JOURNAL OF VIROLOGY, Dec. 1996, p. 9069–9073 Vol. 70, No. 12
0022-538X/96/$04.00+0

Influence of Subterminal Viral DNA Nucleotides on Differential Susceptibility to Cleavage by Human Immunodeficiency Virus Type 1 and Visna Virus Integrases

MICHAEL KATZMAN[1,2]* AND MALGORZATA SUDOL[1]

Departments of Medicine[1] and Microbiology and Immunology,[2] Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033-0850

Received 19 June 1996/Accepted 14 August 1996

A comparison of the extents of site-specific cleavage of U5 and U3 viral DNA termini by the integrases of human immunodeficiency virus type 1 and visna virus guided the quantitative testing of oligonucleotide substrates containing specific base substitutions. The simultaneous exchange of positions 5 and 6 between U3 substrates switched the patterns of differential susceptibility to the two integrases. The activity of visna virus integrase was more dependent on the identity of position 5 adjacent to the invariant CA bases than on position 6, whereas human immunodeficiency virus type 1 integrase appeared to interact even more critically with position 6. Although the paired natural substrates of most lentiviral integrases match at positions 7 and 8, these bases were not important for susceptibility of U5 substrates. In fact, the final six U5 positions contained all of the sequence information necessary for susceptibility. These results suggest that constraints other than integration influence the terminal inverted repeats of retroviral DNA.

Retroviral integrase (IN) catalyzes the incorporation of a DNA copy of the viral RNA genome into host cell DNA, a recombination that is necessary for viral replication and disease pathogenesis. Defining specific interactions between substrate DNA bases and amino acid residues of IN will refine models of integration and contribute to the development of antiviral agents. Although host DNA integration sites do not exhibit any sequence consensus, integration is specific with respect to the U3 and U5 ends of viral DNA that act as attachment (*att*) sites during this reaction. Following reverse transcription in vivo, the site-specific endonuclease activity of IN nicks both termini of linear viral DNA adjacent to invariant CA bases typically found 2 nucleotides from the 3′ end of each DNA strand. The resulting recessed 3′-OH ends of the viral DNA subsequently are inserted by IN into each strand of host DNA at staggered cleavage sites without sequence specificity. Repair of the joined intermediate yields an integrated provirus with a characteristic loss of 2 bp from each end and a short duplication of flanking host DNA (7, 15, 32). The reactions catalyzed by IN can be modeled in vitro by using purified INs and oligonucleotides designed to mimic either end of unintegrated linear viral DNA (Fig. 1A). With these substrates, IN exhibits site-specific endonuclease (or processing) activity that appropriately removes the expected 2 nucleotides following the conserved CA dinucleotide (9) and inserts the processed DNA ends into various sites on other oligonucleotides (6, 8).

When this assay system was introduced (9), structure, size, and sequence requirements for substrates of avian myeloblastosis virus IN, which have been confirmed for other integrases, were defined. For optimal susceptibility to IN, substrates must be double stranded, be at least 15 bp long, and contain authentic viral sequences close to the CA dinucleotide. Several groups subsequently showed the importance of the CA dinucleotide (1, 16, 18, 22, 26, 27, 29, 31) for susceptibility to human immunodeficiency virus type 1 (HIV-1) IN. In addition, the effects of other base substitutions (1, 16, 18, 22, 26, 29, 31, 33), abasic sites (22, 29), mismatched base pairs (22, 29, 33), methylphosphonodiester substitution (21), and adduct interference (2) have been used to explore potentially important interactions between HIV-1 IN and viral U5 (2, 16, 18, 21, 22, 26, 29, 31, 33) or U3 (1, 2, 25) sequences during processing and DNA joining. Other INs have also been studied with altered substrates (6, 10, 13, 23, 24, 28, 30). However, general rules describing which viral DNA bases interact most critically with different integrases have not been defined. Our laboratory has performed comparative studies on the related lentiviral INs of HIV-1 and visna virus to identify regions of the IN protein that interact with different nucleophiles and with features of target DNA during catalysis (10–12). These studies have permitted a directed and quantitative examination of the roles of subterminal U3 and U5 DNA positions in differential susceptibility to the two enzymes.

Effects of exchanging positions 5 and 6 in U3 substrates. We previously reported (10, 11) that HIV-1 and visna virus INs exhibit different levels of site-specific processing activity on 18-mer substrates derived from either end of linear DNA from both viruses (summarized in Fig. 1B). In particular, the substrates derived from the viral U3 termini clearly distinguish between the two enzymes (Fig. 2A, lanes 1 to 10), a difference we exploited when examining chimeric INs made from the two proteins (11). The differential activity on the two U3 substrates is striking, considering that these sequences differ at only 2 of the final 9 bp at their 3′ ends, i.e., positions 5 and 6 just internal to the invariant CA dinucleotide (Fig. 1B). To probe the significance of these positions, double-stranded oligonucleotides with base substitutions that interconvert portions of the two U3 sequences were assayed with each IN.

An HIV-1 U3 substrate with a double substitution at positions 5 and 6 to match the terminal 9 bp of visna virus U3 DNA (designated HIV-1 U3s5,6 [the s indicates an exchange substitution]) acted in these assays similarly to the wild-type visna virus substrate (Fig. 2A [compare specific 16-mer products in lanes 11 to 15 with those in lanes 6 to 10]). Likewise, a visna

* Corresponding author. Mailing address: Department of Medicine, The Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, P.O. Box 850, Hershey, PA 17033-0850. Phone: (717) 531-8881. Fax: (717) 531-4633.

A.

B.

Terminus	Sequence	Specific Cleavage By HIV-1 IN	Visna IN
HIV-1 U5 plus strand	5'TGGAAAATCTCTAGCAGT3'	++	++
Visna U5 plus strand	5'CGGAGCGGATCTCGCAGC3'	+	++
HIV-1 U3 minus strand	5'TGAATTAGCCCTTCCAGT3'	++	+
Visna U3 minus strand	5'GTTCTCTGTCCTGACAGT3'	+	++
	18 14 11 9 8 7 6 5 4 3 2 1		

FIG. 1. (A) In vitro IN assays. Double-stranded oligonucleotides that mimic one end of unintegrated retroviral DNA are 5′ radiolabeled (asterisk) on the strand that contains the conserved CA bases (boldface type). Specific endonucleolytic cleavage or processing (arrow) by IN yields a labeled oligomer 2 nucleotides shorter than the substrate. The DNA-joining activity of IN inserts the recessed CA3′-OH terminus into various sites along other oligonucleotides (shown as dotted lines) to create a set of labeled products longer than the substrate. The target DNA strand can be processed or labeled or not. Processing and DNA joining can occur sequentially in one assay. (B) Differential processing by HIV-1 and visna virus INs on four natural substrates. The terminal 18 bases of the strand that gets cleaved by IN are shown in parallel 5′-to-3′ orientation, with the conserved CA dinucleotide shown in boldface type; the numbering used throughout the text is indicated. The extent of specific cleavage by each IN in the presence of 10 mM Mn^{2+} is represented as +, ≤10%, or ++, >10%. Results for this and other figures were quantified from radioactive 20% polyacrylamide–7 M urea gels with a Betascope (Betagen, Waltham, Mass.). Specific cleavage was calculated for each lane as (counts per minute of 16-mers + counts per minute of >18-mers)/(total counts per minute in the lane), with background corrections from analogous parts of a negative control lane; >18-mers are products longer than substrate length, which form only following specific cleavage (1, 6, 8). The use of total counts per minute in the denominator best reflects the conversion of the substrate to a specific product but yields calculated specific cleavages lower than those that would be suggested by comparing 16-mer products with remaining 18-mer substrates.

virus U3 substrate with the reciprocal double substitution (visna U3s5,6) to match the terminal 9 bp of HIV-1 U3 DNA acted similarly to the wild-type HIV-1 substrate (Fig. 2A [compare lanes 16 to 20 with lanes 1 to 5]). Similar results were obtained when early time points on the linear portions of the reaction curves were examined (data not shown). A longer exposure of the upper part of the same gel (Fig. 2B) shows that each enzyme performed the DNA joining reaction on each substrate and that the extent of this reaction (i.e., the total amount of longer products) was roughly similar for substrates that matched at the terminal nine positions (compare lanes 1 to 5 with lanes 16 to 20 and lanes 6 to 10 with lanes 11 to 15). As previously noted, the patterns of joined products differed as a function of the target DNA sequence and the source of IN (11). Substrate sequences and quantitation of specific cleavage for multiple replicate reactions are shown in Fig. 2E, in which the distinct sensitivity patterns for a given substrate to the two INs can be appreciated. In particular, the patterns for HIV-1 U3 and visna U3s5,6 match, as do those of HIV-1 U3s5,6 and visna virus U3. Thus, the exchange of positions 5 and 6 between U3 substrates switched the patterns of susceptibility to the two INs, an observation that will be useful for analyzing specific protein-DNA contacts when the viral DNA binding site of IN is identified and structural information becomes available.

The results described above strongly suggest that only the final 9 bp of the U3 substrates specifically interact with these enzymes. However, positions 11 and 14 of the two wild-type U3 sequences are the same (Fig. 1B) and could have contributed to the susceptibility of the different substrates. To test this possibility, we used an HIV-1 U3 substrate with the double substitution at positions 5 and 6 that additionally contained other changes at positions 11 and 14 (HIV-1 U3s5,6,m11,14 [the m indicates mutation rather than exchange substitution]). This substrate also acted like the visna virus wild-type U3 substrate (summarized as the third entry in Fig. 2E). The high level of susceptibility of this substrate to visna virus IN and its susceptibility to HIV-1 IN that was comparable to that of HIV-1 U3s5,6 indicate that positions 11 and 14 do not interact in a sequence-specific manner with these enzymes. We conclude that all of the sequence information necessary for susceptibility of U3 substrates to specific cleavage by either IN is contained in the final nine positions and that positions 5 and 6 together are critical for susceptibility. Since the double substitution of positions 5 and 6 in the avian system (albeit in the U5 sequence) hinders cleavage in vitro and impairs integration in vivo (5, 9), it could be that all INs interact specifically with these bases.

Effects of independently exchanging position 5 or 6 in U3 substrates. Surprisingly, HIV-1 IN and visna virus IN were found to interact with positions 5 and 6 differently when potential interactions with these positions were tested individually. We anticipated that altering position 5, which is closer to the CA dinucleotide, would have a greater effect on specific cleavage by either enzyme than would altering position 6. This result is exactly what was found for visna virus IN, which was very sensitive to substitution of position 5 but not position 6 in the visna virus U3 sequence (visna U3s5 and visna U3s6, respectively [Fig. 2C, lanes 14, 15, 19, and 20]). However, HIV-1 IN exhibited relatively poor processing activity on substrates with a single substitution of either position 5 or 6 in the HIV-1 U3 sequence (HIV-1 U3s5 and HIV-1 U3s6, respectively [Fig. 2C, lanes 2, 3, 7, and 8]). Moreover, HIV-1 IN seemed at least as sensitive to substitution of position 6 as it did to position 5, if not more so (Fig. 2C [compare lanes 7 and 8 with lanes 2 and 3]). We know that the enzymes used in this experiment were active, since they exhibited high levels of processing on the HIV-1 U5 substrate in concurrent reactions (not shown). A darker exposure of the top portion of a similar gel (Fig. 2D) showed that each IN created longer joined DNA products with HIV-1 U3s5 and visna U3s6, which have the identical terminal 9 bp (Fig. 2E). Trace amounts of such products for HIV-1 U3s6 (better appreciated on other gels) and visna virus U3s5, which also match at their final nine positions, were detected.

Sequences of the singly substituted substrates and quantitation of specific cleavage for multiple reactions are shown in Fig. 2E, in which the extents of cleavage for a given IN on different substrates can be compared directly. In particular, the activity of HIV-1 IN was affected more by substitution of HIV-1 U3 position 6 than position 5, whereas visna virus IN was hindered more by substitution of visna virus U3 position 5 than position 6. These observations must be accommodated as models of retroviral integration are refined for each IN. It also is evident that results with HIV-1 U3s6 and visna virus U3s5 are similar, as are those with HIV-1 U3s5 and visna virus U3s6, as expected if all of the sequence information necessary for cleavage is contained in the final nine positions of substrate DNA. The relatively insensitive oligonucleotides (HIV-1 U3s6 and visna virus U3s5) were at least 95% double stranded under these reaction conditions, as assessed by nondenaturing gel electrophoresis (data not shown). In addition, labeling these substrates more than once and resynthesizing the HIV-1 U3s6 oligonucleotides yielded similar data, providing confidence for these observations. Similar results also were obtained with different preparations of purified IN.

It is likely that the tolerance of INs for substrate base substitutions is limited such that some bases disrupt important interactions and are unacceptable at certain positions. For

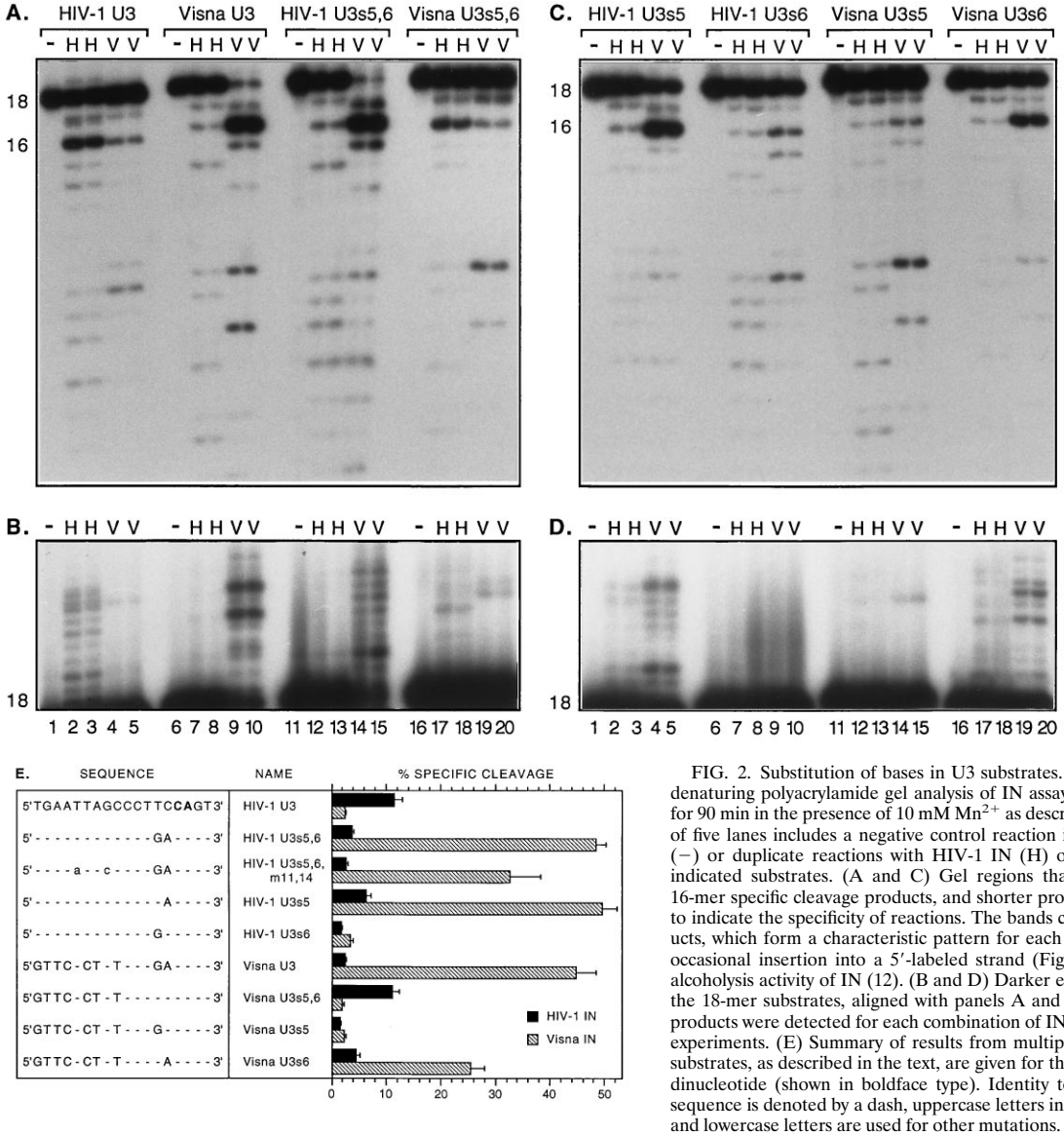

FIG. 2. Substitution of bases in U3 substrates. (A to D) Autoradiograms of denaturing polyacrylamide gel analysis of IN assays. Reactions were conducted for 90 min in the presence of 10 mM Mn^{2+} as described previously (10). Each set of five lanes includes a negative control reaction incubated with protein buffer (−) or duplicate reactions with HIV-1 IN (H) or visna virus IN (V) for the indicated substrates. (A and C) Gel regions that include 18-mer substrates, 16-mer specific cleavage products, and shorter products, to the levels of 4-mers, to indicate the specificity of reactions. The bands containing these shorter products, which form a characteristic pattern for each substrate and IN, are due to occasional insertion into a 5′-labeled strand (Fig. 1A) and to the nonspecific alcoholysis activity of IN (12). (B and D) Darker exposures of gel regions above the 18-mer substrates, aligned with panels A and C, respectively. Joined DNA products were detected for each combination of IN and substrate in this or other experiments. (E) Summary of results from multiple experiments. Sequences of substrates, as described in the text, are given for the strand that contains the CA dinucleotide (shown in boldface type). Identity to the base in the HIV-1 U3 sequence is denoted by a dash, uppercase letters indicate exchange substitutions, and lowercase letters are used for other mutations. Specific cleavage measured in replicate experiments is shown as the mean ± standard error (error bar); each reaction was conducted an average of 20 times.

example, our data suggest that HIV-1 IN cannot tolerate G at position 6 in the U3 substrate. Reicin et al. found that a U3 substrate with three Gs substituted at positions 6 to 8 was a poor cleavage substrate for HIV-1 IN, and a virus containing this mutation replicated poorly (25). However, Sherman et al. (26) reported that single base substitutions at positions 5 or 6 in the context of HIV-1 U5 substrates did not hinder processing by HIV-1 IN and Mazumder and Pommier (22) found that a mismatched deoxyuridine at position 6 in a U5 substrate was tolerated. The intrinsically higher susceptibility of U5 substrates to HIV-1 IN (see below) could explain these differences, since small changes might be better tolerated within that context.

Effects of base substitutions in U5 substrates. Given the importance of U3 positions 5 and 6, it is surprising that these bases are not conserved between the two natural substrates of HIV-1 IN (i.e., the HIV-1 U5 and U3 termini within a single virus [Fig. 1B]). The same observation holds for the visna virus U5 and U3 termini. In contrast, positions 7 and 8 of the paired natural substrates of these (and most lentiviral) INs do match, suggesting an important interaction for each protein with those bases. We tested this hypothesis using substrates derived from the HIV-1 U5 sequence, which is very sensitive to either enzyme (Fig. 3A, lanes 1 to 5). We first mutated positions 10 to 18 to bases that appear in none of the four natural substrates (HIV-1 U5m10-18) (Fig. 3A, lanes 6 to 10, and Fig. 3B) and found only minimal effects on susceptibility, confirming that the identity of bases at positions 10 to 18 of the U5 terminus does not contribute significantly to specific cleavage by IN. However, a substrate with the mutation of positions 7 and 8 to bases found in none of the four substrates also remained highly sensitive to each IN (HIV-1 U5m7,8) (Fig. 3A, lanes 11 to 15, and Fig. 3B). Thus, positions 7 and 8 are not critical for susceptibility to either IN.

To be sure that position 9 of the U5 sequence was not playing an important role in susceptibility to IN, we tested an additional substrate, HIV-1 U5m7-18. This substrate has a purine at position 9 of the strand that is specifically nicked by IN (the four natural substrates shown in Fig. 1B have pyrimi-

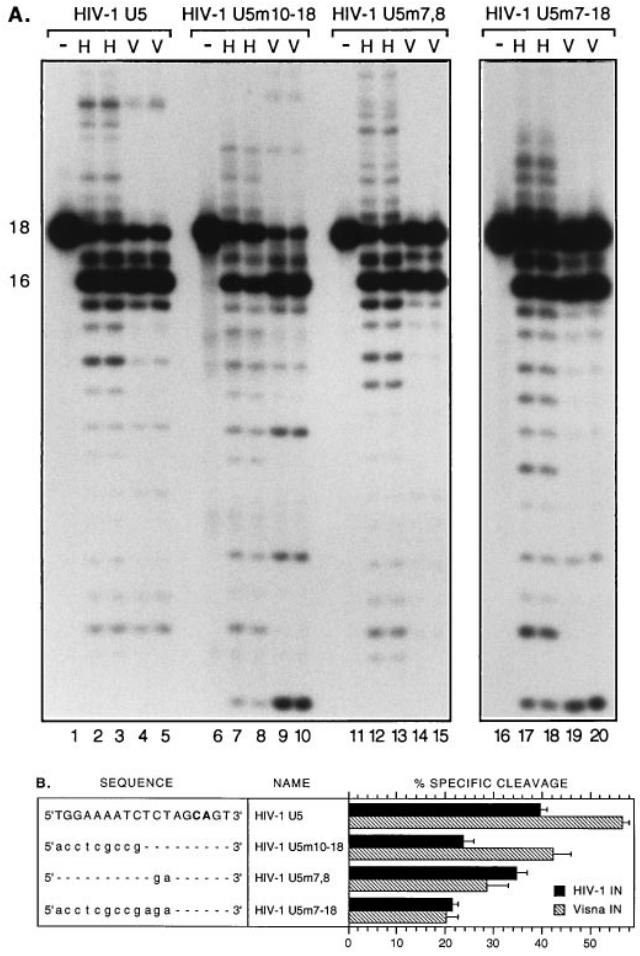

FIG. 3. Substitution of bases in U5 substrates. (A) Autoradiograms of denaturing polyacrylamide gel analysis of IN assays on the indicated substrates are shown. Details are given in the legend to Fig. 2. These exposures adequately demonstrate most of the DNA-joining products; longer exposures confirmed such products for all reactions that contained IN. (B) Sequences of substrates are shown relative to the HIV-1 U5 sequence; details are given in the legend to Fig. 2E. Specific cleavage measured in replicate experiments is shown as the mean ± standard error (error bar); each reaction was conducted an average of 18 times.

dines at this position) and retains only the terminal 6 bp of the HIV-1 U5 sequence. This substrate was also very sensitive to each IN (Fig. 3A, lanes 16 to 20, and Fig. 3B). Thus, all of the U5 sequence information necessary for specific cleavage by IN is contained in the final 6 bp. Some investigators have suggested that bases between HIV-1 U5 positions 11 and 17 can influence susceptibility to IN (18, 31, 34). Indeed, our data cannot exclude the possibility of a minor effect resulting in approximately 50% less cleavage of some of the altered U5 substrates. However, compared with the susceptibility of many wild-type terminal DNA substrates to different retroviral INs, these sequences (Fig. 3) certainly can be deemed highly susceptible to each IN. The number and choice of base substitutions utilized could also affect results. Our data are consistent with those of others for HIV-1 IN (1, 2, 16, 22, 26, 33) and confirm the importance of the terminal 6 bp for high susceptibility to processing. In particular, Sherman et al. (26) found that the terminal 6 bp from either end of HIV-1 DNA could support near-maximal levels of processing by HIV-1 IN, even though internal bases could compensate for mutations at those positions. Since IN requires that substrates have many more than 6 bp for optimal activity (9, 19, 26), it would not be surprising if particular sequences other than the terminal 6 bp could influence the efficiency of reactions catalyzed by IN.

The finding that U5 positions 7 and 8 are not critical for in vitro susceptibility to HIV-1 IN and visna virus IN raises the question of why terminal matches at these positions are conserved in most lentiviruses. The idea that retroviral DNA contains terminal inverted repeats to facilitate recognition by homomultimeric INs is challenged by the imperfect nature of many of the inverted repeats and by the tolerance for many base substitutions exhibited by IN in in vitro assays. However, differences between the end sequences might promote sequential, rather than simultaneous, recognition and processing since Kukolj and Skalka found that cleavages of the two viral DNA ends could be temporally separated (14). Moreover, in vitro IN assays might not reflect constraints that occur in vivo (3–5, 17, 20). Our results suggest that positions 7 and 8 at the viral DNA termini should be mutated in the context of infectious molecular clones to examine any effects on lentiviral replication.

This work was supported by Public Health Service grant R29 AI30759 from the National Institute of Allergy and Infectious Diseases.

We thank Lynn M. Skinner and John W. Wills for helpful discussions.

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