JOURNAL OF BACTERIOLOGY, Feb. 1981, p. 914-919
0021-9193/81/020914-06$02.00/0

Vol. 145, No. 2

Transductional Mapping of *ksgB* and a New Tn*5*-Induced Kasugamycin Resistance Gene, *ksgD*, in *Escherichia coli* K-12

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We have mapped the *Escherichia coli ksgB* gene to min 36.5, 0.8 min from *man* and 0.7 min from *aroD*. A new kasugamycin resistance (Ksg^r) gene, *ksgD*, has been isolated, using a transposon, Tn*5*. *ksgD*::Tn*5* is 44% cotransducible with *sbcA*, unlinked to *trp*, and unlinked to *man* (by P1 transduction). The *ksgD*::Tn*5* has a late time of entry from HfrB7 (PO43). These data place *ksgD* clockwise from *sbcA* (which enters early from HfrB7) at min 30.4. The resistance of *ksgB ksgD* single and double mutant strains has been quantitated. Single mutations, *ksgB* or *ksgD*, gave resistance to 600 μg of kasugamycin per ml, whereas a *ksgB ksgD* strain was able to grow in the presence of kasugamycin levels in excess of 3,000 μg/ml. This indicates that the mechanisms of resistance coded for by the two genes are independent and synergistic.

Mutations in at least three genes in *Escherichia coli* cause resistance to the aminoglycoside antibiotic kasugamycin. Two of these genes, *ksgA* and *ksgC*, have been characterized as to mechanism of resistance and map position. Mutations in *ksgA* (min 1) eliminate a 16S RNA methylase activity (7, 8, 15, 16). The kasugamycin resistance (Ksg^r) of a *ksgC* strain (min 12) results from an alteration of the amount of ribosomal protein S2 present in the cell (14, 19). Ribosomes from both *ksgA* and *ksgC* strains are resistant to kasugamycin in vitro (14, 16). The mechanism of Ksg^r in *ksgB* mutants has not been elucidated, nor has the gene been well mapped. Ribosomes extracted from *ksgB* strains are not resistant to kasugamycin in vitro (16).

This laboratory has been actively studying the *recE* recombination pathway whose genes are located around min 30 (*recE* and *sbcA*). These genes border on a cotransductional gap of approximately 4 min (min 30 through 34). To more easily manipulate the *sbcA* and *recE* genes, we attempted to find markers that were closely linked. Thus arose our interest in *ksgB*. Since Sparling et al. (16) have presented evidence that *ksgB* maps in this region, we were interested in determining the precise map position of this marker. To aid in this study, we attempted to isolate Tn*5* insertions in *ksgB*. The kasugamycin-resistant mutants obtained in this way did not have Tn*5* inserted into *ksgB*, however, but rather contained insertion mutations in a new locus, *ksgD*.

In this paper, we report transductional mapping of *ksgB*, the isolation by transposon insertion and mapping of an additional Ksg^r gene, *ksgD*, and the quantitation of Ksg^r in *ksgB* and *ksgD* singly and doubly mutant strains.

MATERIALS AND METHODS

Bacterial strains. The strains of *E. coli* K-12 relevant to this paper are listed in Table 1. A characterized colony of each strain was grown in Luria broth and stored at 4°C. This culture served as a working stock.

Media. M9 salts, M9-glucose medium (6), and Luria broth (17) have been described. Luria agar contained 2% agar (Difco Laboratories). When amino acids were required, they were added at a final concentration of 30 to 50 μg/ml. Lambda broth contained 10 g of tryptone (Difco) and 2.5 g of NaCl per liter. Lambda-yeast-maltose was lambda broth with 2 g of maltose and 0.1 g of yeast extract (Difco) per liter. Lambda agar plates were made by adding 1% agar (Difco) to lambda broth. Kanamycin sulfate (Sigma Chemical Co.) was used at concentrations of 30 to 75 μg/ml. Kasugamycin sulfate (Sigma) was used at 600 μg/ml both for selection of mutants resistant to kasugamycin and for screening Ksg^r. Selection of man^+ clones was done on medium composed of M9 salts with 0.4% mannose as the sole carbon source. Selection for $aroD^+$ clones was done on minimal medium (without tryptophan, tyrosine, or phenylalanine). An *aroD* strain was supplemented with 50 μg of shikimic acid and 17 μg of *p*-aminobenzoic acid per ml. Mitomycin C (Sigma) was used at 1 μg/ml. SM buffer for storage of lambda was 0.02 M Tris-hydrochloride–0.1 M NaCl–0.01 M $MgSO_4$ adjusted to pH 7.5. Saline (0.85%) was used as diluent.

P1 transductions. P1 *vir* was used as described by Willetts et al. (17). The Wu formula (18) was used to calculate distances and cotransducibility, with 2.3 min of *E. coli* DNA being the maximum size that can be carried by the P1 phage vector.

Tn*5* mutagenesis. λ *b221 rex*Tn*5* *c*I857 was kindly given to us by Doug Berg. High-titer lysates were made on JC4583 grown on lambda-yeast-maltose. After harvesting, the lysate was stored at 4°C in SM buffer saturated with chloroform.

Mutagenesis was done according to David Botstein (personal communication). An overnight culture of the

TABLE 1. *Strains of E. coli K-12 relevant to this paper*

Strain	Sex	Genotype	Source or reference
CGSC2495	F⁻	*thi-1 thr-1 leuB6 argE3 his-4 trp-35 proA2 thyA20 thyR13 mtl-1 xyl-5 ara-14 galK2 lacY1 tsx-33 rpsL31* λ⁻ *supE44*	*E. coli* Genetic Stock Center
CGSC4237	Hfr(PO43)	*metB1 relA-1* λ^{r-}	Broda (5)
CGSC5496	F⁻	*aroD6 argE3 lacY1 galK2 man-4 mtl-1 rspL700 (=strA700) tsx-29 supE44*	Novel and Novel (13)
CGSC5497	F⁻	*argE3 lacY1 galK2 man-4, mtl-1 tsx-29 supE44 uidA1*	Novel and Novel (13)
CGSC5519	F⁻	*nirA trpA9761 gal-25 rpsL195* λ⁻	Lambden and Guest (10)
FS173	F⁻	*ksgB1 argG6 met-1 leu-2 malA1 xyl mtl lac* Str^r λ⁻	Sparling et al. (16)
JC4583	F⁻	Gal⁻ B_1⁻ EndoI⁻ His⁻	Capaldo-Kimball and Barbour (6)
JC4584	F⁻	*recB21C22* Gal⁻ B_1⁻ EndoI⁻ His⁻	Capaldo-Kimball and Barbour (6)
JC5029	Hfr(PO45)	*thr-300 ilv-318*	A. J. Clark
JC5170	F⁻	*recC22 sbcA4*	Barbour et al. (2)
JC5176	F⁻	*recB21C22 sbcA6*	Barbour et al. (2)
KL16	Hfr(PO45)	*thi-1 relA1* λ⁻	K. B. Low (12)
SDB1041	Hfr(PO45)	Same as JC5029, except *recB21C22 sbcA111*::Tn*5*	Fouts and Barbour (in preparation)
SDB1044	Hfr(PO45)	Same as JC5029, except *recB21C22 sbcA8 ksgD101*::Tn*5 zfi-1*::Tn*10*	This paper
SDB1167	F⁻	Same as JC5176, except *ksgD101*::Tn*5*	This paper (Tn*5* mutagenesis of JC5176)
SDB1168	Hfr(PO45)	Same as KL16, except *sbcA6 ksgD101*::Tn*5*	This paper (P1·SDB1167 → KL16)
SDB1169	Hfr(PO45)	Same as JC5029, except *ksgD101*::Tn*5*	This paper (P1·SDB1168 → JC5029)
SDB1170	Hfr(PO45)	Same as JC5029, except *ksgB1*	This paper (P1·FS173 → JC5029)
SDB1171	Hfr(PO45)	Same as JC5029, except *ksgB1 ksgD101*:: Tn*5*	This paper (P1·SDB1168 → SDB1170)
SDB1172	Hfr(PO43)	Same as CGSC4237, except *ksgD101*::Tn*5*	This paper (P1·SDB1168 → CGSC4237)
SDB1178	F⁻	Same as JC5176, except *ksgD103*, Tn*5* in chromosome	This paper (*ksgD* arising during Tn*5* mutagenesis)
SDB1179	F⁻	Same as JC5176, except *ksgD104*, Tn*5* in chromosome	This paper (*ksgD* arising during Tn*5* mutagenesis)
SDB1180	F⁻	Same as JC5176, except *ksgD102*::Tn*5*	This paper (Tn*5* mutagenesis of JC5176)
SDB1181	F⁻	Same as JC4583, except *ksgD103 zda-1*:: Tn*10*	This paper (P1·SDB1178 → JC4583)

recipient strain, grown on lambda-yeast-maltose, was diluted to 10^8 cells per ml, and 1 ml was infected with phage at a multiplicity of infection of 0.1. This mixture was incubated for adsorption at room temperature for 30 to 45 min. After centrifuging several times to remove unadsorbed phage, the suspension was diluted 1:10 in lambda-yeast-maltose and plated on selective medium (75 μg of kanamycin per ml plus amino acids) with sodium pyrophosphate (0.0025 M). This procedure yielded 100 to 200 independent kanamycin-resistant (Kan^r) clones per plate.

Conjugation. Donor and recipient strains were grown to an optical density at 650 nm of 0.20 in Luria broth, and then 50 ml of each culture was mixed in a 500-ml Erlenmeyer flask. The mating bacteria were incubated without shaking in a 37°C water bath. At various times after mixing 1.0-ml aliquots of the bacteria were sampled, diluted, and blended in a Vortex mixer for 15 s. Dilutions were made in ice-cold saline, and 0.1-ml aliquots were plated on selective media.

Quantitation of Ksg^r. The kasugamycin resistance of a strain was determined by the following procedure. A log-phase culture grown in M9-glucose medium plus amino acids was diluted and plated on the same medium containing various concentrations of kasugamycin (25 to 3,500 μg/ml). Colonies were counted after 48 h of incubation at 37°C. A plate with no kasugamycin served as a control.

RESULTS

P1 transductional mapping of *ksgB1*. The original mutation in *ksgB* isolated by Sparling et al. (16) was used in our P1 transductional mapping of *ksgB*. Table 2 shows the results. *aroD* (min 37.1) is 28% cotransducible with *ksgB1* when *aroD*$^+$ is the selected marker (77 unselected markers of 271 selected markers) and 48% linked when the reciprocal cross is done (82 unselected markers of 171 selected markers). This represents a distance between the markers of 0.7 min. Linkage between *ksgB1* and *man* (min 35.7) is found to be 32%, corresponding to a distance of 0.8 min. Linkage between *aroD* and *man* of 4% (or a distance of 1.5 min) agrees

TABLE 2. *P1 transductional mapping of ksgB1*[a]

Donor	Recipient	Selected Marker	Unselected Marker	Cotransducibility (%)	Distance apart (min)
FS173	CGSC5496	*aroD*$^+$	*ksgB*	28 (77/271)[b]	0.8
		aroD$^+$	*man*$^+$	4 (11/271)	1.5
		ksgB	*aroD*$^+$	48 (82/171)	0.5
		ksgB	*man*$^+$	30 (50/171)	0.8
FS173	CGSC5497	*ksgB*	*man*$^+$	38 (34/89)	0.6
		man$^+$	*ksgB*	28 (156/549)	0.8
FS173	SDB1041	*ksgB*	Kan^s (loss of Tn*5* in *sbcA*)	<1 (0/250)	≥2.0
SDB1041	FS173	Kan^r (Tn*5* in *sbcA*)	Ksg^s (*ksgB*$^+$)	<1 (0/157)	≥2.0

[a] Conclusions: *ksgB* is 0.7 min away from *aroD* and 0.8 min away from *man* at min 36.5.
[b] Number of unselected markers/selected markers tested is given in parentheses.

closely with the current *E. coli* genetic map (1). No linkage is found between *ksgB1* and *sbcA* when a Tn*5* insert in *sbcA* is used as a selection for that gene. These P1 transductional results place *ksgB* halfway between *aroD* and *man* at min 36.5.

Isolation of Ksg^r strains by Tn*5* mutagenesis. Strain JC5176 was subjected to Tn*5* mutagenesis as outlined in Materials and Methods. The initial selection was for Kan^r (75 μg/ml). These clones arose at a frequency of 1 per 10^3 cells infected with the lambda vector. Colonies exhibiting Kan^r were screened for Ksg^r (600 μg/ml). Of the Kan^r clones, 1 in 1.1×10^3 was also Ksg^r. A total of 108 Kan^r Ksg^r clones were isolated. Seven independent isolates obtained from separate experiments were mapped by P1 transduction. Five were found to have Tn*5* in *ksgD*. The other two, also with *ksgD* mutations, did not have a complete Tn*5* in *ksgD* but were Kan^r, indicating that an intact Tn*5* was present somewhere in the chromosome. It is interesting that all seven Kan^r Ksg^r strains were mutations in *ksgD*. None were found in *ksgA*, *ksgB*, or *ksgC* (see below).

Conjugational mapping of the Tn*5* element in a Kan^r Ksg^r strain. Initial mapping of Ksg^r::Tn*5* was done by conjugation. HfrSDB1044 (PO45, transferring counterclockwise from min 61) was mated with F$^-$ JC4583. The time of entry of tetracycline resistance (Tet^r) located at min 59 (*zfil*::Tn*10*) from SDB1044 was 4 min. This served as an internal control. Ksg^r::Tn*5* entered at 30 min, placing the Tn*5* element at about min 30. A total of 99% of exconjugants receiving Kan^r (Tn*5*) also became Ksg^r.

Further conjugational mapping was done by transducing the Ksg^r::Tn*5* out of SDB1167 into strain CGSC4237 (PO43, transferring counterclockwise from min 30). The resulting strain, SDB1172, was used in a mating with CGSC2495 (Trp$^-$ Ksg^s Kan^s). We predicted that if Ksg^r::Tn*5* were counterclockwise from min 30, it would enter very early from SDB1172. If it were clockwise from min 30, it would enter very late. Ksg^r::Tn*5* entered after 70 min of mating, demonstrating that it is clockwise from min 30. *trp* was included as a control marker and had a time of entry of 3 to 5 min.

These data are inconsistent with the Tn*5* being inserted into either *ksgA* or *ksgC* since these genes are located at min 1 and 12, respectively (1). It appeared possible that the Ksg^r mutants had a Tn*5* inserted into *ksgB*. However, by data presented below, this was determined not to be the case. The Tn*5* was inserted into a previously unknown Ksg^r gene, *ksgD*. Therefore, in all figures and tables, as well as in the text below, we refer to these strains as carrying *ksgD*::Tn*5*.

P1 transductional mapping of *ksgD101*::Tn*5*. Based on the data from conjugational mapping of *ksgD101*::Tn*5* and transductional mapping of *ksgB*, we selected markers to test for P1 transductional linkage with *ksgD*. Results are shown in Table 3. There is no linkage between *man* (min 35.7) and *ksgD* (0 of 493), nor is there linkage between *trp* (min 27.5) and *ksgD* (0 of 1,000). When *ksgD* and *sbcA* (min 29.8) are tested for cotransducibility, 44% linkage is found. These data show that Ksg^r is not in the same position as *ksgB* and place the newly isolated gene, *ksgD*, 0.6 min away from *sbcA*. But this does not indicate on which side of *sbcA* the *ksgD* is located. However, it is known that *sbcA* is transferred early from HfrB7 (11; Fouts and Barbour, manuscript in preparation) and that *ksgD* is transferred late (see above). Therefore, *ksgD* is clockwise from *sbcA* at min 30.4.

Isolation of Tn*5*-induced *ksgD* strains in which the Tn*5* is not cotransducible with *ksgD*. In two Ksg^r strains isolated during Tn*5* mutagenesis, Tn*5* was not cotransducible with Ksg^r (SDB1178 and SDB1179). When the Ksg^r genes of these two strains were mapped with respect to a Tn*10* at min 31, they were found to be mutations in *ksgD* (*ksgD103* and *ksgD104*,

TABLE 3. *P1 transductional mapping of ksgD101::Tn5*[a]

Donor	Recipient	Selected marker	Unselected marker	Cotransducibility (%)	Distance apart (min)
SDB1167 and SDB1168	CGSC5497	*man*$^+$	Kan^r (in *ksgD*)	<1 (0/184)[b]	≥1.9
		man$^+$	*ksgD*	<2 (0/81)	≥1.8
		ksgD	*man*$^+$	<2 (0/81)	≥1.8
		Kan^r (in *ksgD*)	*man*$^+$	<1 (0/147)	≥1.9
SDB1167 and SDB1168	AB2495	*trp*$^+$	Kan^r (in *ksgD*)	<1 (0/250)	≥1.9
		Kan^r (in *ksgD*)	*trp*$^+$	<1 (0/750)	≥2.0
SDB1167	JC4584	Kan^r (in *ksgD*)	UV^r/Mito^r (*sbcA*)	57 (31/54)	0.4
SDB1172	JC5170	Kan^r (in *ksgD*)	UV^s/Mito^s (*sbcA*$^+$)	53 (23/43)	0.4
SDB1172	JC5176	Kan^r (in *ksgD*)	UV^s/Mito^s (*sbcA*$^+$)	40 (106/267)	0.6

[a] Conclusions: *ksgD* is 44% cotransducible with *sbcA*, placing it at either min 30.4 or min 29.2. Mito, Mitomycin C.

[b] Number of unselected markers/selected markers tested is given in parentheses.

respectively). Since spontaneous mutation to Ksg^r occurs at a very low frequency in JC5176 (0 of 2,000 random colonies tested were Ksg^r), it is likely that these two mutations not containing Tn*5* within *ksgD* were derived from the Tn*5* mutagenesis. These strains do have an intact Tn*5* elsewhere in the chromosome as judged by the criterion of being Kan^r, and they may be carrying IS*50* in *ksgD* derived from Tn*5* (see below).

Quantitation of Ksg^r in *ksgB*, *ksgD*, and *ksgB ksgD* strains. To differentiate whether *ksgB* and *ksgD* act through the same or different mechanisms to give Ksg^r, an isogenic strain series was constructed, and Ksg^r of each was quantitated. *ksgB1* was transduced into JC5029 from FS173 by selection for Ksg^r. The resultant strain is called SDB1170. *ksgD101*::Tn*5* was transduced into JC5029 via selection for Kan^r and screening for Ksg^r. This strain is SDB1169. Finally, a strain carrying both *ksgB1* and *ksgD101*::Tn*5* was constructed by transducing *ksgD101*::Tn*5* into SDB1170. Selection for Kan^r enabled us to avoid the problem of selecting for possible high-level Ksg^r in a strain already exhibiting Ksg^r. This strain is SDB1171.

Once these strains were constructed, the Ksg^r of each was quantitated as described in Materials and Methods. Results are displayed in Fig. 1. The wild-type strain formed colonies only at very low levels of kasugamycin. It was inhibited down to a surviving fraction of 10^{-4} when kasugamycin was present at 50 μg/ml. A strain carrying either *ksgB* or *ksgD* was resistant to kasugamycin at concentrations up to 600 μg/ml. Ksg^r was the same in three different *ksgD* strains, one with Tn*5* inserted in *ksgD* (SDB1167), one with Tn*5* not in *ksgD* but somewhere else in the chromosome (SDB1178), and a strain with a *ksgD* mutation and no intact Tn*5*, by the criterion of being Kan^s (SDB1181). When the *ksgB* and *ksgD* markers were in the same strain, there was no reduction in viable cell count at 2,400 μg of kasugamycin per ml. In fact, at the highest level of kasugamycin tested, 3,500 μg/ml, there was no significant inhibition. These data indicate that *ksgB* and *ksgD* give resistance to kasugamycin by different mechanisms and when present in the same strain act synergistically to give a very high level of Ksg^r.

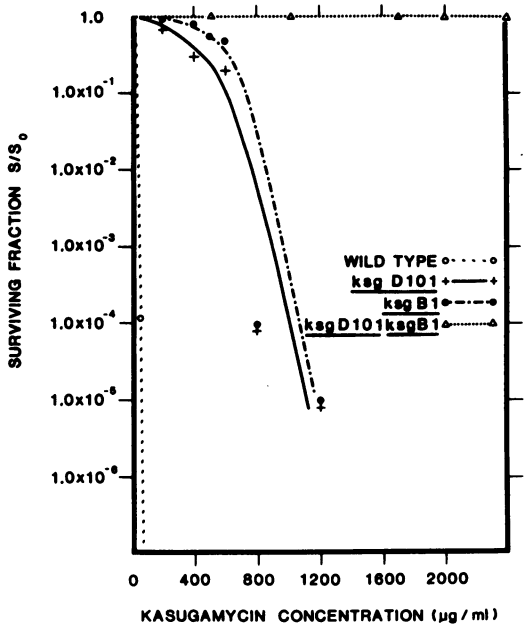

FIG. 1. *Quantitation of Ksg^r as determined by plate assay. Symbols:* ○, *wild type, JC5029;* +, *ksgD101::Tn5, SDB1169;* ●, *ksgB1, SDB1170;* △, *ksgD101::Tn5 ksgB1, SDB1171.*

DISCUSSION

The *ksgD* mutations described are of two types; those having Tn*5* tightly linked with and presumably in *ksgD*, and those having Tn*5* unlinked with *ksgD*. Both classes of mutations exhibit Kan^r. All seven of the Ksg^r strains char-

acterized were found to be mutations in *ksgD*. This is curious since there are at least three other genes that can give Ksg^r to strains carrying them. Other laboratories (14, 16) that have isolated Ksg^r genes by chemical mutagenesis have not found *ksgD* mutants. These data suggest that some process other than simple inactivation of *ksgD* is necessary to get Ksg^r at this locus. Recently, Berg et al. (4) have found that Tn*5* is able to constitutively promote low-level expression of *lacY* when inserted into *lacZ*. Their results suggest that the promoter is within 186 base pairs of the ends of Tn*5*. We have found (in preparation) that both Tn*5* and IS*50* can promote the expression of *recE*. Berg et al. (3) have found that at least one of the IS*50* repeats on Tn*5* can itself transpose. It is possible, therefore, that Tn*5* is promoting expression of *ksgD* and, when Tn*5* is not linked to that gene, the same function is being supplied by an IS*50*.

The Tn*5* insert into *ksgD* was very helpful in the construction of strains carrying both *ksgB* and *ksgD*. These strains were made in a manner similar to that reported by Kleckner et al. (9) in constructing a strain with multiple mutations in the *his* operon. P1 grown on a *ksgD*::Tn*5* donor was transduced into a strain already carrying *ksgB*. Since we were able to select indirectly for *ksgD* by plating on kanamycin, we could avoid using Ksg^r as a selectable phenotype. Once the *ksgB* strain became Kan^r, it was simple to quantitate Ksg^r, which turned out to be quite high (3,500 μg/ml). In retrospect, we can say that the *ksgB ksgD* strain could also have been constructed by doing the same transduction and selecting for high-level Ksg^r and then screening for Kan^r.

When *ksgB* or *ksgD* is present in an *E. coli* strain, the bacteria are resistant to 600 μg of kasugamycin per ml (Fig. 1). A strain carrying both mutations becomes resistant to very high levels to kasugamycin (3,500 μg/ml). If the mutations were acting by similar mechanisms, one would expect at most an additive effect on Ksg^r when both were present in a strain (i.e., resistance to 1,000 to 1,500 μg/ml). Since the resistance is much higher, it is reasonable to assume that *ksgB* and *ksgD* are acting independently and synergistically.

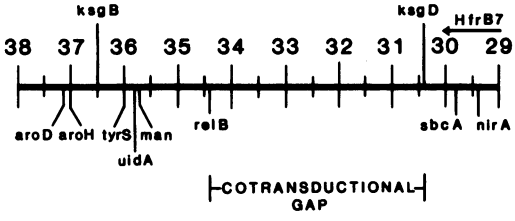

FIG. 2. *Segment of the E. coli genetic map from min 29 to 38 (1). Markers placed above the horizontal line are mapped by data from this paper. HfrB7 transfers sbcA early and ksgD late.*

Figure 2 represents a segment of the *E. coli* genetic map from min 29.0 to 37.5 (1). Markers designated above the horizontal line are mapped by data in this paper; *ksgB* at min 36.5 and *ksgD* at min 30.4. *sbcA* is placed at min 29.8 by transductions to be reported in another publication (Fouts and Barbour, in preparation). HfrB7 has its origin between *ksgD* and *sbcA*, since it transfers *sbcA* early and *ksgD* late.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Teresan Wasie-Gilbert. Laurette Martens and Carol Johnson were helpful in preparing the manuscript. Also, there is much appreciation for the insight and input of Charles G. Miller.

This investigation was supported by Public Health Service research grant GM17329 (awarded to S.D.B.) from the National Institutes of Health, Public Health Service research career development award GM38140 (awarded to S.D.B.) from the National Institute of General Medical Sciences, and Public Health Service training grant GM07250. K.E.F. is a scholar of the Insurance Medical Scientist Scholarship Fund.

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