JOURNAL OF VIROLOGY, Aug. 2011, p. 8449–8452 Vol. 85, No. 16
0022-538X/11/$12.00 doi:10.1128/JVI.05054-11

Reassortment between Seasonal H1N1 and Pandemic (H1N1) 2009 Influenza Viruses Is Restricted by Limited Compatibility among Polymerase Subunits▽

Cássio Pontes Octaviani,[1] Hideo Goto,[1] and Yoshihiro Kawaoka[1,2,3,4]*

Division of Virology, Department of Microbiology and Immunology,[1] and Department of Special Pathogens, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan[2]; ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama 332-0012, Japan[3]; and Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53711[4]

Received 7 May 2011/Accepted 1 June 2011

Reassortment is important for influenza virus evolution and the generation of novel viruses with pandemic potential; however, the factors influencing reassortment are still poorly understood. Here, using reverse genetics and a replicon assay, we demonstrated that a mixed polymerase complex containing a pandemic (H1N1) 2009 influenza virus PB2 on a seasonal H1N1 virus background has reduced polymerase activity, leading to impaired virus viability. Adaptation of viruses containing the mixed polymerase complex resulted in compensatory mutations in PB1. Taken together, our results identify the cooperation between PB2 and PB1 as an important restricting factor for reassortment of influenza viruses.

Influenza A viruses are enveloped viruses of the family *Orthomyxoviridae* and have a genome composed of eight single-stranded, negative-sense RNA segments. This segmented genome allows reassortment, which is an important mechanism for the generation of new viruses, such as those that sporadically cause pandemics in humans (6).

Influenza pandemics are caused by the introduction of a virus with a hemagglutinin (HA) subtype that is new to human populations, which can result from reassortment (15). However, at least the three last pandemic viruses, i.e., the 1957 "Asian influenza," the 1968 "Hong Kong influenza," and the pandemic (H1N1) 2009 influenza viruses, contained, besides the HA gene, one or more polymerase subunits from a nonhuman source (4, 11, 14), which suggests the importance of these genes for the generation of pandemic viruses through reassortment. However, limited compatibility among polymerase subunits from different viruses is also a restricting factor for reassortment (2, 7, 8).

The emergence and establishment of the pandemic (H1N1) 2009 influenza virus in the human population have created a new scenario of possibilities of reassortment with the contemporary, cocirculating viruses, including seasonal H3N2 and H1N1 viruses (3, 12). Therefore, knowledge of the factors limiting reassortment between the pandemic (H1N1) 2009 influenza virus and contemporary human viruses is important for a complete understanding of the factors that will shape the evolution and emergence of future epidemic and pandemic viruses.

In previous studies, when we used reverse genetics (9) to generate a series of single-gene reassortants containing each of the A/California/04/2009 (H1N1) (CA04) genes on an A/Kawasaki/UT-SAI-23/2008 (H1N1) (k23) genetic background, we found that some of the reassortants could not be rescued after three independent attempts; notably, reassortants containing the PB2, PA, or NP genes derived from CA04 on a k23 background could not be generated. As this suggested a limited level of genetic compatibility among ribonucleoprotein (RNP) components between the two viruses, we wished to investigate further this finding, by means of a luciferase-based replicon assay. Plasmid pPolINP(0)luc2(0) (50 ng), which contains the firefly luciferase gene flanked by the noncoding regions of the NP gene derived from A/WSN/33 (WSN), was cotransfected into human embryonic kidney (HEK) 293 cells grown in 48-well plates, along with the protein expression plasmids (pCAGGS/MCS [5, 10]) containing the PB2, PB1, PA, and NP genes derived from k23 and CA04 (50 ng each) in all possible 16 combinations, by using the Trans IT 293 transfection reagent (Mirus, Madison, WI) (3 μl of transfection reagent per microgram of plasmid); 24 h posttransfection, the cells were assayed for luciferase activity by using a Bright-Glo luciferase assay system and a GloMax 96 microplate luminometer (Promega, Madison, WI). We found that the activities of the heterogeneous RNP complexes varied substantially. Although the introduction of CA04 PB1, PA, or NP genes in the k23 RNP background caused some reduction in polymerase activity relative to the all-k23 RNP complex, the introduction of CA04 PB2 caused the greatest reduction in the polymerase activity (Fig. 1A). Interestingly, all mixed RNP complexes that contained a PB2 derived from CA04 in combination with a PB1 derived from k23 showed markedly reduced activity, indicating a possible incompatibility between these two genes.

Next, we wanted to investigate the relevance of this genetic incompatibility in the context of virus growth. However, most of the single gene reassortants containing CA04 RNP compo-

* Corresponding author. Mailing address: The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-03-5449-5310. Fax: 81-03-5449-5408. E-mail: kawaoka@ims.u-tokyo.ac.jp.

▽ Published ahead of print on 15 June 2011.

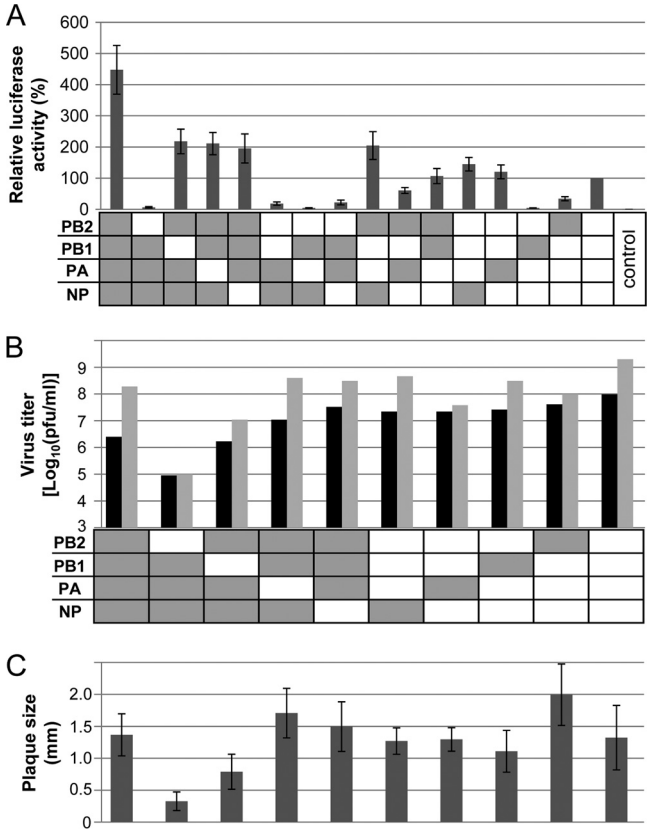

FIG. 1. (A) Polymerase activities of 16 RNP gene combinations measured in a replicon assay. Four expression plasmids (PB2, PB1, PA, and NP) for the 16 RNP gene combinations between k23 and CA04, together with pPolINP(0)luc2(0) for the production of virus-like RNA encoding the reporter luciferase gene, were transfected into HEK 293 cells and assayed for luciferase activity after 24 h of incubation at 37°C. The values shown are means ± standard deviations for three independent experiments and are standardized to the activities of the expression plasmids for the CA04 RNP complex proteins (100%). Genes derived from k23 and CA04 are shown in gray-shaded and open boxes, respectively. (B) Rescue efficiency and titers of stock viruses. Virus titers in the supernatant of transfected HEK 293T cells at 48 h post-transfection (black columns) and in the supernatant of MDCK cells at 48 h postinfection (multiplicity of infection = 0.001) (gray columns), measured by plaque assay in MDCK cells. Genes derived from k23 and CA04 are shown in gray-shaded and open boxes, respectively; the remaining genes are derived from WSN. (C) Plaque size in MDCK cells. Plaques were measured at 48 h postinfection; values represent means ± standard deviations (n = 50). The genetic composition of viruses is the same as that indicated in panel B.

nents on a k23 background could not be rescued. This led us to try a different approach for the generation of the reassortant viruses, by using the HA, NA, M, and NS genes derived from WSN, which is a virus with high rescue efficiency by reverse genetics.

Reassortant viruses containing different combinations of RNP components between k23 and CA04 on a WSN background could be rescued and had their rescue efficiency by reverse genetics as well as growth capability and plaque morphology in MDCK cells assessed (Fig. 1B and C). Intriguingly, the pattern of polymerase activity found in the replicon assay did not match that of the virus growth of the corresponding viruses; the reason for this discrepancy is unclear. Interestingly, however, the virus containing the PB2 gene from CA04 with the remaining RNP components from k23 (designated CA04PB2-k23RNP-WSN) showed substantially lower rescue efficiency by reverse genetics, growth capability, and plaque size in MDCK cells than did all other reassortant viruses.

The results from the replicon assay, combined with the differential growth capability of reassortant viruses, point to a limited compatibility of the CA04 PB2 with the remaining polymerase subunits from k23. To determine the region in PB2 responsible for this deficiency, we constructed a series of chimeric PB2 expression plasmids in which different extensions of the genes derived from k23 and CA04 were exchanged (Fig. 2A). The chimeric plasmids, along with expression plasmids for PB1, PA, and NP derived from k23, were used in the replicon assay, as described above. Recombinant RNP complexes containing the chimeric PB2s on a k23 background showed substantial variation in polymerase activity: while substitution for either the N- or C-terminal region of the k23 PB2 by CA04 did not appreciably reduce the activity, constructs containing the central region derived from CA04 PB2 showed a substantial loss of activity of the recombinant RNP complexes (Fig. 2A), suggesting that this region of CA04 PB2 is responsible for its relative inability to function in combination with the remaining RNP components derived from k23.

Reassortant viruses containing the CA04 PB2 with the remaining RNP components derived from k23 showed substantially impaired growth capability, compared with viruses containing either pure k23 or CA04 RNP complexes (Fig. 1B and C). Therefore, we wished to investigate the possibility of adaptation of viruses containing the hybrid RNP complexes. To this end, we subjected the virus CA04PB2-k23RNP-WSN to serial passage in MDCK cells. After five passages, partial restoration of virus growth capability and plaque formation ability was observed (Fig. 2B and C). Sequencing analysis of the adapted viruses showed compensatory mutations in the PB1 gene in three independent experiments (V285I in one experiment and I423T in two experiments) and also a mutation in NP (T423R) in one experiment (Fig. 2B). No mutation was observed in any other genes. To identify the relevance of each of the compensatory mutations for the polymerase activity, we cloned each of the adapted genes into pCAGGS/MCS and used them in combination with the plasmids for the remaining RNP components of CA04PB2-k23RNP-WSN in the replicon assay. As shown in Fig. 2D, only the mutation PB1 I423T corresponded with a significant increase in polymerase activity of the mixed RNP complex. Moreover, a reassortant virus containing the CA04 PB2 on a k23 background containing the PB1 I423T mutation (designated CA04PB2-k23PB1[I423T]-k23) could be generated by reverse genetics. These findings, combined with the severely impaired activity of all mixed RNP complexes containing a CA04 PB2 in combination with a k23 PB1 (Fig. 1A), indicate that the interaction between PB2 and PB1 is an important restricting factor for influenza virus reassortment and that an I423T mutation in PB1 can overcome the restriction of polymerase activity of the hybrid RNP complex. Intriguingly, the mutation PB1 V285I, despite improving the growth of CA04PB2-k23RNP-WSN, did not confer substantial polymerase activity in the replicon assay. The mechanism for

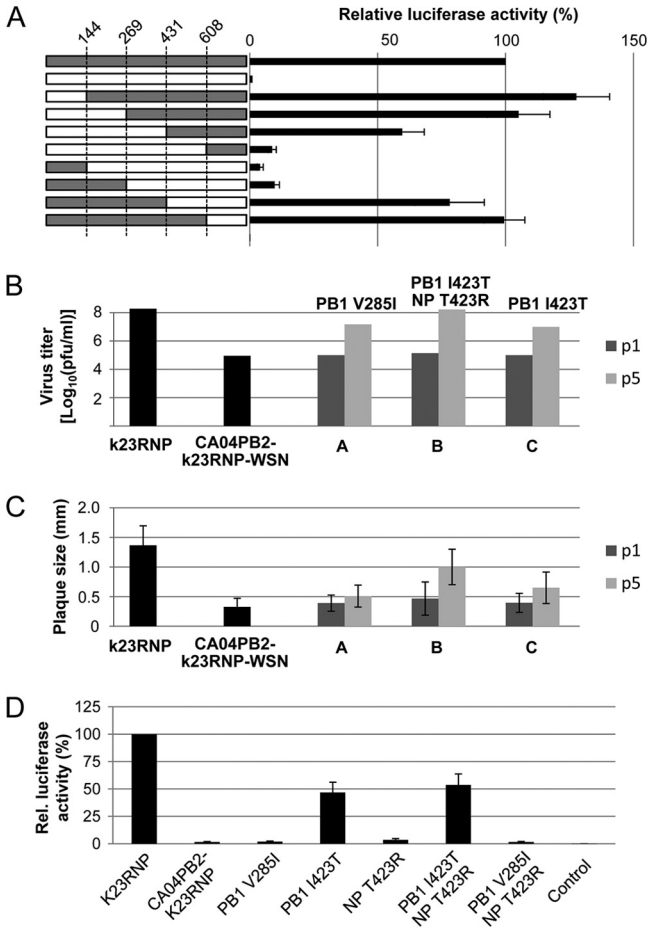

FIG. 2. (A) Schematic diagram of CA04/k23 PB2 chimeras and their effects on RNP activities in a replicon assay. pCAGGS/MCS plasmids for the expression of chimeric PB2s were constructed, making use of restriction endonuclease sites naturally present in the k23 PB2 open reading frame corresponding to the amino acid positions indicated above the diagram. The coding regions of k23 and CA04 PB2 are represented by gray and white bars, respectively. Plasmids for expression of k23 PB2, CA04 PB2, or chimeric PB2 plasmids, together with expression plasmids for PB1, PA, and NP derived from k23, were used in a replicon assay using the same methodology described for Fig. 1A. The values shown are means ± standard deviations for three independent experiments and are standardized to the activities of the expression plasmids for the k23 RNP complex proteins (100%). (B) Virus titers of serially passaged CA04PB2-k23RNP-WSN. Virus CA04PB2-k23RNP-WSN contains the PB2 gene derived from CA04, PB1, PA, and NP genes from k23, and its remaining genes are derived from WSN. Virus titers in supernatants of infected MDCK cells at passages 1 and 5 are represented in dark gray and light gray, respectively. The mutations observed at passage 5 for each virus are indicated above the relevant columns. A, B, and C represent three independent adaptation experiments. Stock titers of k23RNP (RNP components derived from k23 with the remaining genes derived from WSN) and CA04PB2-k23RNP-WSN are given for reference. (C) Plaque size of serially passaged CA04PB2-k23RNP-WSN in MDCK cells. Values represent means ± standard deviations, measured at 48 h postinfection; $n = 50$. (D) Effect of mutations observed in serially passaged CA04PB2-k23RNP-WSN on RNP activity in a replicon assay. Genes with mutations acquired upon five passages in MDCK cells were cloned into pCAGGS/MCS expression vector and used in conjunction with plasmids for remaining RNP components of CA04PB2-k23RNP-WSN in a replicon assay using the same methodology described for Fig. 1A. The mutations tested are indicated below the relevant columns.

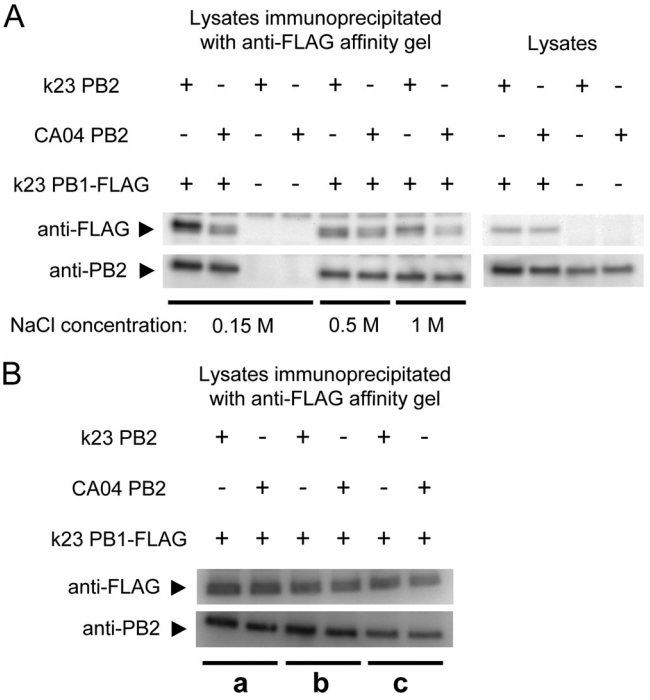

FIG. 3. Immunoprecipitation of polymerase subunits at different salt and detergent concentrations. HEK 293 cells grown in 6-well plates were cotransfected with 750 ng each of expression plasmids for k23 PA, k23 PB1-FLAG, and k23 PB2 or CA04 PB2, as indicated (+). At 24 h posttransfection, cells were lysed and the cleared lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma, St. Louis, MO). The agarose-bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with mouse monoclonal anti-PB2 or anti-FLAG antibodies. (A) Cell lysis and immunoprecipitation were carried out in buffer (50 mM Tris-HCl, pH 7.8, 1% NP-40, 1 mM EDTA, protease inhibitor cocktail) with different salt concentrations, as indicated. The cell lysates were also directly Western blotted with mouse monoclonal anti-PB2 or anti-FLAG antibodies. (B) Cell lysis and immunoprecipitation were carried out in buffer containing 0.15 M NaCl, and 1% NP-40 and 0.1% SDS (a), 2% NP-40 and 0.2% SDS (b), or 5% NP-40 and 0.5% SDS (c), as indicated.

the partial restoration of viral growth by this mutation remains unclear.

Inability to form a heterotrimer is one possible mechanism for incompatibility among polymerase subunits between different viruses (7), so we wondered whether this could explain the incompatibility between CA04 PB2 and k23 PB1. To investigate this, we performed immunoprecipitation assays. Lysates prepared from HEK 293 cells transfected with expression plasmids for k23 PB2, k23 PB1-FLAG (a k23 PB1 with a C-terminal FLAG tag), and k23 PA, or CA04 PB2, k23 PB1-FLAG, and k23 PA, were immunoprecipitated by use of an anti-FLAG M2 affinity gel (Sigma, St. Louis, MO), at different salt and detergent concentrations. However, no difference in the coimmunoprecipitation pattern could be seen between CA04 PB2 and k23 PB2 (Fig. 3). Our results suggest that the functional incompatibility between the polymerase subunits cannot be explained by lack of ability to form a heterotrimer at least as demonstrated by the methods described here. Our mapping of the region of CA04 PB2 responsible for the functional incompatibility with k23 RNP components using chimeric CA04/k23

PB2s pointed to the central region of the gene (Fig. 2A), while adaptation of virus CA04PB2-k23RNP-WSN caused a compensatory mutation in amino acid 423 of PB1 that was able to restore the functionality of the mixed RNP complex to some extent (Fig. 2B, C, and D). However, this position has not been identified as an interaction site between these two proteins in immunoprecipitation experiments, as reported by other groups (reviewed in reference 1), which is in agreement with our finding that CA04 PB2 interacted with k23 PB1 as well as k23 PB2. The reason for the limited activity of the mixed RNP complex therefore remains unclear and is probably due to subtler functional constraints: that is, the level of incompatibility between the two proteins was not explained by the lack of heterotrimer formation, yet the resulting hybrid complex was nonetheless functionally impaired. Isoleucine at position 423 is highly conserved (approximately 99.7%) among influenza A viruses, and although analysis of 3156 unique influenza A virus PB1 sequences, including H1N1, H3N2, and H5N1 subtypes, showed two amino acid substitutions at this position (leucine and valine), threonine was not found. While the significance of this is not clear, the high degree of conservation of isoleucine at this position may indicate that adaptation through an I423T mutation could be restricted due to additional constraints.

The findings reported here contrast with previous observations by our group, in which a high degree of genetic compatibility was found between pandemic (H1N1) 2009 influenza virus and a contemporary H5N1 virus, with all combinations of RNP components between those two viruses showing substantial polymerase activity and producing viruses with high growth capability (13). Further efforts are required for a thorough understanding of the factors governing reassortment in influenza viruses, as this will improve our ability to understand, predict, and control influenza epidemics and pandemics in the future.

We thank Susan Watson for scientific editing.

This work was supported by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, and in part by Grants-in-Aid for Specially Promoted Research and for Scientific Research, by ERATO (Japan Science and Technology Agency), by the National Institute of Allergy and Infectious Diseases Public Health Service research grants, and by the Center for Research on Influenza Pathogenesis (CRIP) funded by the National Institute of Allergy and Infectious Diseases (contract HHSN266200700010C).

REFERENCES

1. **Boulo, S., H. Akarsu, R. W. H. Ruigrok, and F. Baudin.** 2007. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. Virus Res. **124:**12–21.
2. **Chen, L., C. T. Davis, H. Zhou, N. J. Cox, and R. O. Donis.** 2008. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza viruses. PLoS Pathog. **4:**e1000072.
3. **Herfst, S., et al.** 2010. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. J. Virol. **84:**3752–3758.
4. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. **63:**4603–4608.
5. **Kobasa, D., M. E. Rodgers, K. Wells, and Y. Kawaoka.** 1997. Neuraminidase hemadsorption activity, conserved in avian influenza A viruses, does not influence viral replication in ducks. J. Virol. **71:**6706–6713.
6. **Laver, W. G., and R. G. Webster.** 1973. Studies on the origin of pandemic influenza. 3. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology **51:**383–391.
7. **Li, C., M. Hatta, S. Watanabe, G. Neumann, and Y. Kawaoka.** 2008. Compatibility among polymerase subunit proteins is a restricting factor in reassortment between equine H7N7 and human H3N2 influenza viruses. J. Virol. **82:**11880–11888.
8. **Naffakh, N., A. Tomoiu, M. A. Rameix-Welti, and S. van der Werf.** 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. Annu. Rev. Microbiol. **62:**403–424.
9. **Neumann, G., et al.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. **96:**9345–9350.
10. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–200.
11. **Novel Swine-Origin Influenza A (H1N1) Investigation Team.** 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. **360:**2605–2615.
12. **Octaviani, C. P., C. Li, T. Noda, and Y. Kawaoka.** 2011. Reassortment between seasonal and swine-origin H1N1 influenza viruses generates viruses with enhanced growth capability in cell culture. Virus Res. **156:**147–150.
13. **Octaviani, C. P., M. Ozawa, S. Yamada, H. Goto, and Y. Kawaoka.** 2010. High level of genetic compatibility between swine-origin H1N1 and highly pathogenic avian H5N1 influenza viruses. J. Virol. **84:**10918–10922.
14. **Smith, G. J. D., et al.** 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature **459:**1122–1125.
15. **Wright, P. F., G. Neumann, and Y. Kawaoka.** 2007. Orthomyxoviruses, p. 1691–1740. *In* D. M. Knipe et al. (ed.), Fields virology, 5th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.