived vaccine genomes have been established for the first time. Sequence analysis indicates 36 nucleotide differences between the two vaccine strains throughout the entire genome, among which only 14 are involved in unique amino acid substitutions. These results demonstrate that, although Oka-V_{GSK} and Oka-V_{Merck} vaccine strains are not identical, they are very similar, which supports the clinical data showing that both vaccines are well tolerated and elicit strong immune responses against varicella.

Varicella-zoster virus (VZV) is a human alphaherpesvirus that causes chicken pox (varicella) and shingles (herpes zoster) (75). VZV has a linear, double-stranded DNA genome of approximately 125 kb that encodes at least 71 proteins (12). Primary infection with VZV results in varicella, which is a widespread, highly contagious disease. Varicella is commonly regarded as a mild childhood illness, but it may lead to serious complications, such as secondary bacterial infection, pneumonia, encephalitis, congenital infection, and death (76).

Like other herpesviruses, VZV has the capacity to persist in the body after the primary acute infection as a latent infection in sensory nerve ganglia. This lifelong latent infection commonly reactivates to cause herpes zoster, typically in elderly or immunocompromised patients (65).

In 1974, Takahashi et al. reported the development of a live-attenuated varicella vaccine through serial passages of wild-type virus in cell culture (67). The parental virus, Oka-P, was isolated in primary human embryo lung cell culture from vesicle fluid from a 3-year-old boy with typical varicella. The virus was attenuated by 10 passages in HEL and 12 passages in guinea pig embryo cells, plaque-purified, and passaged five times further in human diploid cells (WI38) to prepare a strain suitable for use as a vaccine (Oka-V) (Fig. 1) (67).

The Oka-V strain was first supplied in 1976 under license

from the Biken Institute in Japan. Several manufacturers (SmithKline RIT, Merck Sharp & Dohme, and Pasteur Mérieux) subsequently used the Oka-V strain in the development of proprietary vaccines. A product license was obtained for Varilrix (frozen formulation) in 1984 by SmithKline RIT for use in groups at high risk for severe varicella and their healthy close contacts. SmithKline RIT—now GlaxoSmithKline (GSK) Biologicals—subsequently developed a refrigerator-stable formulation of this varicella vaccine. Varilrix is indicated in many countries for use in healthy and immunocompromised subjects from 9 months of age. GSK Biologicals' varicella vaccine production is based on the seed lot system (6, 14) using classical cell culture methods (Fig. 1). A manufacturer's working cell bank of human diploid cells, MRC-5, was prepared and tested according to World Health Organization requirements.

The Biken vaccine was licensed in Japan and Korea, in 1986 and 1988, respectively, for use in healthy subjects, and a license for Varivax with the same indication was granted in the United States in 1995 (1). In 1993, the vaccine manufactured by Pasteur Mérieux was licensed in France for use in potentially immunocompromised subjects.

Although the varicella vaccine is licensed in many countries, it is not routinely used because complications associated with varicella disease are often underestimated. Universal mass vaccination against varicella is implemented only in few countries; however, it is under consideration in many others (38, 40, 54, 72). The incidence of varicella disease and the rate of varicella-related hospitalizations in the United States have declined by about 80% since implementation of universal mass vaccination against varicella (using Varivax) in 1996 (8, 16, 81). A similar

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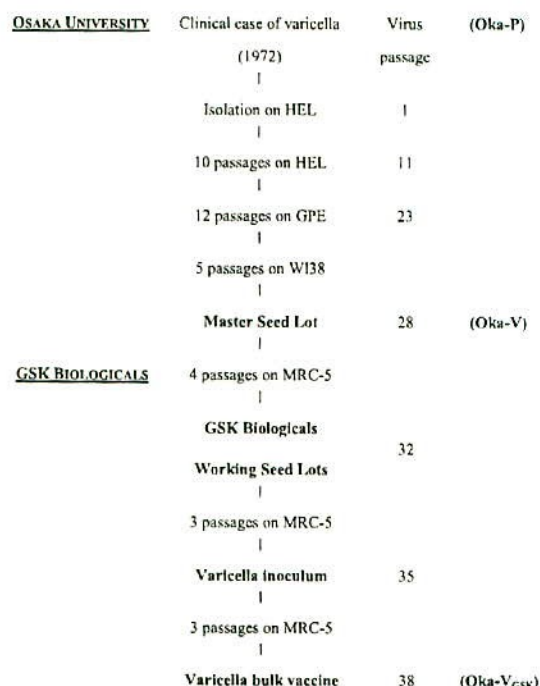


FIG. 1. Passage history of the live attenuated Oka varicella vaccine. HEL, human embryonic lung cells; GPE, guinea pig embryo cells; WI38 and MRC-5, human diploid cells.

decrease was observed in Uruguay since the introduction of varicella vaccination (using Varilrix) into the routine childhood immunization program in 1999, with the greatest reduction in children aged 1 to 4 years (51). Most pre- and postlicense studies showed that vaccination with one dose of varicella-containing vaccine provides 70% to 90% protection from chicken pox and over 95% protection against the most severe forms of the disease for a 7- to 10-year period after vaccination (2, 17, 33, 33a, 34, 40, 61). However, vaccine-induced immunity wanes over time (9), leading countries such as the United States to recommend a two-dose schedule for varicella vaccination (40). This strategy aims to overcome primary vaccine failures and to improve long-term protection, thereby reducing the risk of breakthrough varicella (4). Combined vaccine products containing the VZV Oka strain have been developed as well. For instance, GSK Biologicals and Merck & Co., Inc., developed combined tetravalent measles-mumps-rubella-varicella vaccines (Priorix-Tetra and ProQuad, respectively), providing the benefits of measles-mumps-rubella and varicella vaccination in a single injection (19, 30, 35, 48, 71, 72, 79).

Different sets of serological readouts have been used to characterize the adaptive humoral immune response after varicella vaccination or infection (4, 13, 26, 31, 34, 58, 73, 74). Comparative analysis has raised the possibility that differences in the genetic code between the vaccine strains could be responsible for disparity in vaccine-induced humoral responses (36).

Oka-V, and presumably its derivative vaccine strains, was not cloned during the development and the preparation of vaccine (67). Sequencing of the complete genome of the orig-

inal Oka-V vaccine preparation revealed that it contained multiple variants that could be separated in cell culture (20, 22).

The aim of the present study was to analyze the complete consensus nucleotide sequences of Oka-V strain viruses contained in Varilrix (GSK Biologicals; Oka-V_{GSK}) and Varivax (Merck & Co., Inc.; Oka-V_{Merck}) and to compare them to the published sequences of Oka-V and Oka-P (22). The full-length genomic sequences were also compared to published partial sequencing information on Oka-V_{GSK} and Oka-V_{Merck} (3, 32, 60, 63).

MATERIALS AND METHODS

Nucleic acid extraction. Total DNA was extracted from a single vial of recent production lots of Varilrix (lot VAV10118, produced in April 2002; GSK Biologicals, Rixensart, Belgium) and Varivax (lot 0895 M, purchased in 2003; Merck & Co., Inc., Whitehouse Station, NJ) vaccines using a High Pure viral nucleic acid kit from Roche (Basel, Switzerland). In brief, 100 μ l of sample was lysed in a lysing-binding buffer in the presence of proteinase K. The lysis mixture was then applied to a glass fiber filter, which binds the nucleic acids in the presence of the lysis and binding buffer containing chaotropic salts. Bound nucleic acids were eluted in 50 μ l of nuclease-free water by centrifugation and stored at -70°C .

PCR. Around 540 primers were designed using Primer D software (GSK in-house software) and the nucleotide sequence of the Dumas strain (GenBank accession no. X04370) (12). Overlapping primers were designed approximately 500 bases apart to cover the entire genomic sequence of VZV. Sequences of primers used for amplification and sequencing are available upon request. The reaction mixtures for PCR contained 15 μ l of HotStarTaq Plus Master Mix solution (Qiagen, Valencia, CA), 0.3 μ M of each primer, and 5 ng of template DNA. A Tetrath thermal cycler (MJ Research, Waltham, MA) was used for all amplifications. An initial hot-start PCR step of 96°C for 15 min was followed by 35 cycles of amplification (95°C for 20 s, 55°C for 30 s, and 72°C for 45 s) and a final elongation step at 72°C for 3 min. All amplified products were then purified using QIAquick PCR purification kit (Qiagen). Direct sequencing of both DNA strands was performed on the generated amplicons.

Sequencing. Direct sequencing of purified PCR products and plasmid DNA was performed with BigDye Terminator cycle sequencing kit and a 3730XL genetic analyzer (both from Applied Biosystems, Foster City, CA). The viral sequences were compiled and analyzed with Sequencher software (Gene Codes Corp, Ann Arbor, MI). The following GenBank sequences were used for comparison: for the European (The Netherlands) reference strain (Dumas), X04370 (12); for Oka-P, AB097933 (22); and for Oka-V, AB097932 (22), AF206304 (3), AY016450 (15), and the sequencing information provided by Schmidt et al. (60). Unless otherwise stated, all described nucleotide sequence positions in this paper correspond to the genome of Dumas strain, X04370 (12).

Cloning of PCR products. When direct sequencing did not generate information of sufficient quality or when particular single nucleotide polymorphisms (SNPs) could not be reliably confirmed, additional subcloning was performed, followed by sequencing of numerous generated clones to confirm the consensus sequence of the region. Direct sequencing of the PCR products derived from regions with highly complex secondary structure (flanking regions between internal repeat long and internal repeat short regions, and the R3 repeat region) was complemented by subcloning of amplicons and sequencing of plasmid clones. PCR products containing these regions were individually inserted into a pCR2.1 vector (Invitrogen, Carlsbad, CA) and then transformed into competent *Escherichia coli* by the TOPO TA cloning method (Invitrogen). The plasmid DNAs were purified from cultured bacteria with a QIAprep spin kit (Qiagen). DNA sequences of the cloned inserts were determined using vector-specific sequencing primers.

Sequencing of ends of the viral genomes. The direct sequencing data for viral genome ends were complemented by sequencing of overlapping amplicons generated after circularization using T4 DNA ligase (Roche). The PCR mixtures contained 500 μ M of each deoxynucleoside triphosphate, 10 pmol of each primer, and 2.5 U high-fidelity Platinum Taq polymerase (Invitrogen). PCR products were inserted into a pCR4-TOPO vector and transformed into competent *E. coli* TOP10 bacteria by the TOPO TA cloning method (Invitrogen). The plasmid DNAs were purified with a QIAprep spin kit (Qiagen). The consensus sequence of the cloned amplicons was confirmed by sequencing and alignment of multiple *E. coli* plasmid clones.

Nucleotide sequence accession numbers. The complete nucleotide consensus sequences of the Oka-V_{GSK} (Varilrix) and Oka-V_{Merck} (Varivax) strains are

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TABLE 1. Complete Oka-V_{GSK} genome annotation

Start	Stop	Feature ^a	ORF	Function or comment	Start	Stop	Feature ^a	ORF	Function or comment
88	89	Miscellaneous		TRL/UL boundary	65795	65800	Poly(A) signal		
914	587	Gene	1		64743	65768	CDS	36	Thymidine kinase
592	587	Poly(A) signal			65817	65821	3' end of dPyKmRNA		
914	588	CDS	1		66010	68552	Gene	37	
1133	1861	Gene	2		68747	68552	Poly(A) signal		
1133	1849	CDS	2		66010	68535	CDS	37	gH
1856	1861	Poly(A) signal			70229	68583	Gene	38	
2446	1889	Gene	3		68588	68583	Poly(A) signal		
1894	1889	Poly(A) signal			70229	68604	CDS	38	
2446	1907	CDS	3		70569	71305	Gene	39	
4140	2781	Gene	4		71300	71305	Poly(A) signal		
2781	2776	Poly(A) signal			70569	71291	CDS	39	
4140	2782	CDS	4	Transactivator, tegument protein	71476	75699	Gene	40	
5273	4251	Gene	5		75694	75699	Poly(A) signal		
5273	4251	CDS	5	gK	71476	75666	CDS	40	Major nucleocapsid protein
8576	5325	Gene	6		75783	76748	Gene	41	
8606	9398	Gene	7		76743	76748	Poly(A) signal		
9393	9398	Poly(A) signal			75783	76733	CDS	41	
8606	9385	CDS	7		77974	76791	Gene	42	
10666	9425	Gene	8		76786	76791	Poly(A) signal		ORF 45 + ORF 42
9430	9425	Poly(A) signal			77974	76787	CDS	42	
10666	9476	CDS	8	Deoxyuridine triphosphatase	78105	80136	Gene	43	
10641	10904	CDS	9A ^b	gN	80131	80136	Poly(A) signal		
11008	11963	Gene	9		78105	80135	CDS	43	
11958	11963	Poly(A) signal			80295	81449	Gene	44	
11008	11916	CDS	9	Synecium formation, virion protein	81444	81449	Poly(A) signal		
12159	13420	Gene	10		80295	81386	CDS	44	
13415	13420	Poly(A) signal			82529	81474	CDS	45	
12159	13391	CDS	10	Transactivator, tegument protein	82654	83253	CDS	46	
13589	16076	Gene	11		83103	84635	CDS	47	Protein kinase, tegument protein
13936	14196	Repeat region		Reiteration R1	84602	86257	CDS	48	
16071	16076	Poly(A) signal			86161	86429	Gene	49	
13589	16003	CDS	11		86424	86429	Poly(A) signal		
16168	18153	Gene	12		86161	86406	CDS	49	
18695	19350	Gene	13		87807	86466	Gene	50	
19345	19350	Poly(A) signal			86471	86466	Poly(A) signal		
18395	19300	CDS	13		87807	86500	CDS	50	
21067	19296	Gene	14		87806	90313	CDS	51	Origin binding protein
19301	19296	Poly(A) signal			90418	92771	Gene	52	
20526	20851	Repeat region		Reiteration R2	92766	92771	Poly(A) signal		
21067	19385	CDS	14		90418	92733	CDS	52	
22432	21198	Gene	15		93775	92775	Gene	53	
21203	21198	Poly(A) signal			92780	92775	Poly(A) signal		
22432	21212	CDS	15		93775	92780	CDS	53	
23748	22522	Gene	16		95909	93600	CDS	54	
24103	25468	Gene	17		95921	98566	CDS	55	
25463	25468	Poly(A) signal			98493	99280	Gene	56	
24103	25467	CDS	17		99275	99280	Poly(A) signal		
26444	25501	Gene	18		98493	99224	CDS	56	
25506	25501	Poly(A) signal			99548	99309	Gene	57	
26444	25524	CDS	18	Ribonucleotide reductase, small subunit	99314	99309	Poly(A) signal		
28796	26469	Gene	19	Ribonucleotide reductase, big subunit	99548	99333	CDS	57	Cytoplasmic protein
30426	28956	Gene	20		100194	99529	CDS	58	
28961	28956	Poly(A) signal			101141	100224	CDS	59	Uracil-DNA glycosylase
30426	28975	CDS	20		101574	101092	CDS	60	gL, chaperone for gH
30710	33856	Gene	21		104410	102926	Gene	61	
33851	33856	Poly(A) signal			102931	102926	Poly(A) signal		
30710	33826	CDS	21	Nucleocapsid	104410	103007	CDS	61	Transactivator, transrepressor
34034	42341	Gene	22	Reiteration R3	104849	104850	Miscellaneous		UL/IRL boundary
41405	41470	Repeat region			104938	104939	Miscellaneous		IRL/IRS boundary
42336	42341	Poly(A) signal			109061	105065	Gene	62	
34034	42325	CDS	22		105071	105065	Poly(A) signal		
43090	42378	Gene	23		109061	105129	CDS	62	Transactivator, tegument protein
42383	42378	Poly(A) signal			109693	109718	Repeat region		Reiteration 4
43090	42383	CDS	23		110017	110278	Origin of replication		Origin of replication
43973	43163	Gene	24		110507	111359	Gene	63	
43168	43163	Poly(A) signal			111352	111357	Poly(A) signal		
43973	43164	CDS	24		110507	111343	CDS	63	Tegument protein
44570	44083	Gene	25		111491	112072	Gene	64	
44088	44083	Poly(A) signal			112067	112072	Poly(A) signal		
44570	44100	CDS	25		111491	112033	CDS	64	
44458	46125	Gene	26		112571	112107	Gene	65	
46079	47195	Gene	27		112112	112107	Poly(A) signal		
47190	47195	Poly(A) signal			112571	112263	CDS	65	Virion protein
46079	47080	CDS	27		112263	112264	Miscellaneous		IRS/US boundary
50588	46983	Gene	28		112968	114172	Gene	66	
46988	46983	Poly(A) signal			114167	114172	Poly(A) signal		
50588	47004	CDS	28	DNA polymerase	112968	114149	CDS	66	Protein kinase
50809	54460	Gene	29		114427	115523	Gene	67	
54455	54460	Poly(A) signal			115518	115523	Poly(A) signal		
50809	54408	CDS	29	ssDNA binding protein	114427	115491	CDS	67	gI
54587	56899	Gene	30		115739	117652	Gene	68	
56944	59584	Gene	31		117647	117652	Poly(A) signal		
59579	59584	Poly(A) signal			115739	117610	CDS	68	gE
56944	59550	CDS	31	gB, fusogen	117498	117499	Miscellaneous		US/TRS boundary
59703	60150	Gene	32		118266	117690	Gene	69	
60145	60150	Poly(A) signal			117495	117490	Poly(A) signal		
59703	60134	CDS	32	Substrate for ORF 47 kinase	118266	117724	CDS	69	
62074	60245	Gene	33		119250	118400	Gene	70	
60250	60245	Poly(A) signal			118405	118400	Poly(A) signal		
62074	60257	CDS	33	Protease	119250	118414	CDS	70	Tegument protein
63846	62107	Gene	34		119479	119742	Origin of replication		Origin of replication
64689	63913	CDS	35		119921	120066	Repeat region		Reiteration R4
64300	64306	promoter		TATA element	120698	124694	Gene	71	
64321	64325	5' end of dPyKmRNA			124689	124694	Poly(A) signal		
64743	65800	Gene	36		120698	124630	CDS	71	Transactivator, tegument protein

^a CDS, coding sequence; dPyKmRNA, deoxypyrimidine kinase mRNA.
^b ORF was annotated according to the work of Gomi et al. (20)

TABLE 2. Complete Oka-V_{Merck} genome annotation

Start	Stop	Feature ^a	ORF	Function or comment	Start	Stop	Feature ^a	ORF	Function or comment
88	89	Miscellaneous		TRL/UL boundary	65793	65797	Poly(A) signal		
914	587	Gene	1		64741	65765	CDS	36	Thymidine kinase
592	587	Poly(A) signal			65814	65818	3' end of dPyKmRNA		
914	588	CDS	1		66007	68549	Gene	37	
1133	1861	Gene	2		68744	68549	Poly(A) signal		
1133	1849	CDS	2		66007	68532	CDS	37	gH
1856	1861	Poly(A) signal			70226	68580	Gene	38	
2446	1889	Gene	3		68585	68580	Poly(A) signal		
1894	1889	Poly(A) signal			70226	68601	CDS	38	
2446	1907	CDS	3		70566	71302	Gene	39	
4140	2781	Gene	4		71297	71302	Poly(A) signal		
2781	2776	Poly(A) signal			70566	71288	CDS	39	
4140	2782	CDS	4	Transactivator, tegument protein	71473	75696	Gene	40	
5273	4251	Gene	5		75691	75696	Poly(A) signal		
5273	4251	CDS	5	gK	71473	75663	CDS	40	Major nucleocapsid protein
8576	5325	Gene	6		75780	76745	Gene	41	
8606	9398	Gene	7		76740	76745	Poly(A) signal		
9393	9398	Poly(A) signal			75780	76730	CDS	41	
8606	9385	CDS	7		77971	76788	Gene	42	
10666	9425	Gene	8		76783	76788	Poly(A) signal		ORF 45 + ORF 42
9430	9425	Poly(A) signal			77971	76784	CDS	42	
10666	9476	CDS	8	Deoxyuridine triphosphatase	78102	80133	Gene	43	
10641	10904	CDS	9A ^b	gN	80128	80133	Poly(A) signal		
11008	11963	Gene	9		78102	80132	CDS	43	
11958	11963	Poly(A) signal			80292	81446	Gene	44	
11008	11916	CDS	9	Synctium formation, virion protein	81441	81446	Poly(A) signal		
12159	13420	Gene	10		80292	81383	CDS	44	
13415	13420	Poly(A) signal			82526	81471	CDS	45	
12159	13391	CDS	10	Transactivator, tegument protein	82651	83250	CDS	46	
13589	16076	Gene	11		83100	84632	CDS	47	Protein kinase, tegument protein
13936	14196	Repeat region		Reiteration R1	84599	86254	CDS	48	
16071	16076	Poly(A) signal			86158	86426	Gene	49	
13589	16003	CDS	11		86421	86426	Poly(A) signal		
16168	18153	Gene	12		86158	86403	CDS	49	
18695	19350	Gene	13		87804	86463	Gene	50	
19345	19350	Poly(A) signal			86468	86463	Poly(A) signal		
18395	19200	CDS	13		87804	86497	CDS	50	
21067	19296	Gene	14		87803	90310	CDS	51	Origin binding protein
19301	19296	Poly(A) signal			90415	92768	Gene	52	
20526	20851	Repeat region		Reiteration R2	92763	92768	Poly(A) signal		
21067	19385	CDS	14		90415	92730	CDS	52	
22432	21198	Gene	15		93772	92772	Gene	53	
21203	21198	Poly(A) signal			92777	92772	Poly(A) signal		
22432	21212	CDS	15		93772	92777	CDS	53	
23748	22522	Gene	16		95906	93597	CDS	54	
24103	25468	Gene	17		95918	98563	CDS	55	
25463	25468	Poly(A) signal			98490	99277	Gene	56	
24103	25467	CDS	17		99272	99277	Poly(A) signal		
26444	25501	Gene	18		98490	99221	CDS	56	
25506	25501	Poly(A) signal			99545	99306	Gene	57	
26444	25524	CDS	18	Ribonucleotide reductase, small subunit	99311	99306	Poly(A) signal		
28796	26469	Gene	19	Ribonucleotide reductase, big subunit	99545	99330	CDS	57	Cytoplasmic protein
30426	28956	Gene	20		100191	99526	CDS	58	
28961	28956	Poly(A) signal			101138	100221	CDS	59	Uracil-DNA glycosylase
30426	28975	CDS	20		101571	101089	CDS	60	gL, chaperone for gH
30710	33856	Gene	21		104407	102923	Gene	61	
33851	33856	Poly(A) signal			102928	102923	Poly(A) signal		
30710	33826	CDS	21	Nucleocapsid	104407	103004	CDS	61	Transactivator, transrepressor
34034	42341	Gene	22		104846	104847	Miscellaneous		UL/IRL boundary
41405	41470	Repeat region		Reiteration R3	104935	104936	Miscellaneous		IRL/IRS boundary
42336	42341	Poly(A) signal			109058	105062	Gene	62	
34034	42325	CDS	22		105068	105062	Poly(A) signal		
43088	42376	Gene	23		109058	105126	CDS	62	Transactivator, tegument protein
42381	42376	Poly(A) signal			109690	109715	Repeat region		Reiteration 4
43088	42381	CDS	23		110014	110277	Origin of replication		Origin of replication
43971	43161	Gene	24		110506	111356	Gene	63	
43166	43161	Poly(A) signal			111351	111356	Poly(A) signal		
43971	43162	CDS	24		110506	111342	CDS	63	Tegument protein
44568	44081	Gene	25		111490	112067	Gene	64	
44086	44081	Poly(A) signal			112062	112067	Poly(A) signal		
44568	44098	CDS	25		111490	112032	CDS	64	
44456	46123	Gene	26		112566	112102	Gene	65	
46077	47193	Gene	27		112107	112102	Poly(A) signal		
47188	47193	Poly(A) signal			112566	112258	CDS	65	Virion protein
46077	47078	CDS	27		112258	112259	Miscellaneous		IRS/US boundary
50586	46981	Gene	28		112963	114167	Gene	66	
46986	46981	Poly(A) signal			114162	114167	Poly(A) signal		
50586	47002	CDS	28	DNA polymerase	112963	114144	CDS	66	Protein kinase
50807	54458	Gene	29		114422	115518	Gene	67	
54453	54458	Poly(A) signal			115513	115518	Poly(A) signal		
50807	54406	CDS	29	Single-stranded-DNA binding protein	114422	115486	CDS	67	gI
54585	56897	Gene	30		115734	117647	Gene	68	
56942	59582	Gene	31		117642	117647	Poly(A) signal		
59577	59582	Poly(A) signal			115734	117605	CDS	68	gE
56942	59548	CDS	31	gB, fusogen	117490	117491	Miscellaneous		US/TRS boundary
59701	60148	Gene	32		118260	117682	Gene	69	
60143	60148	Poly(A) signal			117687	117682	Poly(A) signal		
59701	60132	CDS	32	Substrate for ORF 47 kinase	118260	117718	CDS	69	
62071	60242	Gene	33		119244	118394	Gene	70	
60247	60242	Poly(A) signal			118399	118394	Poly(A) signal		
62071	60254	CDS	33	Protease	119244	118408	CDS	70	Tegument protein
63843	62104	Gene	34		119473	119736	Origin of replication		Origin of replication
64686	63910	CDS	35		119915	120060	Repeat region		Reiteration R4
64297	64303	Promoter		TATA element	120692	124688	Gene	71	
64321	64325	5' end of dPyKmRNA			124683	124688	Poly(A) signal		
64740	65797	Gene	36		120692	124624	CDS	71	Transactivator, tegument protein

^a CDS, coding sequence; dPyKmRNA, deoxypyrimidine kinase mRNA.

^b ORF was annotated according to the work of Gomi et al. (20).

available in GenBank under the accession numbers DQ008354 and DQ008355, respectively.

RESULTS

Oka-V_{GSK} and Oka-V_{Merck} genome organization. The full-length consensus sequence of Oka-V_{GSK} and Oka-V_{Merck} vaccine strains was essentially determined by bidirectional sequencing of overlapping PCR-amplified fragments. Occasionally, when the amplified region contained SNPs that could not be conclusively resolved, the amplified fragments were subcloned and a consensus sequence was derived from multiple plasmid clones. The obtained sequences were assembled and the complete genomes of the vaccines were annotated using the VZV sequence of the Dumas strain published by Davison and Scott as a template (12). The full annotations for Oka-V_{GSK} and Oka-V_{Merck} are presented in Tables 1 and 2, respectively.

The complete genomes of Oka-V_{GSK} and Oka-V_{Merck} strains are comprised of 124,821 and 124,815 bp, respectively. Like the wild-type Dumas strain and the parental Japanese Oka-V strain, the Oka-V_{GSK} and Oka-V_{Merck} genomes consist of a unique long region flanked by terminal repeat long and internal repeat long inverted repeat regions, as well as a unique short region flanked by internal repeat short (IRS) and terminal repeat short (TRS) inverted repeat regions. An origin of replication was found in both the IRS and TRS regions. Four unique reiteration regions (R1 to R4) were found along the genome, with R4 duplicated in the IRS and TRS regions.

All the open reading frames (ORFs) described for the Dumas VZV strain (12) and the Oka vaccine parental strain (22) were found in the two Oka-derived vaccine strains (Tables 1 and 2). The 72 ORFs predicted to encode proteins were evenly distributed on both DNA strands. Three genes were located within the repeat sequences and were therefore duplicated within the VZV genome, so that ORFs 69 to 71 in the IRS region correspond to ORFs 62 to 64 in the TRS region.

Comparison of Oka strain genomes to the Dumas strain genome. The obtained sequences of Oka-V_{GSK} and Oka-V_{Merck} were aligned with the full-length VZV genomes of Oka-P, Oka-V, and Dumas strains. All sequence differences between the four Oka strains and the Dumas strain are given in Table 3. A total of 326 nucleotide positions displaying differences relative to the genome of Dumas strain (X04370 [12]) were identified. Among these, 228 were common to the four Oka strains, and the remaining 98 were specific to one, two, or three of the Oka strains. Several deletions or insertions were found, but most mutations were substitutions of one nucleotide, i.e., SNPs. Frequently, the original nucleotide was nonetheless preserved, resulting in a mixture of two nucleotides present at the same position (Table 3). Because, to our knowledge, the vaccine strains were never cloned, this is consistent with the existence of multiple viral species that evolved during the attenuation process. Multiple SNPs were found to still contain the original Oka-P-specific nucleotide. This supports the cooperative effect of the overall pattern of nucleotide substitutions in the expression of the attenuation phenotype and, to a lesser extent, the contribution of individual SNPs.

The 98 differences between the Oka-V_{GSK} (124,821 bp), Oka-V_{Merck} (124,815 bp), Oka-P (125,125 bp), and Oka-V (125,078 bp) genomes were found in 25 ORFs (ORFs 1, 2,

6, 9A, 10, 11, 14, 18, 21, 22, 31, 35, 39, 45, 47, 48, 50, 51, 52, 54, 55, 62, 64, and 71), the R1 and R3 repeat regions (in ORFs 11 and 22, respectively), and one origin of replication (Table 3).

The total number of differences between the four Oka strains was determined (Table 4). Of the 98 differences identified, 69 were found between Oka-P and Oka-V, 51 between Oka-V and Oka-V_{GSK}, and 68 between Oka-V and Oka-V_{Merck}. Consequently, Oka-V_{Merck} contains 17 more differences that discriminate it from Oka-V compared with Oka-V_{GSK}.

Although the highest convergence was found for Oka-V_{GSK} and Oka-V_{Merck}, they still had 36 nucleotide differences (Table 4). For 12 of these positions, Oka-V_{Merck} had nucleotides matching the Oka-P strain, whereas the Oka-V_{GSK} strain had only a single position (119683) where the sequence was Oka-P-like. Overall, for the positions in which Oka-V_{GSK} differed from Oka-V_{Merck}, the Oka-V_{GSK} sequence was closer to Oka-V, whereas the Oka-V_{Merck} sequence was closer to Oka-P.

Sixty-nine nucleotide changes between the Oka-V and the Oka-P strains were identified (Table 5). Among these 69 differences, 56 positions in Oka-P were identical to the reference Dumas strain, whereas only 11 positions in Oka-V were identical to the Dumas strain. Identical nucleotides for many of these positions were also present in Oka-V_{GSK} and Oka-V_{Merck}.

To better characterize the observed differences, the substitution spectra were analyzed (Fig. 2). The large majority of mutations were SNPs and only partial, with two different nucleotides at the same position. Compared to Oka-P, transitions (i.e., mutations resulting in substitution of a purine for a purine [A↔G] or a pyrimidine for a pyrimidine [C↔T]) were more frequently (64% to 69%) observed for the Oka-V_{GSK} and Oka-V_{Merck} strains than transversions (i.e., mutations resulting in substitution of a purine for a pyrimidine and vice versa; 13% to 17%). Transversions were more common than insertions or deletions (≤10%). The majority of the identified mutations were silent mutations, either because they were located in intergenic regions or because of the degenerated genetic code. A significant proportion of mutations in intragenic regions (~45%) caused single amino acid substitutions in both the Oka-V_{GSK} and Oka-V_{Merck} strains (Fig. 2). No stop or frame-shift mutations were identified. All deletions and insertions either were located in intergenic regions or, when located within coding regions, were multiples of three bases.

Comparison of Oka-V_{GSK} and Oka-V_{Merck} genomes. Sequence differences observed between the Oka-V_{GSK} and Oka-V_{Merck} strains are described in Table 6. Only 36 differences were found throughout the complete genomes (i.e., ~125 kb), three of which were repeated in ORF 62 and its duplicate, ORF 71. These 33 nucleotide unique position changes resulted in 14 amino acid changes, 1 each in ORFs 6, 9A, 10, 31, 39, and 52 and 2 each in ORFs 14, 55, and 62/71 and the R3 repeat region.

Among these 36 position differences between Oka-V_{GSK} and Oka-V_{Merck}, Oka-V_{GSK} had 23 nucleotide sequences identical to Oka-V but only 3 identical to Oka-P. In contrast, Oka-V_{Merck} had 18 positions identical to Oka-P but only 6 identical to Oka-V (Table 6 and Fig. 3).

TABLE 3. Comparison of complete genomic sequences of Dumas and Oka strains of VZV^a

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
A→G	1	X	X	X	X	1	1
G→C	3	X	X	X	X	3	3
Deletion of C (from WT)	109	X	X	X	X	109	109
G→C	178	-	-	X	X	177	177
A→G	236	X	X	X	X	235	235
C→T	262	X	X	X	X	261	261
T→C	560	-	X	X	X	559	559
G→A (ORF 1), N, silent	685	X	X	X	X	684	684
T→T/C (ORF 1), Q, silent	703	-	X	C	C	702	702
T→T/C (ORF 1), P, silent	763	-	X	C	C	762	762
T→C (ORF 1), T→A	789	X	X	X	X	788	788
T→C (ORF 1), Q→R	790	X	X	X	X	789	789
T→C (ORF 1), Q→R	791	X	X	X	X	790	790
C→G (ORF 2), G, silent	1838	-	X	-	-	1837	1837
T→T/C	2515	-	X	C	-	2514	2514
A→G (ORF 4), T, silent	3764	X	X	X	X	3763	3763
C→T (ORF 5), K, silent	4258	X	X	X	X	4257	4257
A→G (ORF 6), S→P	5745	-	X	X	A/G	5744	5744
G→T (ORF 6), H→Q	6853	X	X	X	X	6852	6852
C→A (ORF 6), G→V	7091	X	X	X	X	7090	7090
C→T (ORF 6), P, silent	7753	X	X	X	X	7752	7752
T→C	9460	X	X	X	X	9459	9459
G→A (ORF 8), P→S	10079	X	X	X	X	10078	10078
T→C/T (ORF 9A), W→R	10900	-	X	X	-	10899	10899
T→G (ORF 9), S, silent	11890	X	X	X	X	11889	11889
A→G (ORF 9), T→A	11906	X	X	X	X	11905	11905
C→A (ORF 10), P→H	12188	X	X	X	X	12187	12187
T→C (ORF 10), F→S	12284	X	X	X	X	12283	12283
T→C (ORF 10), F→S	12285	X	X	X	X	12284	12284
C→C/T (ORF 10), A→V	12779	-	X	X	-	12778	12778
T→G (ORF 10), G, silent	13173	X	X	X	X	13172	13172
G→A	13407	X	X	X	X	13406	13406
Deletion (ORF 11, R1), ATTGACGACGAGG GAGAGGCGGAGGA GGGAGAGGCGGA GGAGGGAGAGGC GGAGGAGGGA GAG, IDDEGEAEEG EAEEGEAEEGE	14088	X	X	X	X	14086	14086
Deletion (ORF 11, R1), GCGGAGGAGGAC GCG, AEEDA	14199–213	X	X	X	X	14134–48	14134–48
Insertion (ORF 11, R1), CGCGATCGACGAC GAGGGAGAGGCG GAGGAGGA	14242	X	-	X	X	14164–96	14164–96
T→C	14390	X	X	X	X	14344	14344
C→T (ORF 12), V, silent	17404	X	X	X	X	17358	17358
C→T (ORF 12), L, silent	17834	X	X	X	X	17788	17788
C→T (ORF 12), T, silent	18082	X	X	X	X	18036	18036
G→A (ORF 13), K, silent	18467	X	X	X	X	18421	18421
T→T/C (ORF 14), stop	19431	-	X	-	-	19385	19385
A→G (ORF 14), I, silent	19719	X	X	X	X	19673	19673
T→A (ORF 14), Y→F	20656	X	X	X	X	20610	20610
T→C (ORF 14), T→A	20684	X	X	X	X	20638	20638
C→C/T (ORF 14), K, silent	20703	-	-	X	-	20657	20657
A→T (ORF 14), E→V	20711	X	X	-	-	20665	20665
C→A (ORF 14), C→A	20745	X	X	-	-	20699	20699
T→A (ORF 14), T→S	20753	X	X	X	X	20707	20707

Continued on following page

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TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
C→A (ORF 14), K→N	20787	X	X	C/A	-	20741	20741
C→A (ORF 14), K→N	20829	X	X	C/A	C/A	20783	20783
T→A/T (ORF 14), T→S	20837	-	-	X	X	20791	20791
C→A (ORF 14), K→N	20871	-	-	C/A	C/A	20825	20825
A→T (ORF 14), S→T	20879	-	-	A/T	-	20833	20833
C→A (ORF 14), K→N	20913	-	-	A/C	A/C	20867	20867
T→A (ORF 14), T→S	21005	X	X	X	X	20959	20959
G→A (ORF 15), L, silent	21371	X	X	X	X	21325	21325
G→T (ORF 15), R, silent	21734	X	X	X	X	21688	21688
G→A (ORF 15), S, silent	22311	X	X	X	X	22265	22265
A→G	22504	X	X	X	X	22458	22458
A→G (ORF 16), M→T	22794	X	X	X	X	22748	22748
A→G (ORF 16), F, silent	23294	X	X	X	X	23248	23248
Deletion (ORF 17), dCAT (delS)	24516	X	X	X	X	24469	24469
A→G (ORF 17), T→A	24578	X	X	X	X	24529	24529
C→T (ORF 17), T→M	24654	X	X	X	X	24605	24605
G→A (ORF 17), V→I	25067	X	X	X	X	25018	25018
A→G (ORF 18), N, silent	26125	-	X	X	A/G	26076	26076
A→G (ORF 19), H, silent	27523	X	X	X	X	27474	27474
T→G (ORF 20), G, silent	29201	X	X	X	X	29152	29152
C→T/C (ORF 21), T→I	31732	-	X	-	-	31683	31683
A→G (ORF 21), T→A	32274	X	X	X	X	32225	32225
T→C (ORF 21), H, silent	33722	X	X	X	X	33673	33673
T→C (ORF 21), D, silent	33725	X	X	X	X	33676	33676
T→C (ORF 21), N, silent	33728	X	X	X	X	33679	33679
T→C (ORF 22), V, silent	35543	X	X	X	X	35494	35494
A→G (ORF 22), L, silent	37649	X	X	X	X	37600	37600
A→G (ORF 22), I→V	37902	X	X	X	X	37853	37853
T→C (ORF 22), T, silent	38036	-	-	C/T	C/T	37987	37987
T→C (ORF 22), Y→H	38055	X	X	X	X	38006	38006
A→C (ORF 22), P, silent	38081	X	X	X	X	38032	38032
G→A (ORF 22), E, silent	38177	X	X	X	X	38128	38128
G→T (ORF 22), T, silent	38714	X	X	X	X	38665	38665
C→T (ORF 22), A, silent	38717	X	X	X	X	38668	38668
A→G (ORF 22), R, silent	39023	X	X	X	X	38974	38974
T→T/G (ORF 22), P, silent	39227	-	X	X	-	39178	39178
G→A (ORF 22), Q, silent	39263	X	X	X	X	39214	39214
G→A (ORF 22), R→H	39394	X	X	X	X	39345	39345
A→G (ORF 22), V, silent	39530	X	X	X	X	39481	39481
A→G (ORF 22), Q, silent	40388	X	X	X	X	40339	40339
T→C (ORF 22), P, silent	41057	X	X	X	X	41008	41008
G→A	41452	X	X	X	X	41403	41403
C→T (R3 repeat), A→V	41458	X	X	X	X	41409	41409
G→C (R3 repeat), A→V	41459	X	X	X	X	41410	41410
C→T (R3 repeat), A→V	41476	X	-	X	X	41427	41427
Deletion, GCGCAGCCCC	41475–83	-	X	-	-	41426–34	41426–34
G→C (R3 repeat), A→V	41476	X	-	X	X	41427	41427
Deletion, GCGCAGCCCC GCGCAGACCGTCC AGCCCGCGCAG CCC, AQPAQTVO PAQP	41484–519	X	X	-	-	41435–70	41435–70

Continued on following page

TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
C→T (R3 repeat), A→V	41485	-	-	X	-	41436	41436
C→C/T (R3 repeat), A→V	41494	-	-	X	-	41445	41445
A→C (R3 repeat), T→P	41499	-	-	X	X	41450	41450
C→T (ORF 22), T, silent	41618	X	X	X	X	41569	41569
G→A (ORF 22), S→N	41764	X	X	X	X	41715	41715
C→G (ORF 22), Q→E	42069	X	X	X	X	42020	42020
C→T (ORF 22), R, silent	42176	X	X	X	X	42127	42127
A→C (ORF 22), A, silent	42242	X	X	X	X	42193	42193
	42403	Del AAA	Del AA	Ins A	Del A	42355	42353
T→G (ORF 23), S, silent	42476	X	X	X	X	42428	42426
T→C (ORF 24), I→V	43262	X	X	X	X	43214	43212
C→T (ORF 26), C, silent	44835	X	X	X	X	44787	44785
A→G (ORF 28), C→R	47162	X	X	X	X	47114	47112
C→T (ORF 28), L, silent	47940	X	X	X	X	47892	47890
T→C (ORF 28), S→G	48050	X	X	X	X	48002	48000
G→A (ORF 28), T, silent	48825	X	X	X	X	48777	48775
G→A (ORF 28), L, silent	49535	X	X	X	X	49487	49485
C→A (ORF 28), G→C	50081	X	X	X	X	50033	50031
C→T (ORF 29), S, silent	51168	X	X	X	X	51120	51118
A→G (ORF 29), Q, silent	52917	X	X	X	X	52869	52867
A→C (ORF 29), I→L	53482	X	X	X	X	53434	53432
G→A (ORF 29), A→T	53938	X	X	X	X	53890	53888
Deletion (ORF 29), ACA TTTCAGGGTCAA, NISGS	54359–73	X	X	X	X	54310	54308
Deletion, T	54562	X	X	X	X	54498	54496
T→C	54564	X	X	X	X	54500	54498
A→G (ORF 30), P, silent	55820	X	X	X	X	55756	55754
A→C (ORF 31), T→P	57224	X	X	X	X	57160	57158
A→C (ORF 31), A, silent	57301	X	X	X	X	57237	57235
G→T (ORF 31), A, silent	57397	X	X	X	X	57333	57331
A→G (ORF 31), I→V	58595	-	A/G	A/G	X	58531	58529
A→A/G (ORF 31), P, silent	59287	-	X	X	X	59223	59221
Insertion, G	59760	X	X	X	X	59697	59695
Deletion	60278	Del5A	Del5A	Del A	Del AA	60214	60211
A→C	60279	X	X	X	X	60215	60212
C→A (ORF 33), A, silent	60405	X	X	X	X	60341	60338
T→G (ORF 33), Y→S	60781	X	X	X	X	60717	60714
G→A (ORF 33), P→L	61018	X	X	X	X	60954	60951
G→A (ORF 33), P→L	61019	X	X	X	X	60955	60952
T→C (ORF 33), N→G	61201	X	X	X	X	61137	61134
T→C (ORF 33), N→G	61202	X	X	X	X	61138	61135
A→G (ORF 35), A, silent	64067	-	X	X	A/G	64003	64000
A→G (ORF 35), C, silent	64136	X	X	X	X	64072	64069
T→C (ORF 35), P, silent	64259	X	X	X	X	64195	64192
T→C (ORF 35), M→V	64375	X	X	X	X	64311	64308
C→T (ORF 36), A, silent	64989	X	X	X	X	64925	64922
C→T (ORF 36), S→L	65669	X	X	X	X	65605	65602
G→T (ORF 37), L, silent	66646	X	X	X	X	66582	66579
C→T (ORF 37), P→L	66879	X	X	X	X	66815	66812
G→A (ORF 37), R→K	68172	X	X	X	X	68108	68105
A→G (ORF 38), T, silent	69349	X	X	X	X	69285	69282
T→C (ORF 38), S→G	69756	X	X	X	X	69692	69689

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TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
T→C (ORF 39), M→T	71252	-	X	X	C/T	71188	71185
C→T (ORF 40), V, silent	72997	X	X	X	X	72933	72930
T→C (ORF 40), T, silent	73993	X	X	X	X	73929	73926
C→T (ORF 41), V, silent	76530	X	X	X	X	76466	76463
Deletion, T	78144	X	X	X	X	78079	78076
G→T	80244	X	X	X	X	80179	80176
A→G (ORF 44), N→D	80840	X	X	X	X	80775	80772
C→T (ORF 44), A, silent	81187	X	X	X	X	81122	81119
A→A/G (ORF 45), P, silent	82225	-	X	-	-	82160	82157
G→A/G (ORF 47), E, silent	84091	-	X	-	-	84026	84023
A→G (ORF 47), T, silent	84616	X	X	X	X	84551	84548
G→A (ORF 48), R→H	84983	X	X	X	X	84918	84915
C→T (ORF 48), D, silent	85563	X	X	X	X	85498	85495
A→A/G (ORF 48), T→A	85594	-	-	X	X	85529	85526
C→A (ORF 48), Q→K	86170	X	X	X	X	86105	86102
Deletion, CCTGAT AAAC	86484–93	X	X	X	X	86418	86415
T→G	86556	X	X	X	X	86481	86478
A→A/G (ORF 50), C, silent	87280	-	X	-	-	87205	87202
T→C/T (ORF 50), S→G	87306	-	X	-	-	87231	87228
C→T (ORF 50), S, silent	87841	X	X	X	X	87766	87763
G→T (ORF 51), S, silent	88477	X	X	X	X	88402	88399
A→G (ORF 51), T, silent	89734	-	X	X	-	89659	89656
T→C (ORF 51), T, silent	89905	X	X	X	X	89830	89827
G→T (ORF 51), Q→H	90202	X	X	X	X	90127	90124
T→C (ORF 51), S, silent	90217	X	X	X	X	90142	90139
G→A	90392	X	X	X	X	90317	90314
A→A/G (ORF 52), I→V	90535	-	X	X	-	90460	90457
C→T (ORF 52), G, silent	91191	X	X	X	X	91116	91113
A→G (ORF 52), T→A	92026	X	X	X	X	91951	91948
A→G (ORF 52), T→A	92092	X	X	X	X	92017	92014
A→G (ORF 52), H→R	92375	X	X	X	X	92300	92297
T→C (ORF 53), V, silent	92999	X	X	X	X	92924	92921
T→C (ORF 54), L, silent	94167	-	X	X	T/C	94092	94089
A→G (ORF 54), V, silent	94632	X	X	X	X	94557	94554
A→T (ORF 54), T, silent	94641	X	X	X	X	94566	94563
T→C (ORF 54), G, silent	95241	X	X	X	X	95166	95163
G→A (ORF 54), L, silent	95546	X	X	X	X	95471	95468
T→G (ORF 54), E→D	95601	X	X	X	X	95526	95523
T→C (ORF 55), L, silent	97141	X	X	X	X	97066	97063
T→T/C (ORF 55), V→A	97479	-	-	-	X	97404	97401
C→T (ORF 55), I, silent	97591	X	X	X	X	97516	97513
G→A/G (ORF 55), A→T	97748	-	X	X	X	97673	97670
T→C/T (ORF 55), C→R	97796	-	X	X	-	97721	97718
T→C (ORF 55), G, silent	98437	X	X	X	X	98362	98359
T→C (ORF 56), V, silent	98765	X	X	X	X	98690	98687
A→C (ORF 56), T, silent	98807	X	X	X	X	98732	98729
Deletion (ORF 56), TTC, S	99227–29	X	X	X	X	99148	99145
T→G (ORF 57), H→P	99421	X	X	X	X	99343	99340
A→G (ORF 58), Y, silent	99709	X	X	X	X	99631	99628
C→T (ORF 58), V→I	99981	X	X	X	X	99903	99900
T→A (ORF 58), K→N	100114	X	X	X	X	100036	100033
T→G (ORF 58), N→T	100151	X	X	X	X	100073	100070
A→G	100283	X	X	X	X	100205	100202
A→A/G (ORF 59), L→P	101089	X	X	X	X	101011	101008
C→T (ORF 60), A→T	101331	X	X	X	X	101253	101250

Continued on following page

TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
Insertion (ORF 60), ATC	101623	X	X	X	X	101543–101545	101540–101542
T→C	101886	X	X	X	X	101811	101808
C→T	101991	X	X	X	X	101916	101913
G→A	102192	X	X	X	X	102117	102114
A→G	102203	X	X	X	X	102128	102125
Insertion, TCAAGCTTT	102219	X	X	-	-	102144	102141
AAAAACGTACCCCA							
AACTTAAAACGCTC							
AAATTGCCTTTTGG							
AGGCCTGCCCAACG							
GCCATTATCCCTTG							
GATCTAAGATTGAT							
TTGCGGTAACGTTT							
GCCAA							
C→A	102309	X	X	X	X	102234	102231
A→C	102351	X	X	X	X	102276	102273
A→G	102458	X	X	X	X	102383	102380
T→G	102601	X	X	X	X	102526	102523
T→C	103043	X	X	X	X	102968	102965
A→G	104898	X	X	X	X	104823	104820
C→G	105010	X	X	X	X	104935	104932
T→C	105012	X	X	X	X	104937	104934
T→C	105015	X	X	X	X	104940	104937
T→C	105017	X	X	X	X	104942	104939
Insertion, C	105020	X	-	X	X	104946	104943
Deletion, G	105054	X	X	X	X	104979	104976
Deletion, G	105071	X	X	X	X	104995	104992
Insertion, ACAA	105145	X	X	X	X	105075	105072
A→A/G	105169	-	X	X	X	105097	105094
A→A/G (ORF 62), L→S	105310	-	X	X	X	105238	105235
A→G (ORF 62), G, silent	105312	X	X	X	X	105240	105237
T→C (ORF 62), I→V	105356	-	X	X	T/C	105284	105281
A→G (ORF 62), L→P	105451	X	X	X	X	105379	105376
A→C (ORF 62), S→A	105512	X	X	X	X	105440	105437
A→G (ORF 62), V→A	105544	-	X	X	X	105472	105469
T→C (ORF 62), A, silent	105705	-	X	X	X	105633	105630
T→C (ORF 62), R→G	106262	-	X	X	X	106190	106187
T→C (ORF 62), A, silent	107136	-	X	X	T/C	107064	107061
C→T (ORF 62), A→T	107165	X	X	X	X	107093	107090
T→C (ORF 62), S→G	107252	-	X	X	X	107180	107177
T→C (ORF 62), R, silent	107307	X	X	X	X	107235	107232
A→A/G (ORF 62), V→A	107599	-	X	X	-	107527	107524
C→A (ORF 62), T, silent	107607	X	X	X	X	107535	107532
T→C (ORF 62), A, silent	107715	X	X	X	X	107643	107640
T→C (ORF 62), P, silent	108111	-	X	X	X	108039	108036
A→G (ORF 62), L, silent	108747	X	X	X	X	108675	108672
A→A/G (ORF 62), M→T	108838	-	X	X	X	108766	108763
G→A (ORF 62), H, silent	108951	X	X	X	X	108879	108876
C→G (ORF 62), A, silent	109044	X	X	X	X	108972	108969
A→A/G	109137	-	X	X	X	109065	109062
A→A/G	109200	-	X	X	-	109128	109125
T→C/T	109546	-	-	X	-	109474	109471
G→T	109654	X	X	X	X	109582	109579
Insertion, CAT	109696	X	X	X	X	109625–109627	109622–109624
Insertion, GGGAGGGG	109907	X	X	-	-	109838	109835
GCGCGGTACCCCGC							
CGATGGGGAGGGG							
GCGCGGTACCCCGC							
CGATGGGGAGGGG							
GCGCGGTACCCCGC							
CGATGGGGAGGGG							
GCGCGGTACCCCGC							
CGATG							
Insertion, GGGAGGGG	109907	X	-	-	-	109838	109835
GCGCGGTACCCCGC							
CGATG							

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TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
G→A	110003	X	X	X	X	109934	109931
Deletion, G	110058	X	X	X	X	109988	109985
G→A (Ori), -, silent	110112	X	X	X	X	109934	109931
Deletion, AT	110212	-	X	X	-	110142	110140–110141
T→G	110214	-	-	-	X		110141
Insertion, ATATAG	110214	X	-	-	-	110142	110141
T→G (Ori)	110216	X	X	X	X	110144	110143
T→G (Ori)	110218	X	X	X	X	110146	110145
T→G (Ori)	110220	X	X	X	X	110148	110147
T→G (Ori)	110222	X	X	X	X	110150	110149
T→G (Ori)	110224	X	X	X	X	110152	110151
T→G (Ori)	110226	X	X	X	X	110154	110153
A→G (Ori)	110232	X	X	X	X	110160	110159
A→G (Ori)	110235	X	X	X	X	110163	110162
Deletion, GC	110378–110379	X	X	X	X	110305	110304
A→G (ORF 63), T, silent	111312	X	X	X	X	110238	110237
A→G (ORF 64), Q→R	111650	-	X	A/G	A/G	111576	111575
T→C (ORF 64), Y→H	112093	X	X	X	X	112019	112018
Deletion/insertion	112128	Del A	Del A	Ins 5a	Ins A	112064–112068	112063
G→A	112198	X	X	X	X	112129	112124
A→G (ORF 66), S, silent	114140	X	X	X	X	114071	114066
G→A (ORF 67), P, silent	115041	X	X	X	X	114072	114967
C→T (ORF 68), T→I	115926	X	X	X	X	115857	115852
C→T	117699	X	X	X	X	117630	117625
Deletion/insertion	117769	Del T	Del T	Ins 5T	Ins T	117701–117705	117696
A→G (ORF 69), Y→H	117804	X	X	X	X	117740	117731
T→C (ORF 69), Q→R	118247	-	X	T/C	T/C	118183	118174
T→C (ORF 70), T, silent	118585	X	X	X	X	118521	118512
Deletion, GC	119518–119519	X	X	X	X	119453	119444
Insertion, CTCTCT	119654	X	X	-	-	119588	119579
T→C (Ori)	119656	X	X	-	-	119590	119581
T→C (Ori)	119665	X	X	X	X	119599	119590
A→C (Ori)	119671	X	X	X	X	119605	119596
A→C (Ori)	119673	X	X	X	X	119607	119598
A→C (Ori)	119675	X	X	X	X	119609	119600
A→C (Ori)	119677	X	-	X	X	119611	119602
Deletion, ATATATAT	119677–119684	-	X	-	-	119611–119618	119602–119609
A→C (Ori)	119679	X	-	X	X	119613	119604
A→C (Ori)	119681	X	-	X	X	119615	119606
A→C (Ori)	119683	X	-	X	A/C	119617	119608
C→T (Ori)	119785	X	X	X	X	119719	119710
Deletion, C	119847	X	X	X	X	119780	119771
C→T,	119894	X	X	X	X	119827	119818
Insertion, TACCGCGCC	120135	X	X	-	-	120068	120059
CCCTCCCCATCGGC							
GGGGTACCGCGCCC							
CCTCCCCATCGGCG							
GGGTACCGCGCCCC							
CTCCCCATCGGCGG							
GGTACCGCGCCCC							
TCCCCATCGGCGGG							
GTACCGCGCCCCCT							
CCCCATCGGCGGGG							
Insertion, TACCGCGCC	120135	X	-	-	-	120068	120060
CCCTCCCCATCGGC							
GGGG							
Insertion, GAT	120202	X	X	X	X	120136–120138	120127–120129
C→A	120243	X	X	X	X	120179	120170
A→A/G	120351	-	-	X	-	120287	120278
T→T/C	120697	-	X	X	-	120633	120624
T→T/C	120760	-	X	X	X	120696	120687
G→C (ORF 71), A, silent	120853	X	X	X	X	120789	120780
C→T (ORF 71), H, silent	120946	X	X	X	X	120882	120873
T→C/T (ORF 71), M→T	121059	-	X	X	X	120995	120986
T→C (ORF 71), L, silent	121150	X	X	X	X	121086	121077
A→G (ORF 71), P, silent	121786	-	X	X	X	121722	121713

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TABLE 3—Continued

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
A→G (ORF 71), A, silent	122182	X	X	X	X	122118	122109
G→T (ORF 71), T, silent	122290	X	X	X	X	122226	122217
T→C/T (ORF 71), V→A	122298	-	X	X	-	122234	122225
A→G (ORF 71), R, silent	122590	X	X	X	X	122526	122517
A→G (ORF 71), S→G	122645	-	X	X	X	122581	122572
G→A (ORF 71), A→T	122732	X	X	X	X	122668	122659
A→G (ORF 71), A, silent	122761	-	X	X	A/G	122697	122688
A→G (ORF 71), R→G	123635	-	X	X	X	123571	123563
A→G (ORF 71), A, silent	124192	-	X	X	X	124128	124119
T→C (ORF 71), V→A	124353	-	X	X	X	124289	124280
T→G (ORF 71), S→A	124385	X	X	X	X	124321	124312
T→C (ORF 71), L→P	124446	X	X	X	X	124382	124373
A→G (ORF 71), I→V	124541	-	X	X	A/G	124477	124468
T→C (ORF 71), G, silent	124585	X	X	X	X	124521	124512
T→C (ORF 71), L→S	124587	-	T/C	T/C	T/C	124523	124514
T→T/C	124728	-	X	X	X	124664	124655
Insertion, TGTT	124750	X	X	X	X	124687–124690	124678–124681
Deletion, C	124834	X	X	X	X	124773	124764
Deletion, C	124851	X	X	X	X	124789	124780
A→G	124880	NA	NA	X	X	124818	124809
A→G	124882	NA	NA	X	X	124820	124811

* A partial analysis of 20 of these nucleotide differences was published previously (70). Nucleotide positions within ORFs are indicated, as well as the encoded amino acids. Ori, origin of replication; WT, wild type. X, difference relative to Dumas strain; -, identical nucleotide relative to Dumas strain; NA, not applicable; Del, deletion; Ins, insertion. Where applicable, the resulting codon switch is specified.

DISCUSSION

In this study, we compared the complete genomes of the varicella vaccine strains Oka-V_{GSK} and Oka-V_{Merck}, both derived from the original attenuated Oka-V strain (67). Phylogenetic analyses of these sequences along with 16 other complete VZV genomes were recently reported (50, 69), providing new insight into strain variability (69) and evidence of recombination between major circulating VZV clades (50).

Although VZV is a monotypic virus with a very low rate of interstrain sequence variations (0.061%) compared to other members of the *Herpesviridae* family of viruses (between 0.32% and 3.0% [47]), the sequence analysis of the Oka vaccine strains is not straightforward due to the presence of heterogeneous genomes with distinct sequences (21). Therefore, consensus sequencing provides only an indication of the most prevalent bases for each position. In the present study, we determined the full-length sequences of both Oka-V_{GSK} and Oka-V_{Merck} largely by bidirectional sequencing of overlapping PCR fragments, but when direct sequencing did not generate results of sufficient quality, fragments were subcloned and the

consensus sequence was derived from numerous plasmid clones. All sequences obtained were confirmed on both DNA strands. This approach gave a high-quality assessment of the whole genomes of Oka-V_{GSK} and Oka-V_{Merck}, and this is, to our knowledge, the first published comparative analysis of the complete genomes of these two strains.

Comparison with partial sequencing information published on these strains and the other Oka strains, Oka-P and Oka-V, is shown in Table 7 (3, 22, 32, 59, 60, 63, 69). Argaw et al. sequenced approximately 34 kb from the 3' ends of Oka-V, Oka-P, and Oka-V_{Merck} strains, and Schmidt et al. sequenced approximately 26 kb of the Oka-V_{GSK} strain (3, 60). Two sequence differences were found for Oka-V_{Merck} in ORF 59 (position 101089; A versus A/G) and ORF 62 (position 105310; G versus A/G) (3). Six differences were observed between the present results and those previously published for Oka-V_{GSK}, and 13 differences were observed for Oka-V_{Merck} (60). Finally, comparison between the present study and a previous one (32) revealed quantitative (number of sequence differences between Oka-V_{GSK} and Oka-V_{Merck} strains) and qualitative (ORFs involved) discrepancies.

Our analysis of Oka-V_{GSK} and Oka-V_{Merck} sequences revealed that they have very few nucleotide differences. When we compared their complete genomic sequences (i.e., ~125 kb), we found that only 36 positions were different between Oka-V_{GSK} and Oka-V_{Merck}. These differences lead to 14 unique amino acid substitutions, which suggests that although these two vaccine strains are not identical, they are very similar. The differences resulting in amino acid substitutions were found in 10 different ORFs and in the R3 repeat region, while silent

TABLE 4. Numbers of genomic sequence differences between the four Oka strains

Strain	No. of differences from:		
	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}
Oka-P	69 (Table 5)	79	64
Oka-V		51	68
Oka-V _{GSK}			36 (Table 6)

TABLE 5. Comparison of complete genomic sequences of Oka-P and Oka-V strains of VZV^a

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
T→C	560	-	X	X	X	559	559
T→T/C (ORF 1), Q, silent	703	-	X	C	C	702	702
T→T/C (ORF 1), P, silent	763	-	X	C	C	762	762
C→G (ORF 2), G, silent	1838	-	X	-	-	1837	1837
T→T/C	2515	-	X	C	-	2514	2514
A→G (ORF 6), S→P	5745	-	X	X	A/G	5744	5744
T→C/T (ORF 9A), W→R	10900	-	X	X	-	10899	10899
C→C/T (ORF 10), A→V	12779	-	X	X	-	12778	12778
Insertion (ORF 11, R1), CGCGATCGA CGACGAGGGAGAGGCGGAGG AGGA	14242	X	-	X	X	14164–14196	14164–14196
T→T/C (ORF 14), stop	19431	-	X	-	-	19385	19385
A→G (ORF 18), N, silent	26125	-	X	X	A/G	26076	26076
C→T/C (ORF 21), T→I	31732	-	X	-	-	31683	31683
T→T/G (ORF 22), P, silent	39227	-	X	X	-	39178	39178
C→T (R3 repeat), A→V	41476	X	-	X	X	41427	41427
Deletion, GCGCAGCCCC	41475–83	-	X	-	-	41426–41434	41426–41434
G→C (R3 repeat), A→V	41476	X	-	X	X	41427	41427
Deletion/insertion	42403	Del AAA	Del AA	Ins A	Del A	42355	42353
A→G (ORF 31), I→V	58595	-	A/G	A/G	X	58531	58529
A→A/G (ORF 31), P, silent	59287	-	X	X	X	59223	59221
A→G (ORF 35), A, silent	64067	-	X	X	A/G	64003	64000
T→C (ORF 39), M→T	71252	-	X	X	C/T	71188	71185
A→A/G (ORF 45), P, silent	82225	-	X	-	-	82160	82157
G→A/G (ORF 47), E, silent	84091	-	X	-	-	84026	84023
A→A/G (ORF 50), C, silent	87280	-	X	-	-	87205	87202
T→C/T (ORF 50), S→G	87306	-	X	-	-	87231	87228
A→G (ORF 51), T, silent	89734	-	X	X	-	89659	89656
A→A/G (ORF 52), I→V	90535	-	X	X	-	90460	90457
T→C (ORF 54), L, silent	94167	-	X	X	T/C	94092	94089
G→A/G (ORF 55), A→T	97748	-	X	X	X	97673	97670
T→C/T (ORF 55), C→R	97796	-	X	X	-	97721	97718
Insertion, C	105020	X	-	X	X	104946	104943
A→A/G	105169	-	X	X	X	105097	105094
A→A/G (ORF 62), L→S	105310	-	X	X	X	105238	105235
T→C (ORF 62), I→V	105356	-	X	X	T/C	105284	105281
A→G (ORF 62), V→A	105544	-	X	X	X	105472	105469
T→C (ORF 62), A, silent	105705	-	X	X	X	105633	105630
T→C (ORF 62), R→G	106262	-	X	X	X	106190	106187
T→C (ORF 62), A, silent	107136	-	X	X	T/C	107064	107061
T→C (ORF 62), S→G	107252	-	X	X	X	107180	107177
A→A/G (ORF 62), V→A	107599	-	X	X	-	107527	107524
T→C (ORF 62), P, silent	108111	-	X	X	X	108039	108036
A→A/G (ORF 62), M→T	108838	-	X	X	X	108766	108763
A→A/G	109137	-	X	X	X	109065	109062
A→A/G	109200	-	X	X	-	109128	109125
Insertion, GGGAGGGGGCGCGGTAC CCCGCCGATG	109907	X	-	-	-	109838	109835
Deletion, AT	110212	-	X	X	-	110142	110140–110141
Insertion, ATATAG	110214	X	-	-	-	110142	110141
A→G (ORF 64), Q→R	111650	-	X	A/G	A/G	111576	111575
T→C (ORF 69), Q→R	118247	-	X	T/C	T/C	118183	118174
A→C (Ori)	119677	X	-	X	X	119611	119602
Deletion, ATATATAT	119677–119684	-	X	-	-	119611–119618	119602–119609
A→C (Ori)	119679	X	-	X	X	119613	119604
A→C (Ori)	119681	X	-	X	X	119615	119606
A→C (Ori)	119683	X	-	X	A/C	119617	119608
Insertion, TACCGCGCCCCCTCCCCA TCGGCGGGG	120135	X	-	-	-	120068	120060
T→T/C	120697	-	X	X	-	120633	120624
T→T/C	120760	-	X	X	X	120696	120687
T→C/T (ORF 71), M→T	121059	-	X	X	X	120995	120986
A→G (ORF 71), P, silent	121786	-	X	X	X	121722	121713
T→C/T (ORF 71), V→A	122298	-	X	X	-	122234	122225
A→G (ORF 71), S→G	122645	-	X	X	X	122581	122572
A→G (ORF 71), A, silent	122761	-	X	X	A/G	122697	122688
A→G (ORF 71), R→G	123635	-	X	X	X	123571	123563
A→G (ORF 71), A, silent	124192	-	X	X	X	124128	124119
T→C (ORF 71), V→A	124353	-	X	X	X	124289	124280
A→G (ORF 71), I→V	124541	-	X	X	A/G	124477	124468
T→C (ORF 71), L→S	124587	-	T/C	T/C	T/C	124523	124514
T→T/C	124728	-	X	X	X	124664	124655

^a Ori, origin of replication; WT, wild type. X, difference relative to Dumas strain; -, identical nucleotide relative to Dumas strain; Del, deletion; Ins, insertion. Where applicable, the resulting codon switch is specified. Boldface highlights homologies between genomic sequences of Oka-V and genomic sequences of Oka-V_{GSK} and/or Oka-V_{Merck}.

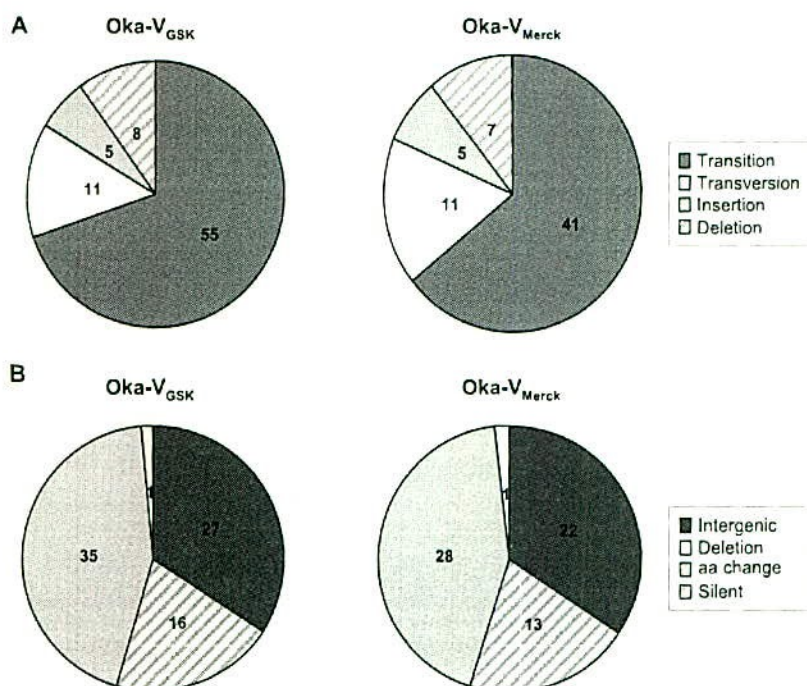


FIG. 2. Type (A) and function (B) of the mutations between Oka-P and the Oka-V_{GSK} and Oka-V_{Merck} vaccine strains of VZV. The numbers indicate the number of events identified for each category of mutations. aa, amino acid.

nucleotide substitutions were found in 8 different ORFs, and 1 noncoding substitution was found in the origin of replication.

Transactivation. ORF 62 encodes immediate early protein 62 (IE62), also known as a transcription regulator, which is the major component of the virion tegument and an important transactivating protein for all classes of VZV promoters (28, 49, 56). It is located in the short repeat sequences and has therefore a duplicate gene, ORF 71. These two duplicated genes cover 7% of the whole VZV genome. Recent studies suggested that ORF 62 could play a central role in the attenuated phenotype of the Oka vaccine strains (3, 20–22). Defined amino acid substitutions in ORF 62 that are associated with individual virus variants purified from the vaccine mixture have been linked to enhanced virus growth and spread in monolayer cell culture (22).

The present analysis of vaccine strains confirmed that a high number of mutations could be detected within ORF 62 (20–22). As previously discussed, these SNPs in ORF 62/71 may be important for attenuation of VZV (50, 69). The current analysis identified a nucleotide transition (position 105356 in ORF 62, corresponding to 124541 in ORF 71) that altered an Ile of the IE62 protein to a Val only partially in Oka-V_{Merck} and completely in Oka-V_{GSK} and Oka-V. Because Oka-P encodes only Ile at this position, it is likely that the Oka-V_{Merck} passaging history has selected for minor Oka-P-related species that might be present in the Oka-V vaccine. The second substitution (position 107599 in ORF 62 and 122298 in ORF 71) partially changed a Val to Ala in Oka-V_{GSK} and Oka-V; for Oka-V_{Merck}, this position is identical to the one found in Oka-P and encodes Val only. In both cases, the amino acids involved are small hydrophobic residues. Gomi et al. demonstrated that five amino acid substitutions, including the 105356

mutation, in the carboxyl terminus of IE62 directly reduced transactivational activity (22). Experiments with recombinant VZV will be required to determine how these mutations in ORF 62 modulate VZV gene expression and which amino acid substitutions are responsible for the differences in viral spreading.

The product of ORF 10, the virion-associated transactivator, is a tegument protein that regulates the IE62 promoter (28, 46). A nucleotide substitution (C→C/T) at position 12779 results in a conversion of an Ala in the Dumas, Oka-P, and Oka-V_{Merck} strains to a mixture of Ala and Val in the Oka-V and Oka-V_{GSK} strains. Similarly, minor subspecies from Oka-V that were originally present in Oka-P may have been selected in Oka-V_{Merck}. This position, which corresponds to a location in the middle of the protein, in Oka-V and Oka-V_{GSK} encodes two small hydrophobic amino acids (Val and Ala) that could have similar functions. Indeed, no statistically significant differences in transactivational activity of the ORF 10 gene product could be detected between the wild type and the mutant form, suggesting that this alternative form of ORF 10 has a minimal effect on viral attenuation through modulation of the expression level of IE62 (22). Furthermore, in vitro studies have shown that ORF 10 product was dispensable for VZV replication in vitro (10).

The helicase-primase complex. The helicase-primase complex consists of three proteins encoded by ORF 6 (primase) and ORFs 52 and 55 (helicase). Interestingly, we found four amino acid substitutions in these proteins, three of which were described previously (22).

The first amino acid substitution was located near the C terminus of ORF 6 (position 5745, A→G), which was a Ser in Dumas and Oka-P, a Pro in Oka-V and Oka-V_{GSK}, and a

TABLE 6. Comparison of complete genomic sequences of Oka-V_{GSK} and Oka-V_{Merck} vaccine strains of VZV^a

Feature relative to WT (Dumas)	Position (WT)	Feature in:				Position in:	
		Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
T→T/C	2515	-	X	C	-	2514	2514
A→G (ORF 6), S→P	5745	-	X	X	A/G	5744	5744
T→C/T (ORF 9A), W→R	10900	-	X	X	-	10899	10899
C→C/T (ORF 10), A→V	12779	-	X	X	-	12778	12778
C→C/T (ORF 14), K, silent	20703	-	-	X	-	20657	20657
C→A (ORF 14), K→N	20787	X	X	C/A	-	20741	20741
A→T (ORF 14), S→T	20879	-	-	A/T	-	20833	20833
A→G (ORF 18), N, silent	26125	-	X	X	A/G	26076	26076
T→T/G (ORF 22), P, silent	39227	-	X	X	-	39178	39178
C→T (R3 repeat), A→V	41485	-	-	X	-	41436	41436
C→C/T (R3 repeat), A→V	41494	-	-	X	-	41445	41445
Deletion/insertion	42403	Del AAA	Del AA	Ins A	Del A	42355	42353
A→G (ORF 31), I→V	58595	-	A/G	A/G	X	58531	58529
Deletion	60278	Del 5A	Del 5A	Del A	Del AA	60214	60211
A→G (ORF 35), A, silent	64067	-	X	X	A/G	64003	64000
T→C (ORF 39), M→T	71252	-	X	X	C/T	71188	71185
A→G (ORF 51), T, silent	89734	-	X	X	-	89659	89656
A→A/G (ORF 52), I→V	90535	-	X	X	-	90460	90457
T→C (ORF 54), L, silent	94167	-	X	X	T/C	94092	94089
T→T/C (ORF 55), V→A	97479	-	-	-	X	97404	97401
T→C/T (ORF 55), C→R	97796	-	X	X	-	97721	97718
T→C (ORF 62), I→V	105356	-	X	X	T/C	105284	105281
T→C (ORF 62), A, silent	107136	-	X	X	T/C	107064	107061
A→A/G (ORF 62), V→A	107599	-	X	X	-	107527	107524
A→A/G	109200	-	X	X	-	109128	109125
T→C/T	109546	-	-	X	-	109474	109471
Deletion, AT	110212	-	X	X	-	110142	110140–110141
T→G	110214	-	-	-	X	-	110141
Deletion/insertion	112128	Del A	Del A	Ins 5a	Ins A	112064–112068	112063
Deletion/insertion	117769	Del T	Del T	Ins 5T	Ins T	117701–117705	117696
A→C (Ori)	119683	X	-	X	A/C	119617	119608
A→A/G	120351	-	-	X	-	120287	120278
T→T/C	120697	-	X	X	-	120633	120624
T→C/T (ORF 71), V→A	122298	-	X	X	-	122234	122225
A→G (ORF 71), A, silent	122761	-	X	X	A/G	122697	122688
A→G (ORF 71), I→V	124541	-	X	X	A/G	124477	124468

^a WT, wild type. X, difference relative to Dumas strain; -, identical nucleotide relative to Dumas strain; Ori, origin of replication; Del, deletion; Ins, insertion. When applicable, the resulting codon switch is specified. Boldface highlights homologies between genomic sequences of Oka-V and genomic sequences of Oka-V_{GSK} and/or Oka-V_{Merck}.

mixture of both in Oka-V_{Merck}. Pro is a rigid residue that could induce substantial changes in the protein conformation. This nucleotide substitution was also comprised in an AluI restriction site. Interestingly, Quinlivan et al. found no differences between Oka-V_{GSK} and Oka-V_{Merck} by AluI restriction analysis: both Oka-V_{GSK} and Oka-V_{Merck} were A/G ($\pm$ AluI), whereas Oka-V was G ($\pm$ AluI) (52).

The second substitution occurred in ORF 52 (position 90535, A→A/G). In this case, the amino acid residue in Oka-P and Oka-V_{Merck} was Ile, which was partially changed to Val in Oka-V and Oka-V_{GSK}.

The last two substitutions were located in ORF 55. Val at position 97479 was partially replaced by Ala in Oka-V_{Merck}, and Cys at position 97796 was partially replaced by Arg in Oka-V and Oka-V_{GSK}.

Gomi et al. demonstrated that pathogenicity and spreading of VZV were affected by mutations in ORFs 6, 10, and 62, whereas ORFs 52 and 55 did not seem to be important for efficient VZV spreading (22). Because these substitutions were only partial, they could result in the coexistence of different helicase-primase activities resulting from different isomeric

complexes, as shown by restriction fragment length polymorphism analysis (22). For most of these positions Oka-V_{GSK} was similar to Oka-V, whereas Oka-V_{Merck} was similar to Oka-P.

Envelope glycoproteins. VZV produces at least seven glycoproteins, gK, gC, gB, gH, gL, gI, and gE, which are the products of ORFs 5, 14, 31, 37, 60, 67, and 68, respectively (11). Two putative additional glycoproteins were recently described, gN (ORF 9A) and gM (ORF 50) (55, 77). It is known that VZV glycoproteins induce a strong humoral immune response following either natural infection or vaccination with the Oka strain. The SNPs in the nine VZV glycoproteins were reviewed in a recent comparative analysis (64). Some of them are specific to the vaccine strains and thus could be involved in VZV attenuation.

The product of ORF 68, gE, is the most abundant glycoprotein expressed during infection. A single amino acid substitution in this protein was shown to induce the accelerated replication phenotype of the VZV-MSP mutant strain (57). Recently, Grose et al. reported the sequences of two VZV isolates harboring a D150N mutation within ORF 68 (25). That study identified only one mutation, common to all four Oka

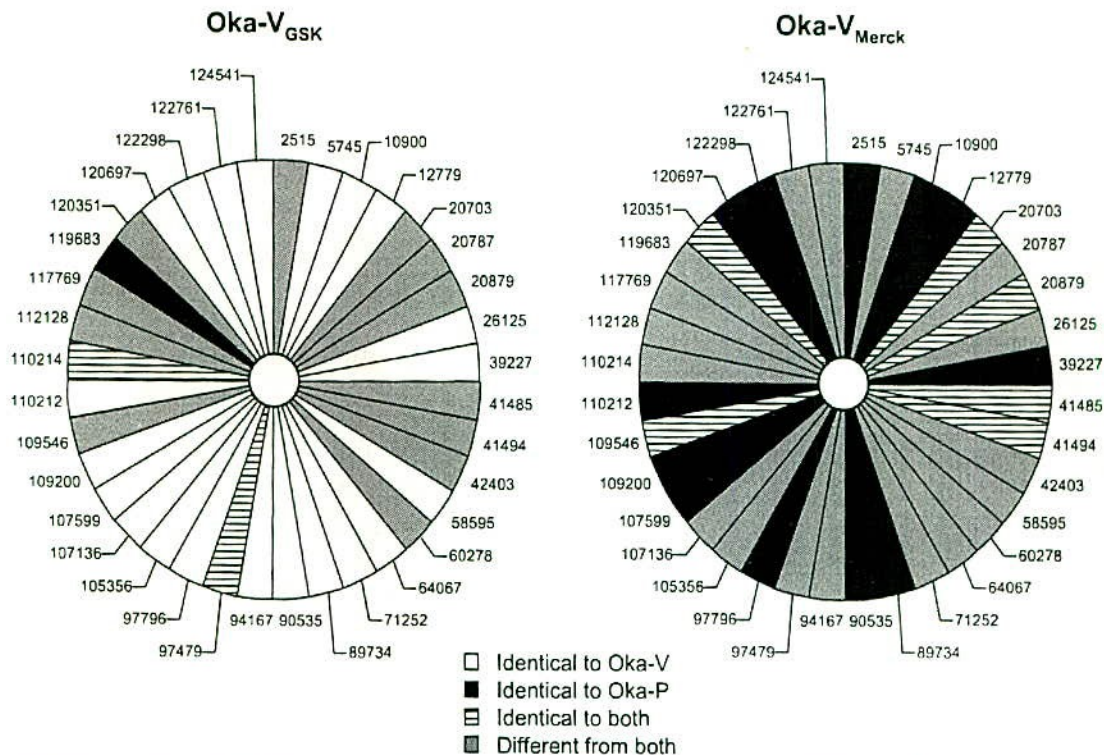


FIG. 3. Sequence comparisons of Oka-V_{GSK} and Oka-V_{Merck} with Oka-P and Oka-V strains of VZV. The 36 nucleotide positions that are different in Oka-V_{GSK} and Oka-V_{Merck} vaccines were compared to the sequence of the original vaccine strain Oka-V and its parental virus, Oka-P.

strains (position 115926, C→T), which induced the replacement of a Thr by Ile.

The product of ORF 31, gB, is the second most abundant and immunogenic envelope glycoprotein of VZV after gE. Along with gH and gC, it seems to play a role in the attachment and penetration of viral particles. It was also shown to have important fusogenic properties in the presence of gE and to be associated with cell-to-cell infection (39, 45). An A→G transition at position 58595 induced a conversion of Ile to Val in Oka-V_{Merck} and a mixture of Ile and Val in both Oka-V_{GSK} and Oka-V. Both amino acids are small and hydrophobic, suggesting that this substitution would probably not affect the properties of this glycoprotein.

ORF 14 exhibited one silent replacement and two amino acid substitutions in its product, gC. At position 20787, a co-existence of C and A induced the partial replacement of a Lys by Asn in Oka-V_{GSK}, whereas the original C residue was completely replaced by A in Oka-V_{Merck}, entirely replacing Lys with Asn. Both residues are hydrophilic; however, Lys is basic and Asn is polar, with an uncharged side chain. Because Asn was not detected in either Oka-P or Oka-V, it is likely that this amino acid substitution evolved as a result of additional vaccine passages and might reflect additional cell culture adaptation. The second modification (position 20879, A→T) was found only in the Oka-V_{GSK} strain. It partially changed a Ser to Thr. Both residues are hydrophilic and polar with an uncharged side chain. Grinfeld et al. showed that products of ORF 14 and ORF 67 were dispensable for the establishment of latency in a rat model (24). However, experiments with

SCID-hu mice showed that gC is important for viral tropism in skin cells and that a decrease in gC plays a critical role in attenuation (44). It was also previously shown that expression of gC is dependent on the strain of VZV, with the gC level of Oka-V being much lower than that of wild-type viruses (29, 37).

A nucleotide substitution at position 71252 induced the replacement of a Met by a Thr in the product of ORF 39, one of the two multiply inserted membrane proteins of VZV. This change was complete in Oka-V_{GSK} and Oka-V but only partial in Oka-V_{Merck}. Although both amino acids are neutral, Thr is polar and Met is hydrophobic. This difference may have some effect on the properties of the protein, although these are unclear at present (23).

We also found a mutation in the gN envelope glycoprotein. This glycoprotein is the product of ORF 9A, a newly identified gene positioned closely upstream of the ORF 9 initiation codon (55). Glycoprotein gN is an 87-amino-acid protein whose amino-terminal extremity overlaps with the first nine amino acids of the ORF 8 product, coded on the complementary strand. The observed T-to-T/C shift at position 10900 involves a Trp-to-Arg switch in the very last amino acid of the protein in Oka-V and Oka-V_{GSK}. This change leads to replacement of a hydrophobic amino acid with a large aromatic side chain at the carboxyl terminus of the protein by a hydrophilic basic amino acid, which could alter the membrane topology of the protein or its stability.

R3 repeat region. The R3 reiteration region is located in ORF 22, the longest ORF in VZV. The product of ORF 22 is

TABLE 7. Comparison of Oka-V_{GSK} and Oka-V_{Merck} genomic sequences with previously published Oka genomic sequences⁶⁸

Reference	Position	ORF	Previously reported feature					Feature in present study	
			Dumas	Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
3	84983	48	G	A	A		A	A	A
	85563	48	C	T	T		T	T	T
	86484		cctgataaac		-			cctgataaac	cctgataaac
	86556		T		G			G	G
	87841	50	C		T			T	T
	88477	51	G		T			T	T
	89734	51	A		G			G	A
	89905	51	T		C			C	C
	90202	51	G		T			T	T
	90217	51	T		C			C	C
	90392		G		A			A	A
	91191	52	C		T			T	T
	92092	52	A		G			G	G
	92375	52	A		G			G	G
	92999	52	T		C			C	C
	94167	54	T		C			C	T/C
	94632	54	T		G			G	G
	94641	54	A		T			T	T
	95241	54	T	C	C		C	C	C
	95546	54	G		A			A	A
	97141	55	T		C			C	C
	97470 ^b	55	G		C			G	G
	97591	55	C		T			T	T
	97748	55	G		A			A/G	A/G
	97834 ^c	55	C		T			C	C
	98437	55	T		C			C	C
	98765	56	T		C			C	C
	98807	56	A		C			C	C
	99227	56	TTC	-	-			TTC	TTC
	99709	58	A	G	G		G	G	G
	99981	58	C	T	T		T	T	T
	100114	58	T	A	A		A	A	A
	100151	58	T	G	G		G	G	G
	100283		A		G			G	G
	101089	59	A	A	G		A	A/G	A/G
	101331	60	C	T	T		T	T	T
	101623	60	-	+ATC	+ATC			+ATC	+ATC
	101886		T		C			C	C
	101991		C		T			T	T
	102192		G		A			A	A
	102203		A		G			G	G
	102219				+112 bp				
	102309		C		A			A	A
	102351		A		C			C	C
	102458		A		G			G	G
	102601		T		G			G	G
	103043		T		C			C	C
	104898		A		G			G	G
	105010		cctcctct	cctcctct	gccttaccct		cctcctct	cctcctct	cctcctct
	105054		G		Del G			Del G	Del G
	105063 ^d		G		Del G			Del G	Del G
	105145			+AACA	+AACA			+AACA	+AACA
	105310	62	A	A	G		G	A/G	A/G
	105312	62	A	G	G		G	G	G
	105356	62	T	T	C		C	C	C
	105451	62	A		G			G	G
	105512	62	A		C			C	C
	105544	62	A		G			G	G
	105705	62	T		C			C	C
	106262	62	T	T	C		C	C	C
	107136	62	T		C			C	C
	107165	62	C		T			T	T
	107252	62	T		C			C	C
	107307	62	T		C			C	C
	107607	62	C		A			A	A
	107715	62	T		C			C	C

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TABLE 7—Continued

Reference	Position	ORF	Previously reported feature					Feature in present study	
			Dumas	Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
	108111	62	T		C			C	C
	108747	62	A		G			G	G
	108951	62	G		A			A	A
	109044	62	C		G			G	G
	109694 ^e				+ATC			+CAT	+CAT
	109762				+27 bp			C	C
	110196				Del TA			No del	No del
	110216		(ga) ₆ gg		(ta) ₆ (gc) ₂ aaga		(ta) ₁₆ gag(ga) ₄		(ta) ₁₀ (ga) ₆ aaa(ga) ₄
	110378				Del GC			Del GC	Del GC
	111312	63	A		G			G	G
	111650	64	A	A	G		A/G	A/G	A/G
	112093	64	T	C	C		C	C	C
	112130 ^f		-	-	+A ₈		+A ₈	+A ₁₂	+A ₉
	112198		G	A	A		A	A	A
	114140	66	A		G			G	G
	115041	67	G		A			A	A
	115926	68	C		T			T	T
60	1		A	G	G	G	A	G	G
	3		G	C	C	C	G	C	C
	178		G	G	G	C	C	C	C
	560		T	T	C	C	C	C	C
	703 ^g	1	T	T	T	C	C	C	C
	82225 ^h	45	A	A	A	G	A	A	A
	86363	49	A	A	A	T	A	A	A
	87677	50	A	A	A	G	A	A	A
	89734	51	A	A	G	A	A	G	A
	90115 ⁱ	51	A	A	stop	A	A	A	A
	105054		G	-	-	-	G	Del G	Del G
	105071		G	G	-	-	G	Del G	Del G
	105145	Poly(A)	-	AACA	AACA	AACA	-	ACAA	ACAA
	105169		A	A	A/G	A/G	A	A/G	A/G
	105310	62	A	A	A/G	G	A	A/G	A/G
	105356	62	T	T	C	C	T	C	T/C
	105544	62	A	A	G	G	G	G	G
	124353	71	T	T	C	C	C	C	C
	124541	71	A	A	G	G	G	G	A/G
	124587	71	T	T	C/T	C	T	C	C/T
	124728		T	T	C/T	C/T	T	C/T	C/T
	124750	pA-71	-	TGTT	TGTT	TGTT	TGTT	TGTT	TGTT
	124834		C	-	-	-	C	Del C	Del C
	124851		C	-	-	-	C	Del C	Del C
22	560			T	C			C	C
	703	1		T	T/C			C	C
	763	1		T	T/C			C	C
	2515			T	T/C			C	T
	5745	6		A	G			G	A/G
	10900	9A		T	T/C			T/C	T
	12779 ^j	10		T	T/C			T/C	C
	19431	14		T	T/C			T	T
	26125	18		A	G			G	A/G
	31732	21		C	T/C			C	C
	38036 ^k	22		T	T/C			T/C	T/C
	39227	22		T	T/G			T/G	T
	58595	31		A	A/G			A/G	G
	59287	31		A	A/G			A/G	A/G
	64067 ^l	35		A	A/G			G	A/G
	71252 ^m	39		T	T/C			C	T/C
	82225	45		A	A/G			A	A
	84091	47		G	A/G			G	G
	87280	50		A	A/G			A	A
	87306	50		T	T/C			T	T
	89734 ⁿ	51		A	A/G			G	A
	90535	52		A	A/G			A/G	A

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TABLE 7—Continued

Reference	Position	ORF	Previously reported feature					Feature in present study	
			Dumas	Oka-P	Oka-V	Oka-V _{GSK}	Oka-V _{Merck}	Oka-V _{GSK}	Oka-V _{Merck}
	94167	54		T	C			C	T/C
	97748	55		G	A/G			A/G	A/G
	97796	55		T	T/C			T/C	T
	101089 ^a	59		A	A/G			A/G	A/G
	105169			A	A/G			A/G	A/G
	105310	62		A	A/G			A/G	A/G
	105356	62		T	C			C	T/C
	105544	62		A	G			G	G
	105705	62		T	C			C	C
	106262	62		T	C			C	C
	106710 ^b	62		A	A/G			A	A
	107136	62		T	C			C	T/C
	107252	62		T	C			C	C
	107599	62		A	A/G			A/G	A
	107797 ^c	62		A	A/G			A	A
	108111	62		T	C			C	C
	108838	62		A	A/G			A/G	A/G
	109137			A	A/G			A/G	A/G
	109200			A	A/G			A/G	A
	111650 ^d	64		A	A/G			A/G	A/G

^a Boldface highlights differences (3, 60) and homologies (22) between results from published studies and results from the present study. Lowercase indicates insertion; -, missing nucleotide position.

^b Indicated as G in the Oka-V GenBank submission AB097932.

^c Indicated as C in the Oka-V GenBank submission AB097932.

^d In the present alignment, this position is 105071.

^e In the present alignment, this position is 109696.

^f In the present alignment, this position is 112128.

^g Indicated as Y in the Oka-V GenBank submission AB097932.

^h Indicated as R in the Oka-V GenBank submission AB097932.

ⁱ Indicated as A in the Oka-V GenBank submission AB097932.

^j Indicated as C in the Oka-P GenBank submission AB097933.

^k Indicated as T in the Oka-V GenBank submission AB097932.

^l Indicated as G in the Oka-V GenBank submission AB097932.

^m Indicated as C in the Oka-V GenBank submission AB097932.

ⁿ Indicated as G in the Oka-V GenBank submission AB097932.

^o Indicated as R in the Oka-P GenBank submission AB097933.

^p Indicated as A in the Oka-V GenBank submission AB097932.

^q Indicated as A in the Oka-V GenBank submission AB097932.

^r Indicated as A in the Oka-V GenBank submission AB097932.

homologous to the UL36 virion tegument phosphoprotein of herpes simplex virus type 1 (41, 42). R3 is a highly variable region consisting of repeated elements that can vary in number and combination (12, 22), but the impact of this region on the function of ORF 22 phosphoprotein remains mostly unknown because the function of the phosphoprotein itself is poorly understood.

Despite the high variability of the R3 region, no frame-shift mutations were detected in ORF 22, because the repeated elements are present as multiples of 3 bp. Nevertheless, two mutations (positions 41485 and 41494, C→T) in the Oka-V_{GSK} strain were identified, both of which convert Ala into Val, another amino acid with similar properties that probably does not affect the function of the ORF 22 protein.

Attenuation and reactivation. Comparison of the four Oka strain sequences of VZV indicated that Oka-V_{GSK} is genetically closer to Oka-V than Oka-V_{Merck} is but that Oka-V_{Merck} is closer to Oka-P than Oka-V_{GSK} is (reference 50 and the present study). In agreement with these findings, Sauerbrei et al. demonstrated that Oka-V_{Merck} is genetically closer to Oka-P than Oka-V_{GSK} is (59). Nevertheless, it is well established that both vaccine strains are attenuated but remain

strongly immunogenic (43, 73). Recently, Quinlivan et al. suggested an association of particular SNPs in the VZV genome with frequency of vaccine-induced rash (53). Four SNP positions were suggested to contain nucleotides specific to Oka-P in most of the viruses isolated from vaccine rashes. The first one is a silent nucleotide change within ORF 51 (A→G at position 89734). The nucleotide at this position is A in Oka-V_{Merck} and Oka-P but G in Oka-V_{GSK} and Oka-V. The second SNP, at position 105169, contains mixed A/G nucleotides for Oka-V_{Merck}, Oka-V_{GSK}, and Oka-V, whereas the parental Oka-P contains only A. The third SNP, at position 105356, is located within ORF 62. The change from T to C is responsible for an amino acid switch (Ile→Val) for both Oka-V_{GSK} and Oka-V. At the same position, the parental Oka-P contains T (Ile), and Oka-V_{Merck} contains a mixture of T and C (Ile/Val). The last position (nucleotide 107797) was not identified as a SNP in our sequencing data, which agrees with a previous study (22).

Although the genetic basis of Oka-V attenuation has not been determined, Oka-V and Oka-P genomes have nucleotide differences predicted to change amino acids in every class of viral proteins (3, 21). VZV attenuation is a multifactorial phe-

nomenon whose mechanism remains unclear (80), but it is conceivable that mutations of the vaccine genome, in particular, mutations resulting in amino acid modifications, could affect virulence or latency of the vaccine strain. Recently, Peters and coworkers sequenced 11 VZV genomes from different clades, bringing the current number of available full-length VZV sequences to 18 (50, 69). To assess variations that can occur during serial passage in cell culture, these studies included the four Oka strains (Oka-P and the three Oka vaccine strains) and a VZV strain sequenced at passages 5, 22, and 72. As discussed by Tyler et al. (69), the SNPs in ORF 62/71 found in the three Oka vaccine strains and in the VZV strain at high passage level (S628G, R958G, and I1260V in IE62) could be involved in the attenuation of VZV. In addition, it was suggested that other mutations could play a role, particularly those in regions containing ORFs 30 to 55 (69, 78, 80). However, we found that numerous SNPs contain the original Oka-P nucleotide, supporting the idea that the attenuated phenotype is the result of a cooperative effect between several SNPs rather than the result of selected mutations. Therefore, our analysis of SNP importance is aligned with the conclusions of Tyler et al. regarding VZV attenuation (69).

VZV remains latent in sensory-nerve ganglia and can reactivate later, causing herpes zoster. It was suggested that herpes zoster is less common after vaccination, because initial access of Oka-V to neural cells is reduced by limited skin replication or because Oka-V reactivation and secondary viral infection of skin are less efficient, rather than because of an intrinsic attenuation of Oka-V neurotropism (5). Indeed, even though the Oka-V strain of VZV can cause herpes zoster, it seems to reactivate less often than the wild-type VZV even in immunocompromised children (18, 27, 68), and increased incidences of reactivation after vaccination have not been demonstrated in either clinical studies or postmarketing surveillance (33a, 43, 62, 66, 70, 73). In addition, the Oka vaccines have been shown to elicit a strong and protective immune response against varicella (7, 43, 73).

Conclusion. Overall, this study shows that, throughout the entire VZV genome, only 36 nucleotide positions differ between the Oka-V_{GSK} and Oka-V_{Merck} vaccine strains. Analysis of the complete genome of VZV also shows that, genetically, Oka-V_{GSK} is closer to Oka-V and that Oka-V_{Merck} is closer to Oka-P. Although Oka-V_{GSK} and Oka-V_{Merck} exhibit differences, there is a high degree of conservation between these strains at both the nucleotide and amino acid levels. This result supports the clinical data showing that both vaccines are well tolerated and elicit strong immune responses against varicella.

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AUTHOR'S CORRECTION

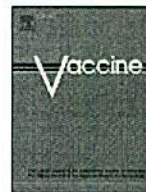
Comparison of the Complete DNA Sequences of the Oka Varicella Vaccine and Its Parental Virus

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Michiaki Takahashi, and Koichi Yamanishi

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Nucleotide sequence accession number. The complete sequences of VZV Oka parental and vaccine have been deposited in the DDBJ Data Library under accession no. AB097932 and AB097933, respectively.



Population diversity in batches of the varicella Oka vaccine

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ABSTRACT

The Oka vaccine is a live attenuated vaccine for the prevention of varicella. Although the vaccine differs from the progenitor virus by over 40 mutations, only three of these are fixed, the rest being a mixture of the wildtype and the vaccine allele. To examine the extent of this variability between two of the three commercially available vaccine preparations, we analysed the vaccine/wildtype allele frequencies present at fifteen vaccine loci in five preparations each from two different manufacturers of the vOka vaccine. Our results suggest that differences in manufacturing processes between the two companies have resulted in significant variation in the frequencies of the vaccine/wildtype alleles in their vaccines. Yet despite these differences, the allele frequencies in the vaccines from the two companies are strongly correlated. We discuss the significance of these findings and the role of evolutionary processes that influence the production of this live attenuated vaccine.

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1. Introduction

The live attenuated Oka vaccine is licensed for the prevention of varicella in children in the USA, Japan, Korea, Germany, Greece, Australia and Canada and for targeted immunisation of seronegative health care workers in several countries, including the UK. Over 15 years of experience has demonstrated that VZV vaccines are safe and well tolerated [1–4]. Universal vaccination in the USA has massively decreased the incidence of varicella in both vaccinated individuals and, by herd immunity, unvaccinated individuals, with significant reduction of associated hospitalization and mortality (reviewed in [5]). The commonest adverse event is a mild rash occurring following vaccination, which occurs in around 5% of children and a higher proportion of adults. Life threatening vOka (vaccine Oka) infection has only been described in subjects with undiagnosed cell mediated immunodeficiencies.

The original Oka vaccine strain was derived by serial passage of a wildtype Japanese VZV isolate, pOka (parental Oka) which had been obtained from a child with chickenpox [6]. Three main preparations derived from the original vaccine seed stock are licensed for use, the Biken vaccine, Varivax (Merck) and Varilrix (GSK). In

addition, combined vOka with live attenuated measles, mumps and rubella, is marketed by both Merck and GSK. Comparative Sanger sequencing of the Biken strain vOka and pOka, the wildtype progenitor, genomes identified 42 loci at which vaccine substations have occurred, 20 of which are non-synonymous [7]. In addition, length polymorphisms in the known variable repeat R1, R3 and R4 repeat regions and the viral origin of replication OriS were observed and five loci identified that were fixed for the vaccine polymorphism, the rest being polymorphic for vaccine and wildtype single nucleotide polymorphisms (SNPs). Sequencing of 10 clones of the Biken vaccine ORF62 confirmed the Biken vaccine to contain a mixture of related but distinct haplotypes [8]. Subsequently, both the Merck and GSK vOka vaccine preparations were also found to be heterogeneous [9–11].

Only three loci in the ORF 62 gene, which codes for the major immediate transactivating protein, are fixed for the vaccine SNP in all three vaccine preparations, the remainder being either wildtype or mixtures of vaccine and wildtype SNPs [7,11–13]. Overall the GSK vaccine has been found to carry more loci which are fixed for vaccine mutations and fewer which are wildtype than either the Merck or Biken vaccines [11]. Merck and Biken vaccines also differ from one another, with the former having at least one SNP that was not previously identified [9]. However, unlike the Merck vaccine [10,13], there has been no analysis of vaccine allele frequencies in the GSK vaccine. Differences between Merck and GSK vaccines could have arisen due to differences in the downstream manufacturing process by each company (Fig. 1). For example,

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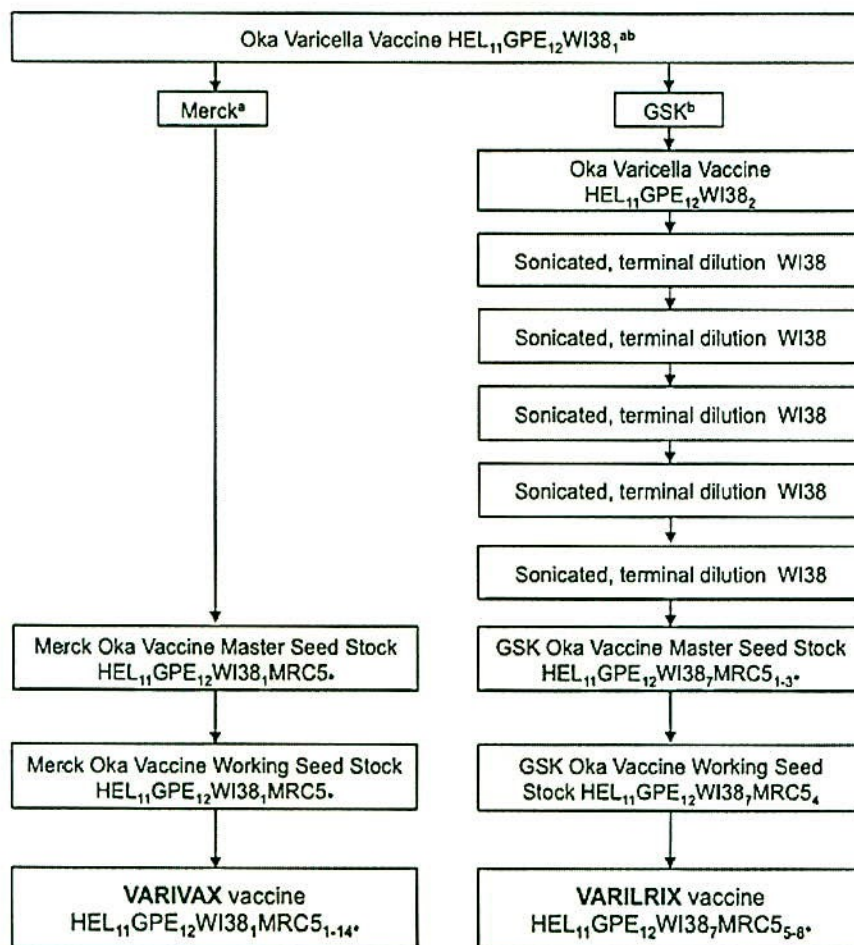


Fig. 1. Overview of varicella vaccine production. HEL, human embryonic lung cell cultures; GPE, embryonic guinea pig cell cultures; WI38, human diploid cell cultures; MRC5, human diploid cell cultures. Subscript numbers indicate passage levels. ^aInformation obtained from Merck Product information IPC-VRV3-I-032008. ^bInformation obtained from D'Hondt et al. [14]. ^{*}Unknown number of passage levels in MRC5 cells. Where passage levels are given next to a "", these have been inferred from ^a and ^b. World Health Organisation guidelines indicate a limit for Oka-strain varicella vaccine at passage level 38, hence final levels of passage in MRC5 cells can be estimated [14].

the GSK vaccine was subjected to five rounds of terminal dilution in an attempt to generate a genetically uniform vaccine strain [14]. Somewhat surprisingly this process did not appear to generate new vaccine mutations [11]. Limited analysis of different batches of vOka vaccines suggests little variation, [13], however, this has not been extensively characterised. To further examine variability between companies and among batches/lots, we analysed vaccine/wildtype allele frequencies present at 15 vaccine loci in five preparations each of the Merck and GSK Oka vaccines, which are currently available for vaccine programmes in Europe and the USA.

2. Methodology

2.1. Vaccine batches and SNPs

Five lots each of GSK and Merck vaccines derived in each case from three batches were available for analysis (Table 1). Fifteen loci at which vaccine substitutions have been identified were selected for analysis. Nine of these were located in ORF62, which contains over 30% of all the vaccine SNPs identified—several of these SNPs (105169, 105705, 105724, 107136 and 107797) have been identified as being of potential biological importance for vaccine attenuation and as immune targets [13,15]. Two of these were previously identified as fixed vaccine SNPs. The remainder were chosen as they have previously been found to be variable and therefore

informative [10,13] and to consistently be present in ORFs across the VZV genome.

2.2. Nucleic acid extractions

Total DNA was extracted using a QIAamp[®] DNA Mini Kit (Qiagen LTD., UK) according to the manufacturers instructions. The lot numbers, their date of issue and the number of loci analysed in each are shown in Table 1. Prior to extraction, lyophilised vaccine preparations were resuspended in the 500 µl of accompanying buffer. DNA from each preparation was extracted from 2 × 200 µl and 1 × 100 µl (mixed with 100 µl of PBS) aliquots. The 3 × 200 µl aliquots of eluted DNA were merged to form a 600 µl DNA pool, which was used for subsequent experiments. A negative control was used during the extraction process by replacing sample material with 200 µl of water.

2.3. PCR reactions

All PCRs were performed in a GeneAmp 2700 thermocycler (Perkin Elmer, UK), initiated by hot start activation at 95 °C for 10 min and ended with a step of 72 °C for 10 min before being incubated at 4 °C. For direct sequencing, reactions included 35 cycles of amplification (95 °C, 1 min; primer annealing temperature (supplementary Table 1), 1 min, 72 °C, 1 min). For pyrosequencing, reactions included 45 cycles of amplification (95 °C, 15 s; primer

annealing temperature (supplementary Table 2), 30 s, 72 °C, 15 s). PCR reactions included 5 µl of template DNA, 5 µl of PCR buffer II (Perkin Elmer, UK), 1.5 mM MgCl₂ (Perkin Elmer, UK), 200 µM of each deoxynucleotide triphosphate (Advanced Biotechnologies, UK), 12 pmol of each primer and 1 unit of AmpliTaq Gold DNA polymerase (Perkin Elmer, UK). Final reaction volumes were adjusted to 50 µl using DNase/RNase free water (Sigma, UK). For PCR reactions with each primer set, two negative controls were employed. The first consisted of PCR reaction mix, in which template DNA was replaced with water. The second consisted of PCR reaction mix, in which template DNA was replaced with the water used as a negative control during the DNA extraction process. PCR with each set of CSA and pyrosequencing primers also included a reaction containing DNA homogenous for the wildtype allele. For pyrosequencing primers a second reaction containing DNA homogenous for the vaccine allele was also included.

2.4. Determining allele frequencies

In the past we have used two methods for determining allele frequencies, comparative sequence analysis (CSA) and pyrosequencing, using primers directed against 35 of 42 vaccine SNPs identified by Gomi et al. [7]. To determine the accuracy of CSA and pyrosequencing, we used primer sets which amplify fragments containing the nucleotide present at position 108838 (supplementary Tables 1 and 2). These fragments were cloned into the *EcoRI* site of a pUC based pCR2.1 vector using a TA Cloning Kit in accordance with the manufacturer's instructions (Invitrogen, UK) and transformed into One Shot competent TOP10 *Escherichia coli* (Invitrogen, UK). Clones containing the vector plus insert were isolated and the DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, UK) according to the manufacturer's instructions. The DNA concentration was measured on a Gene Spec I Spectrophotometer (Nalca Instruments Ltd., UK) and Tris–EDTA (10 mM) was used to normalise the concentrations (i.e. DNA from clones containing the wildtype allele and clones containing the vaccine allele were both diluted to a concentration of 200 ng/µl each). DNA from clones containing the wildtype SNP at 108838, and clones containing the vaccine SNP at 108838, were then mixed in ratios of 100:0, 90:10, 70:30, 50:50, 30:70, 10:90 and 5:95. The allele frequencies at position 108838 were analysed in triplicate by CSA and pyrosequencing

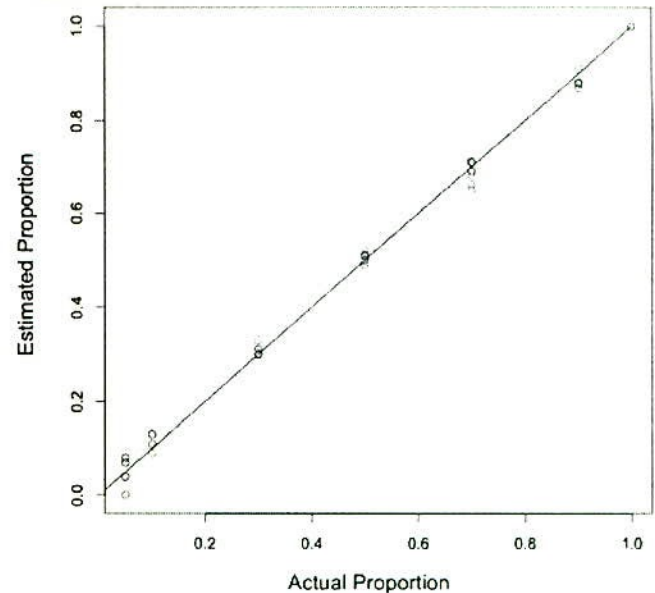


Fig. 2. Calibration of the two different methods for determining the frequency of alleles at polymorphic loci. The three estimates obtained by pyrosequencing (black symbols) and three obtained by CSA (grey symbols) are plotted for each mixture, along with a 1:1 line illustrating the known frequency.

using published methods [13,16]. For mixtures where the one allele was present at <10%, the experiment was repeated, giving six readings per SNP rather than just three. Relative SNP percentages were calculated automatically for the pyrosequencing, by measuring the area under the curve for loci with dual peaks. The relative percentages for CSA were calculated according to the method described by Mattocks et al. [16].

2.5. Statistical methods

The significance of the genetic differences among the various vaccines was evaluated using generalized linear modelling of the arcsine transformed allele frequencies.

3. Results

3.1. Comparison of CSA and pyrosequencing for allelic quantification

The accuracy of CSA and pyrosequencing for allele mixtures were comparable (Fig. 2), but the pyrosequencing was significantly closer to the true proportions ($P=0.012$): the standard deviation around the known frequency being 0.016 (max. 0.03) compared to 0.027 (max. 0.05) for CSA. When the mixtures of allele frequencies were at their most different, the pyrosequencing still detected the lowest frequency allele, whereas CSA did not (Fig. 2). Because of this greater sensitivity and reproducibility, pyrosequencing was used for further analyses. When quantifying alleles in the vaccines, if the wildtype allele was detected at $\leq 10\%$, the PCR and relevant assay was repeated.

3.2. Variation in lots of Oka/GSK and Oka/Merck

The allele frequencies observed at the fifteen loci analysed in all of the GSK and Merck lots are given in Table 2. Three of the loci were fixed for the novel vaccine allele (560, 106262 and 107252). With the exception of positions 89734 which retained the ancestral allele in all Merck batches, and 105724 which retained the ancestral

Table 1
Vaccines used in this study.

Company	Vaccine ^a	Year (country) of purchase	Number of SNPs analysed
Merck	Batch 1:	2003 (U.K.)	35
	Lot 1 (A)		
	Batch 2:	2004 (U.K.)	15
	Lot 1 (B)		
	Batch 2:	2005 (U.K.)	15
	Lot 2 (C)		
GSK	Batch 3:	2005 (U.S.)	15
	Lot 1 (D)		
	Batch 3:	2005 (U.S.)	15
	Lot 2 (E)		
	Batch 1:	2003 (U.K.)	35
	Lot 1 (1)		
	Batch 2:	2008 (U.K.)	15
	Lot 1 (2)		
	Batch 2:	2008 (U.K.)	15
	Lot 2 (3)		
	Batch 2:	2008 (U.K.)	15
	Lot 3 (4)		
	Batch 3:	2010 (U.K.)	15
	Lot 1 (5)		

^a Vaccine lot identifiers were anonymised for the purpose of this study. Numbers and letters in parenthesis are abbreviated identifiers for each vaccine.

Table 2

Variation in lots of Oka/GSK and Oka/Merck.

SNP ^a	ORF	P-Oka ^b	Oka/GSK lots ^{c,e}					Oka/Merck lots ^{d,e}				
			A	B	C	D	E	1	2	3	4	5
560	5' non-cod	T	0	0	0	0	0	0	0	0	0	0
19431	14	T	61	57	57	57	64	79	81	80	85	83
58595 (530)	31	A	71	67	67	68	78	45	46	48	47	49
87280	50	A	100	84	80	74	100	100	85	85	85	87
89734	51	A	24	25	25	25	19	100	100	100	100	100
97748 (585)	55	G	45	56	50	58	30	61	56	64	67	60
105169	61/62	A	50	21	21	21	61	56	50	53	53	51
105356 (1260)	62	T	13	6	0	0	0	34	36	31	33	31
105705	62	T	11	0	0	0	0	51	0	0	0	0
105724 (1137)	62	A	100	100	100	100	100	93	81	82	81	100
106262 (958)	62	T	0	0	0	0	0	0	0	0	0	0
107136	62	T	40	0	32	33	0	50	50	48	48	46
107252 (628)	62	T	0	0	0	0	0	0	0	0	0	0
107797 (446)	62	A	69	55	56	55	55	70	75	68	68	68
108838 (99)	62	A	100	60	60	63	60	100	76	75	77	75

^a Numbers refer to the nucleotide position in Dumas; numbers in parenthesis refer to the affected amino acid residue within a particular ORF.

^b P-Oka bases are identical to those in Dumas at every position; P-Oka sequence is from Gomi et al. [7].

^c Oka/GSK-A was analysed by CSA, Oka/GSK-B, -C & -D were analysed by pyrosequencing.

^d Oka/Merck-1 was analysed by CSA, Oka/Merck-2, -3, -4 & -5 were analysed by pyrosequencing.

^e Numbers refer to the percentage wildtype allele.

allele in all GSK batches, the remaining loci were a mixture of the of the ancestral (wildtype) allele and the vaccine allele in varying proportions.

3.3. Comparison between and within Merck and GSK Oka vaccine preparations

The percentage wildtype alleles present in the vaccine preparations from Merck and GSK for 15 of the 42 loci known to differ between pOka and the Oka Biken vaccine preparation are shown in Fig. 3 (data in Table 2). As can be seen in Fig. 3, there is a strong correlation between the allele frequencies in the vaccines

from the two companies ($r=0.79$, $P=0.0005$). The implications of this result are discussed further (see below). For each vaccine we compared genetic variation between different lots from the same batch, between different batches of the same vaccine and between the two vaccine preparations. There was a significant trend across most loci for the Merck vaccine preparations to have higher frequencies of the wildtype allele ($P=3.422 \times 10^{-5}$), as illustrated in Fig. 3. There was significant batch to batch variation between the three Merck batches ($P=7.181 \times 10^{-7}$) and between the three GSK batches ($P=9.697 \times 10^{-10}$). Although the amount of variation between batches was not significantly different for the two vaccine companies ($P=0.160$), there was significantly more variation in allele frequencies among lots from a single batch of GSK vaccine compared to lots from a single batch of Merck vaccine ($P=0.006$). The implications of these between and within-vaccine variations are discussed.

4. Discussion

We and others [10,11,13] have confirmed that the Oka vaccine comprises multiple haplotypes, each of which contains different numbers of vaccine mutations. Our data show three important findings. First, we confirm previous reports of the loss of wildtype alleles in the GSK as compared to Merck at 4 loci (560, 105705, 106262 and 107252), particularly where the wildtype allele was rare (i.e. at low frequency). Additionally, we show that where loci are mixed for vaccine and wildtype SNPs, the frequency of the wildtype allele at these loci is on average lower in the GSK than in the Merck vaccine. These findings are likely to be explained by production differences. Prior to development of the commercial vaccine in the 1980s, Smith Kline Beecham attempted to produce a genetically uniform vaccine—as a part of this the GSK seed lot underwent serial terminal dilutions in WI38 cells before further passages in MRC5s to generate the GSK working seed lot and vaccine stocks (Fig. 1). At the very least, this would be expected to reduce the amount of variability present in the vaccine mixture, if not indeed succeeding in making the vaccine genetically uniform. In contrast, the Merck vaccines are derived directly from the seed stock without any attempted purification before further passages in MRC5 cells ([7], Merck Product information IPC-VRV3-I-032008—summarised in Fig. 1), and are therefore expected to be more of a mixture of multiple haplotypes, as in the original seed stock.

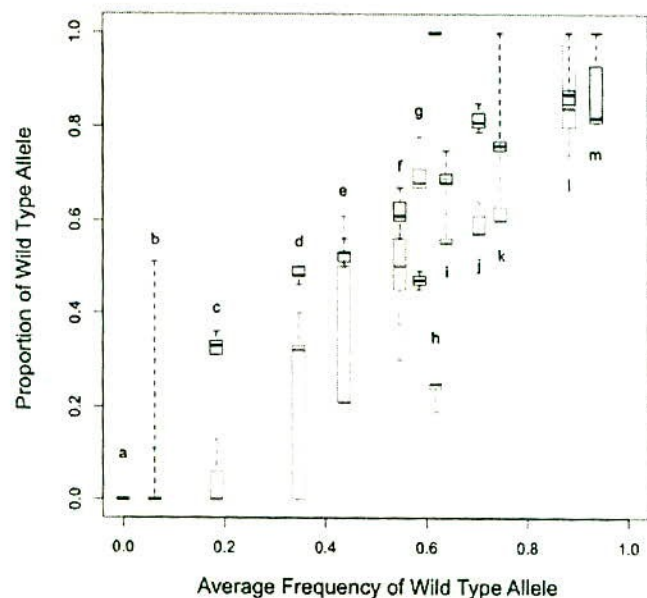


Fig. 3. Comparison of range and average allele frequencies at polymorphic sites in Oka/Merck (black) and Oka/GSK (grey) vaccines. The whiskers on the box plots represent the full range of estimates of the wildtype allele, from the three batches obtained from each company; the box illustrates the inter-quartile range. The band inside the box represents the median value. Letters indicate the SNP position as below: a—560, 106262, 107252; b—105705; c—105356; d—107136; e—105169; f—97748; g—58595; h—89734; i—107797; j—19431; k—108838; l—87280; m—105724.

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Our second observation is that despite the differences in production processes, the allele frequencies in the Merck and GSK vaccines are strongly correlated (Fig. 3). This is an unexpected observation. If the vaccine alleles were simply neutral in the *in vitro* environment of vaccine production one would not expect to see a correlation in allele frequencies between the two vaccines given the differences in the manufacturing process (Fig. 1). Taking into consideration the GSK terminal dilution procedures, there are two possible explanations. If we assume that the GSK process succeeded in producing a genetically uniform vaccine, then the vaccine alleles could have arisen by *de novo* mutation and spread in the GSK production line after purification had occurred. However, two questions then arise: why have the vaccine mutations appeared at the same loci and with approximately the same frequency, as the Merck vaccine, and what would explain the lower frequency of the ancestral alleles in the GSK vaccine? One possibility is that both the Merck and GSK production processes select for the same intermediate allele frequencies, but that the GSK values have had insufficient time to reach this stable “optimum” value through the accumulation of mutations following the loss of the ancestral allele during the process of serial terminal dilutions. An alternative explanation is that the GSK serial dilutions did not result in a genetically uniform population but rather one that remained polymorphic with approximately the same allele frequencies as the original seed mixture. Both explanations would account for the lower frequency of the ancestral allele in GSK batches at those loci where the ancestral allele was initially rare in the seed stock, since it is the rare alleles that would tend to be lost by serial dilution and be prone to ascertainment bias (whereby rare alleles would have been detected if they were slightly more common in the Merck population but not vice versa). The fact that the ancestral allele is rarer in GSK at all but two loci (Fig. 3, 58595 and 87280), suggests a third possibility: that some difference in the Merck production favours selection of the ancestral alleles at the majority of loci.

Finally, we have observed that the Merck vaccine has more similar allele frequencies between lots of the same batch, than does the GSK vaccine. If the variation between lots were due to chance (i.e. random genetic drift), then this observation would imply that the Merck protocols maintain larger sample sizes of virus at each generation than the GSK, as the effects of genetic drift are more pronounced in smaller populations than larger populations. Alternatively, if as suggested earlier, selection is occurring in the Merck production process, this could explain the more similar allele frequencies observed in the Merck vaccines than the GSK.

In summary, our data suggest that evolutionary processes do influence production of live attenuated vaccines and affect their composition. We have focused on variable loci that have previously been identified as being of potential biological importance for vaccine attenuation and as immune targets [13]. However, there is evidence to suggest other loci may also play a key role in attenuation [17]. More extensive sequencing of different batches and lots from each source would give a better idea of the allele frequencies present at all variable loci, allowing us to distinguish between the different hypotheses described here as they make different predictions. If the lower frequency of ancestral alleles in GSK batches were due to ascertainment bias, this trend would disappear once all loci were sequenced. If the trend were due to the loss of rare alleles in the GSK production line, more extensive sequencing would reveal a pattern in which the GSK allele frequencies were more extreme than those in Merck batches, i.e. closer to zero in GSK at loci where they were rare in Merck batches, and closer to one where they were common. Finally, if there were genome-wide selection against ancestral alleles in GSK, that trend should be apparent across most vaccine loci irrespective of their frequency. Our analyses of the differences in allele frequencies between the vaccines provide an insight into the various evolutionary processes that are clearly

affecting the composition of live attenuated vaccines. The data suggest that both batch to batch variation and variation between vaccines manufactured by different companies is not insignificant. To date, there is little evidence that the differences between the GSK and Merck vaccines or the batch to batch variation, affect efficacy or safety. However, a recent paper reported more breakthrough infections in children immunized with one dose of the GSK vaccine as compared with the Merck preparation, although the differences were not significant [18]. A previous publication also reported lower antibody titres following vaccination with the GSK preparation as compared to the Merck, albeit in the context of co administration of MMR vaccine [1]. Whether these data reflects greater variation in batch to batch immunogenicity of the GSK vaccine remains to be established. Nonetheless, understanding the evolutionary processes influencing this vaccine is clearly an important consideration for continued quality assurance of production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.02.021.

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Molecular Analysis of the Oka Vaccine Strain of Varicella-Zoster Virus

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Live attenuated Oka vaccine was subjected to molecular analysis, with the long-term goal of identifying genes in the vaccine strain responsible for its attenuation. Complete genomic sequences for both the Oka parent virus and the Oka vaccine virus were determined and compared. There were differences in only 42 bases between the 2 viruses. More than one-third (15/42) of the nucleotide substitutions in V-Oka were found in open reading frame (ORF) 62. These differences were also observed when sequences of Japanese low-passage clinical isolates of varicella-zoster virus (VZV) were compared with the Oka vaccine strain. The ORF62 gene encodes an immediate early (IE) protein, IE62, that is the major transactivator of VZV and, as such, is critical to initiating the VZV gene expression cascade. Several insertions and deletions were also observed in comparing the 2 sequences, largely in the internal tandem repeat units. Functional differences between the 2 types of Oka virus were also examined. Oka vaccine was found to consist of a mixture of different subpopulations, and the parental Oka virus replicates more efficiently than does the vaccine strain. Development of a bacterial artificial chromosome for VZV expression should lead to the discovery of additional differences in wild and vaccine types and, thus, enhance our understanding of the genetic basis for attenuation of the Oka vaccine strain.

Live attenuated varicella vaccines derived from the parent Oka (P-Oka) strain of varicella-zoster virus (VZV) are used routinely to prevent or modify the severity of varicella in children in Japan and many other countries [1–4]. The vaccine Oka strain (V-Oka) was developed by Takahashi and colleagues, using the empirical approach of passing P-Oka, which is a clinical isolate recovered from skin lesions of a child with varicella, in cultured human and guinea pig cells [5]. Attenuation of V-Oka was established clinically by inoculating susceptible children. These children had no signs of varicella, developed VZV IgG antibodies, and were protected from illness when they were exposed to wild-type VZV. Attenuation was also demonstrated by the reduced virulence of V-Oka in immunocompromised

children with leukemia or other immunosuppressive conditions [5–9]. Finally, V-Oka exhibits a low secondary transmission rate in comparison with wild-type VZV, with secondary transmission occurring only when the vaccine recipient had cutaneous lesions, and cases of varicella resulting from secondary V-Oka transmission are characteristically mild [10, 11]. The development of live varicella vaccine was accomplished before methods were available to investigate the genetic basis for V-Oka attenuation. New tools can now be used to clarify what molecular changes were introduced that distinguish P-Oka and V-Oka and which of those changes are likely to be associated with the reduced pathogenicity of V-Oka. Conceivably, the results from such studies could drive the future development of a molecularly engineered immunogenic vaccine that lacks any functional VZV virulence genes. Here, we summarize our work related to understanding the molecular mechanisms of V-Oka attenuation.

COMPARISON OF THE DNA SEQUENCES OF V-OKA AND P-OKA VIRUSES

The first approach that we used to investigate V-Oka attenuation was to sequence the complete genomes of

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V-Oka and P-Oka, on the basis of the hypothesis that correlations between DNA sequence and virulence could be identified. The full-length DNA sequences of V-Oka and P-Oka were determined by amplifying regions across the whole genome by polymerase chain reaction (PCR), followed by cloning and direct sequencing of the PCR products [12]. When the complete DNA sequences of V-Oka and P-Oka viruses were compared, only 42 bases were found to be different (figure 1). These differences did not include the substitutions in the terminal repeat segment (TRS), because these changes are duplicated in the internal repeat segment (IRS) of the VZV genome.

The DNA sequence information was used to predict amino acid sequences of predicted VZV gene products [12]. This analysis revealed that base substitutions at 20 nucleotide positions in V-Oka would result in amino acid conversions, and base substitutions at 17 nucleotide positions were silent mutations. The remaining 5 base substitutions occurred in noncoding regions. At 30 positions, alternative base substitutions appeared in V-Oka, indicating that V-Oka contained a mixture of genomes that differed with respect to the particular nucleotide (figure 1). Fifteen of these substitutions resulted in differences between the amino acid residues in the respective V-Oka gene products, so that V-Oka was predicted to contain viruses with variations in the amino acid at each of these positions. Surprisingly, more than one-third (15/42) of the nucleotide sub-

stitutions in V-Oka were found in open reading frame (ORF) 62 and therefore in the duplicated gene, ORF71, and its flanking regions. Approximately half (8/20) of the predicted amino acid substitutions resulted from nucleotide substitutions within ORF62. This observation of enhanced variability in the ORF62 region was of particular interest because ORF62 and ORF71 encode the immediate early (IE) 62 protein, which is the major viral transactivator of VZV and directs the transcription of all VZV genes required for its replication and for the production of progeny virions. In addition to the changes in IE62, 12 other amino acid substitutions were identified on the basis of differences in the V-Oka and P-Oka DNA sequences. These mutations occurred in ORFs 6, 9A, 10, 21, 31, 39, 50, 52, 55, 59, and 64. Among these, the gene products for which the characteristics, functions, or predicted functions are known include ORF6 (helicase primase complex), ORF10 (tegument/regulatory protein), ORF21 (nucleocapsid), ORF31 (glycoprotein B), ORF39 (integral membrane protein), ORF50 (glycoprotein M), ORF55 (helicase primase complex), and ORF59 (uracil DNA glycosylase).

In addition to the nucleotide substitutions, the V-Oka sequence had several deletions and insertions, compared with P-Oka in certain regions, including the tandem repeat regions and the origin of DNA replication. The VZV genome has 5 repeat regions, designated R1, R2, R3, R4, and R5, that appear

Position	ORF	Oka vaccine	Oka parent
560	5/1	C (NCR)	T (NCR)
703	1	T/C (Gln)	T (Gln)
763	1	T/C (Pro)	T (Pro)
2515	3/4	T/C (NCR)	T (NCR)
5745	6	G (Pro)	A (Ser)
10300	9A	T/C (Trp/Arg)	T (Trp)
12779	10	T/C (Ala/Val)	T (Ala)
19431	14	T/C (Stop)	T (Stop)
26125	18	G (Asn)	A (Asn)
31732	21	T/C (Thr/Ile)	C (Thr)
39036	22	T/C (Thr)	T (Thr)
39227		T/G (Pro)	T (Pro)
58595	31	A/G (Ile/Val)	A (Ile)
59287		A/G (Pro)	A (Pro)
64067	35	A/G (Ala)	A (Ala)
71252	39	T/C (Met/Thr)	T (Met)
82225	45	A/G (Pro)	A (Pro)
84091	47	A/G (Glu)	G (Glu)
87280	50	A/G (Cys)	A (Cys)
87305		T/C (Ser/Gly)	T (Ser)
89734	51	A/G (Thr)	A (Thr)

Position	ORF	Oka vaccine	Oka parent
90535	52	A/G (Ile/Val)	A (Ile)
94167	54	C (Leu)	T (Leu)
97748	55	G/A (Ala/Thr)	G (Ala)
97796		T/C (Cys/Arg)	T (Cys)
101088	59	A/G (Leu/Pro)	A (Leu)
105189	61/62	A/G (NCR)	A (NCR)
105310	62	A/G (Leu/Ser)	A (Leu)
105356		C (Val)	T (Ile)
105544		G (Ala)	A (Val)
105705		C (Ala)	T (Ala)
106262		C (Gly)	A (Arg)
106710		A/G (Ala)	A (Ala)
107136		C (Ala)	T (Ala)
107252		C (Gly)	T (Ser)
107699		A/G (Val/Ala)	A (Val)
107797		A/G (Leu/Pro)	A (Leu)
108111		C (Pro)	T (Pro)
108838		A/G (Met/Thr)	A (Met)
109137	62/63	A/G (NCR)	A (NCR)
109200		A/G (NCR)	A (NCR)
111650	64	A/G (Gln/Arg)	A (Gln)

Figure 1. Mutations between the Oka vaccine virus (V-Oka) and the parent virus (P-Oka). NCR, noncoding region; ORF, open reading frame. Adapted from [12], with permission from the American Society for Microbiology.

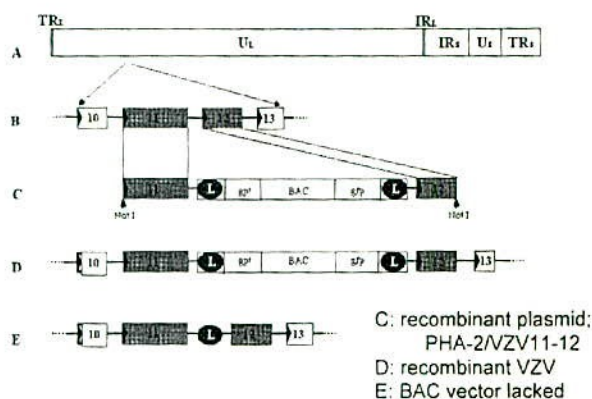


Figure 2. Construction of the varicella-zoster virus (VZV) genome bacterial artificial chromosome (BAC) in *Escherichia coli*. The VZV genome (A) is approximately 125 kbp long and consists of terminal repeat (TR), unique long (UL), internal repeat (IR), and unique short (US) DNA domains. The recombiant plasmid, PHA-2/VZV11-12 (C), contained 2.0 kbp each of 2 sequences from VZV (shaded boxes 11 and 12), as well as the BAC vector, the *gpt* gene, and the *gfp* gene, flanked by *loxP* sites. The open reading frame (ORF) 11 and ORF12 fragments of the genome of the VZV wild-type strain, P-Oka (shaded boxes 11 and 12) were generated by polymerase chain reaction amplification using the appropriate primers. The recombiant VZV rV01 (D) was generated by transfection of the VZV BAC plasmid into HEL cells. The recombiant VZV rV02, which lacked the BAC vector sequences (E), was generated by superinfection of VZV rV01 and a recombiant adenovirus, AxCANCre. The VZV rV02 contains a single *loxP* site. The circle enclosing an L represents the *loxP* site. Adapted from [15].

in ORFs 11, 14, and 22 and in the intergenic regions between ORFs 62 and 63 (and the corresponding region between 70 and 71), and between ORFs 60 and 61, respectively. The length of the repeats in regions R1, R3, and R4 were variable in V-Oka. The VZV genome contains an origin of replication in the IRS, which is duplicated in the TRS; each origin region is ~270 bp in length. The P-Oka genome contained 269 bp in this region and was 8 bp longer than V-Oka. The average full-length genome of V-Oka was slightly longer than that of P-Oka because of these insertions and deletions.

BIOLOGICAL COMPARISON OF V-OKA AND P-OKA VIRUSES

When the sequencing of V-Oka revealed that it consisted of a mixture of subpopulations with some sequence differences, viruses were isolated from V-Oka by plaque purification [13, 14]. Thirteen V-Oka isolates were obtained. When ORF62 was amplified and sequenced from each isolate, one clone, designated S7-01, had all of the 8 predicted amino acid substitutions compared with P-Oka. In contrast, another clone, S7-13, had the minimum number of substitutions compared with P-Oka, with only 5 predicted changes in amino acid residues. Therefore, these 2 clones were chosen to determine whether the ORF62

mutations correlated with differences in the replication of S7-01 and S7-13. A comparison of the plaque sizes of S7-01, S7-13, V-Oka, and P-Oka showed that P-Oka produced the largest plaques. V-Oka and S7-13 showed relatively larger plaques than S7-01. In particular, S7-01 showed significantly smaller plaques compared with P-Oka ($P < .01$). When these V-Oka progeny viruses, V-Oka, and P-Oka were compared by infectious center assay using human embryonic lung fibroblasts, the number of cells infected by P-Oka was 2.1-fold, 4.2-fold, and 2.6-fold higher than the number infected by V-Oka, S7-01, and S7-13, respectively. These results suggested that the efficiency of viral replication differed slightly among the V-Oka clones and that P-Oka replication was relatively more efficient than V-Oka replication.

To further assess the biological significance of differences at ORF62 of the V-Oka clones and P-Oka, the capacity of each version of IE62 to transactivate the ORF28 (VZV polymerase) promoter was evaluated. IE62 has well-established transactivating effects on this promoter. A reporter plasmid, pLuc-Pol, that had the DNA polymerase promoter upstream of the luciferase ORF was constructed and used for transient luciferase assays. The mutant IE62s expressed by the V-Oka variants were predicted to have less transactivating activity than IE62 encoded by P-Oka. The results of the luciferase assays showed trends that were consistent with the differences observed between P-Oka and V-Oka variants in mean plaque size and in infectious center assays. These findings support the possibility that both viral replication and cell-cell spread may be diminished in V-Oka because of altered potency of IE62 for inducing VZV gene transcription.

ORF10 protein, which is a homologue of herpes simplex virus type 1 VP16, is a virion-associated transactivator that can stimulate transcription from the ORF62 promoter. The base substitution identified in ORF10 caused a predicted amino acid conversion. Given the importance of IE62 as the major VZV transactivating protein, this change had the potential to affect V-Oka replication. However, when the ORF10 proteins encoded by V-Oka and P-Oka were compared for their capacities to enhance transcription from the ORF62 promoter, the P-Oka ORF10 gene product was somewhat more potent, but this difference was not significant.

GENETIC DIFFERENTIATION OF V-OKA VIRUS FROM OTHER CLINICAL ISOLATES OF VZV

To demonstrate that the changes in ORF62 were associated with the process of P-Oka attenuation by passage in cultured cells to generate V-Oka, clinical isolates were evaluated to determine the nucleotide sequence of ORF62 [13, 14]. All of the low-passage clinical isolates of VZV differed from V-Oka at the same 15 bases that were found to be different between V-Oka and P-Oka. The differentiation of V-Oka from other clinical

isolates, including P-Oka, was also evident from changes that altered the cleavage sites for *Bss*HII and *Nae*I to a unique pattern in V-Oka. V-Oka was successfully identified using 7 of 8 enzymes, with the exception of *Mae*II. On the basis of PCR and restriction enzyme digestion, all 54 VZV clinical isolates were shown to have sequence differences from V-Oka. Thus, the empirical attenuation of V-Oka introduced genetic changes in V-Oka that resulted in a mixture of genetic variants that appear to differ consistently from wild-type VZV.

DEVELOPMENT OF A BACTERIAL ARTIFICIAL CHROMOSOME (BAC)

A more complete analysis of the mutations in V-Oka that lead to its attenuation may be accomplished by introducing the changes observed by PCR and sequencing as single mutations or combinations of mutations into the P-Oka genome. Toward this end, we have developed a BAC that contains the P-Oka genome (figure 2) [15]. After electroporation into *E. coli* strain DH10B, the VZV BAC was stably propagated over multiple generations of its host. Human embryonic lung cells transfected with VZV BAC DNA that was recovered from DH10B showed cytopathic effects, and spread of the virus to neighboring cells was observed. The BAC vector sequences are flanked by *loxP* sites, and coinfection of the reconstituted virus with a recombinant adenovirus expressing Cre recombinase removed the residual bacterial sequences. The resulting recombinant, designated rV02, replicated as well as the parental virus in cultured cells. This P-Oka BAC should facilitate the next phase of investigations of the molecular mechanisms of V-Oka attenuation.

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