JOURNAL OF BACTERIOLOGY, Mar. 1985, p. 928–932
0021-9193/85/030928-05$02.00/0

Vol. 161, No. 3

# *recA*-Independent Recombination Between Repeated IS*50* Elements Is Not Caused by an IS*50*-Encoded Function

SUHAS H. PHADNIS AND DOUGLAS E. BERG*

*Departments of Microbiology and Immunology and of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110*

Received 9 July 1984/Accepted 30 November 1984

**Certain pBR322-related plasmids containing direct repeats of the insertion element IS*50* appear to be unstable in *recA Escherichia coli* because smaller recombinant derivatives accumulate rapidly in plasmid DNA populations. We show here that (i) this instability is plasmid specific, but not IS*50* specific; (ii) it is due to a detrimental effect exerted by these plasmids on bacterial growth; and (iii) the growth impairment is alleviated in cells harboring the smaller recombinant plasmids. Although a recent report had concluded that accumulation of recombinants reflected an IS*50*-specific recombination function, when correction is made for the relative growth rates of cells containing the parental and recombinant plasmids the evidence for such a recombination function disappears.**

The movement of transposable elements in bacteria is mediated by element-specific proteins called transposases, which probably recognize and cleave distinctive DNA sequences at the ends of their cognate elements. The movement of transposable elements is independent of extensive DNA sequence homology and of the chromosomal *rec* genes whose products promote generalized recombination. Transposable elements are diverse in size, in DNA sequence, and in functional organization. Consequently there may be several mechanisms of transposition (for reviews, see references 6, 16, 19, 20).

Our current understanding of transposition mechanisms relies, in large part, on identifying those elements that form cointegrates (molecules consisting of vector plus target DNAs joined by direct repeats of the mobile element, the result of replication during transposition) (1, 9, 16) and those that generate simple insertions (molecules consisting of the target replicon plus one copy of the element, but lacking vector sequences) (4, 5, 9). Although simple insertions are likely to be the primary products of transposition of certain elements, similar molecules arise from cointegrates by homologus recombination. In the Tn*3* family they also arise by site-specific recombination mediated by an element-encoded protein called resolvase (1, 15, 16).

The discovery of the Tn*3*-encoded resolvase led to the suggestion that all apparently simple insertions might actually arise by the breakdown of cointegrates (20, 22, 24, 26). Transposon Tn*5* and its component insertion element IS*50* share no homology with Tn*3* (6). Our tests had indicated that cointegrate-like structures with direct repeats of IS*50* are stable in RecA$^-$ cells; however, only simple insertions are generated by the movement of IS*50*-based transposons (4, 5, 7, 17, 18). This implies that IS*50* elements are not subject to specific recombination (resolution) in *Escherichia coli*.

The possibility of a resolvase-like recombination function specific for direct repeats of IS*50*, but expressed only under special physiological conditions, was suggested recently by Zupancic et al. (27). They constructed a plasmid with direct repeats of IS*50* similar to our pTn*5*-DR1 (Fig. 1) and observed the accumulation of smaller recombinant plasmids in RecA$^-$ *E. coli*. The recombinants accumulated most dramatically in recently transformed cell populations and in populations maintained for days in the stationary phase. The stimulation by transformation seemed to parallel the higher levels of transposition seen when Tn*5* was introduced into cells that did not already contain Tn*5* (8, 23). These workers generated stable plasmids with mutations in the transposase gene and reasoned that a product of the transposase gene caused recombination between the IS*50* elements. They implied that Tn*5* transposition might always lead to cointegrates (and thus be replicative, not conservative); if so, the seeming absence of cointegrates after Tn*5* transposition would reflect another physiological condition in which the putative IS*50*-specific resolution function was active.

The experiments presented here show that the reported plasmid instability is actually probably due to low levels of spontaneous *recA*-independent homologous recombination, coupled with more rapid growth of cells harboring the recombinant plasmids.

## MATERIALS AND METHODS

*Escherichia coli* K-12 strain DB1891 Δ*trpE5* Δ(*srl-recA306*), used for all studies of plasmid stability, carries a complete deletion of the *recA* gene (17). DB1873 (Δ*trpE5 recA1* and lysogenic for a λ *red* phage; 25) was used as the host in assays of IS*50*-mediated transposition to phage λ. Plasmid pTn*5*-DR1 was derived from a pBR322::Tn*5* plasmid by the in vitro reversal of a restriction (*Bam*HI) fragment containing one IS*50* element, resulting in a plasmid containing direct instead of inverted repeats of IS*50* elements (17). pTn*5*-DR1Δ was generated by homologous recombination between the directly repeated IS*50* elements (17). Dimeric forms of pTn*5*-DR1 and of pTn*5*-DR1Δ were generated by homologous recombination in vivo (25). These plasmids are diagrammed in Fig. 1.

Bacteria were grown in LN broth (10 g of Humko-Sheffield N-Z amine type A, 5 g of Difco yeast extract, and 10 g of NaCl per liter, adjusted to pH 7.2) or on LN agar (LN broth solidified with 1.5% Difco Bacto-agar). Unless otherwise specified, plasmid DNAs were extracted from bacterial clones at a population size of about $2 \times 10^9$ cells, that is, after approximately 31 generations of growth from the founding single cell. Kanamycin and ampicillin were used at 60 and 250 μg/ml, respectively, unless otherwise noted.

* Corresponding author.

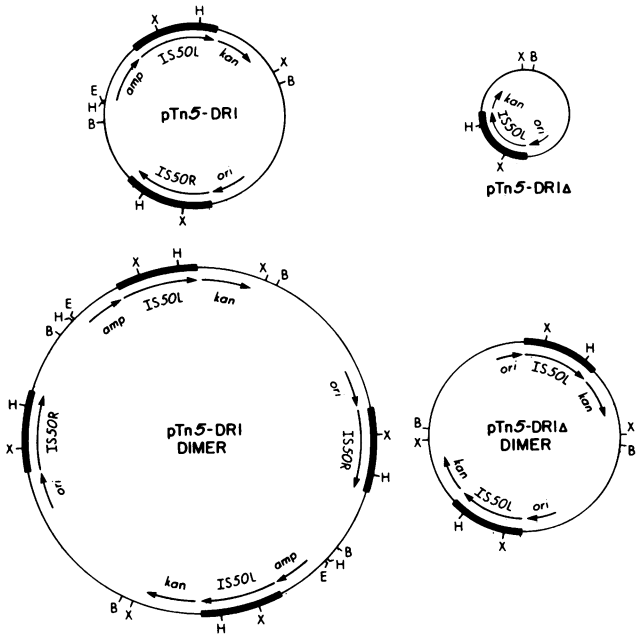


FIG. 1. Plasmid structures. Monomeric pTn5-DR1 (10 kb) contains direct repeats of the insertion elements $IS50_L$ and $IS50_R$, which are present in Tn5 wild type as terminal inverted repeats (17). Monomeric pTn5-DR1Δ (6 kb) was derived from Tn5-DR1 by in vivo recombination between directly repeated IS50 elements (thickened lines) and the loss of one of the elements plus the segment between them, which includes the *amp* gene. Dimeric pTn5-DR1 (20 kb) and dimeric pTn5-DR1Δ (12 kb) plasmids were generated from the monomers depicted directly above them by homologous recombination in vivo (17, 25). The letters B, E, H, and X indicate the sites cleaved by the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Xho*I, respectively.

Standard procedures were used for bacterial growth, plasmid DNA extraction, restriction endonuclease digestions, DNA polymerase (Klenow), and T4 DNA ligase reactions (with enzymes purchased from New England Biolabs, Bethesda Research Laboratories, and P-L Biochemicals), agarose gel electrophoresis, calcium-facilitated transformation, and IS50-mediated gene transposition to bacteriophage lambda (7, 21, 25).

Mutations in the *Hin*dIII and *Xho*I sites of pTn5-DR1 were generated by partial digestion of covalently closed circular plasmid DNA and the copying of sequences in the 4-base 5′ extensions by the Klenow fragment of DNA polymerase I. In the case of mutations at the *Xho*I site, ligation was carried out in the presence of octanucleotide *Sac*I linkers (5′-CGAGCTCG-3′) from New England Biolabs.

## RESULTS

**Choice of plasmids.** We used plasmid pTn5-DR1 (Fig. 1) of Hirschel and Berg (17) instead of plasmid pZ4 of Zupancic et al. (27) to test the idea (27) that an IS50-encoded protein causes IS50-specific recombination between directly repeated IS50 elements. Plasmids pTn5-DR1 and pZ4 are similar; each was generated by the in vitro inversion of an IS50-containing (*Bam*HI) fragment of a pBR322::Tn5 plasmid in which $IS50_R$ was closest to the replication origin and in which $IS50_L$ was closest to the *amp* promoter. However, pZ4 encodes only kanamycin resistance ($Kan^r$), because its parent contained Tn5 inserted in the *amp* gene and inversion of the *Bam*HI fragment split the *tet* gene (27), whereas pTn5-DR1 encodes ampicillin resistance ($Amp^r$) as well as $Kan^r$ because its parent contained Tn5 just distal to *amp* (17). Our choice of pTn5-DR1 was in recognition of potential advantages of using a plasmid containing two markers separated by IS50 elements (Fig. 1).

**Bacterial growth.** The interpretation that IS50 encodes an IS50-specific recombination function was based on the large yield of recombinants relative to the parental pZ4 plasmid extracted from $RecA^-$ cells grown on media containing 60 or 200 μg of kanamycin per ml (27). Ideally, for the rate of accumulation of a recombinant plasmid to accurately reflect the rate of its formation, the parent and recombinant should have similar effects on cell growth (3, 10, 13). Comparison of colony sizes can be a sensitive indicator of growth rate, and Zupancic et al. (27) noted that cells harboring their pZ4 plasmid made smaller colonies than cells harboring its recombinant derivatives. Similarly, the colonies made by strain DB1891 carrying pTn5-DR1 were much smaller than those made by cells harboring any of a series of control plasmids (e.g., the pBR322::Tn5 parent of pTn5-DR1, pTn5-DR1Δ [the recombinant derivative of pTn5-DR1; Fig. 1], or pTn5-DR2 [which contains direct repeats of IS50 elements, but arranged differently vis a vis the rest of the plasmid; 17]). On antibiotic-free media, the pTn5-DR1 plasmid also reduced colony size, but less markedly than on kanamycin-containing medium (17). We found that the plating efficiency of cells carrying pTn5-DR1 relative to cells carrying the control plasmids was reduced to 0.1 on agar containing 60 μg of kanamycin per ml and to 0.01 on agar containing 200 μg of kanamycin per ml. Finally, 18 h of incubation, rather than the usual 12, was required for cells harboring pTn5-DR1 to form visible colonies. These findings suggested that differences in growth rate would contribute to the final yield of recombinant plasmids.

Plasmid pTn5-DR1 was transformed into a derivative of strain DB1891, which already harbored a chromosomal insertion of Tn5 wild type. The transformants (initially selected for $Amp^r$) were inhibited by kanamycin to the same extent as those that did not contain Tn5. Thus the peculiar sensitization to kanamycin caused by pTn5-DR1 is epistatic to the resistance of an unlinked *kan* gene.

**Medium-dependent accumulation of the recombinant plasmid pTn5-DR1Δ.** Plasmid DNAs extracted from clones of the $RecA^-$ strain DB1891 (Tn5 free) carrying pTn5-DR1 and grown for about 31 generations from the founding cell in the absence of antibiotics were generally homogeneous and identical in size to the starting pTn5-DR1 plasmid if grown without kanamycin (Fig. 2, lane 1). Prolonged growth (e.g., another 38 h; Fig. 2, lanes 2 through 5) led to the appearance of pTn5-DR1Δ. This accumulation was most pronounced when kanamycin was included in the growth medium (Fig. 2, lane 4) and was diminished somewhat when ampicillin was also included to kill cells carrying only pTn5-DR1Δ (lane 5). Dimeric pTn5-DR1 also inhibited colony formation on kanamycin-containing agar, and monomeric and dimeric pTn5-DR1Δ recombinant derivatives of dimeric pTn5-DR1 were frequent among the plasmid DNAs extracted from cells grown on kanamycin-containing agar (Fig. 2, lane 8), but not in cells grown on antibiotic-free agar (lane 6).

**Transformation and plasmid stability.** The dimeric form of pTn5-DR1 was used to test whether transformation stimulates recombination as had been postulated (27), or whether the use of kanamycin to select transformants selects preferentially those subclones which carry newly arisen recombinant plasmids. The following three classes of recombinant derivatives of dimeric pTn5-DR1 retain an *amp* gene and

thus can grow on ampicillin-containing medium: monomeric pTn*5*-DR1, a partial dimer containing one copy of the *amp* segment and two copies of *kan*, and a partial dimer containing two copies of *amp* and one copy of *kan* (17). Although selection of clones transformed with dimeric pTn*5*-DR1 on kanamycin agar resulted in the expected appearance of monomeric pTn*5*-DR1Δ (Fig. 2, lane 10), selection on ampicillin led only to dimeric pTn*5*-DR1. None of the Amp$^r$ recombinant species was found (Fig. 2, lane 11). We conclude therefore that transformation per se does not stimulate *recA*-independent crossing over between directed repeated IS*50* elements.

**Transposase and the accumulation of recombinants.** To determine whether the transposase protein (or the transposition inhibitor encoded by the same *tnp* gene; 23) is involved in the accumulation of recombinants as had been postulated (27), we generated two simple mutations in *tnp*. One mutation resulted from duplication of 4 base pairs at the *Hin*dIII site of IS*50*$_R$, and the second resulted from insertion of the octanucleotide 5′-CGAGCTCG-3′ into the filled-in *Xho*I site of IS*50*$_R$ (see above; Fig. 1). Although each mutation in IS*50*$_R$ destroyed transposition activity in our standard tests (17, 25), neither mutation reversed the deleterious effects of pTn*5*-DR1 on bacterial growth. The examination of plasmid DNAs from 10 subclones of DB1891

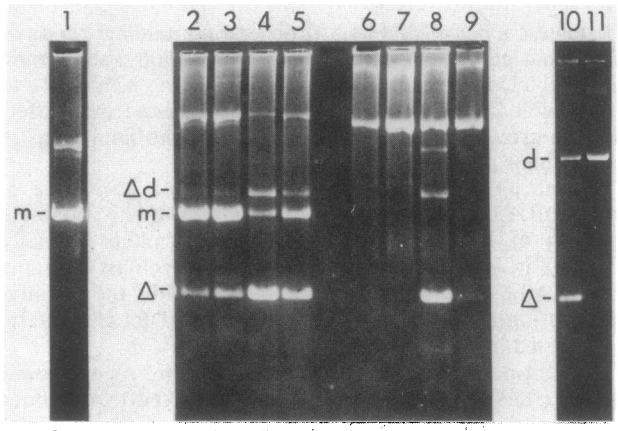


FIG. 2. Accumulation of recombinant derivatives of pTn*5*-DR1 plasmids. Lane 1 shows a typical profile of plasmid DNA from a clone of cells started by DB1891 carrying monomeric plasmid pTn*5*-DR1. A young single colony isolate was picked to 2 ml of antibiotic-free broth and incubated with aeration at 37°C to the early stationary phase (about 8 h), and plasmid DNA was then extracted. Lanes 2 through 5 show plasmid DNAs extracted 38 h after plating samples of $10^8$ cells carrying monomeric pTn*5*-DR1 (lane 1) on agar medium containing no antibiotics (lane 2), 250 μg of ampicillin per ml (lane 3), 200 μg of kanamycin per ml (lane 4), and 200 μg of kanamycin plus 250 μg of ampicillin per ml (lane 5). Lanes 6 through 9 show plasmid DNAs extracted 38 h after plating samples of $10^8$ cells carrying dimeric pTn*5*-DR1 (lane 11) on agar medium containing no antibiotic (lane 6), 250 μg of ampicillin per ml (lane 7), 200 μg of kanamycin per ml (lane 8), and 200 μg of kanamycin plus 250 μg of ampicillin per ml (lane 9). (The two faint bands in lane 8 between monomeric pTn*5*-DR1Δ and dimeric pTn*5*-DR1 probably correspond to dimeric and trimeric pTn*5*-DR1Δ.) Lane 10 shows a typical profile of plasmid DNA from a clone of cells transformed with dimeric pTn*5*-DR1 and selected on medium containing 60 μg of kanamycin per ml. Lane 11 shows a typical profile of plasmid DNA from the same transformation as in lane 10, but with selection for Amp$^r$ instead of Kan$^r$. m, d, Δm, and Δd correspond to the positions of monomeric pTn*5*-DR1, dimeric pTn*5*-DR1, monomeric pTn*5*-DR1Δ, and dimeric pTn*5*-DR1Δ, respectively.

transformed with each mutant revealed an accumulation of pTn*5*-DR1Δ recombinant plasmids during growth with kanamycin which was indistinguishable from that found with the parental pTn*5*-DR1 plasmid (data not shown). Similarly, mutations at the *Hin*dIII and *Xho*I sites in the central region of Tn*5*-DR1 and in the *Xho*I site in IS*50*$_L$ did not alter bacterial growth rates or change plasmid stabilities (12 clones of each mutant were examined; data not shown).

The IS*50* elements are the only components of Tn*5* directly involved in transposition (6, 7). There is only a single large open reading frame in IS*50*; it is translated from two sites, resulting in the transposase and in the shorter inhibitor of transposition. The absence of the other open reading frames in IS*50*, coupled with the stability of those plasmids containing directed repeated IS*50* elements that do not adversely affect cell growth (5–7, 17, 18), make the possibility of a Tn*5*-encoded resolvase function resembling that of Tn*3* extremely remote.

**Other mutations can lead to plasmid stabilization.** When samples of cultures carrying pTn*5*-DR1 that had been grown in kanamycin-free medium were spread on kanamycin- or ampicillin-containing agar, typically about 1% of the resultant colonies grew more rapidly than the majority of colonies. Plasmid DNAs extracted from four of these exceptional subclones taken from kanamycin-containing agar were unchanged in size and in the positions of restriction sites diagrammed in Fig. 1. They efficiently transformed strain DB1891 to an Amp$^r$ Kan$^r$ phenotype, and transformant clones also grew well and contained stable plasmids. Thus, although inactivation of the transposase function does not suppress the tendency of pTn*5*-DR1 to inhibit host cell growth, other uncharacterized mutations in the plasmid can render pTn*5*-DR1 noninhibitory and thereby increase its stability.

**Growth advantage conferred by pTn*5*-DR1Δ.** The selection for cells harboring recombinants was illustrated by changes in the relative proportions of pTn*5*-DR1 and pTn*5*-DR1Δ plasmid DNAs extracted from cell populations grown together for at least eight generations. The dimeric (12-kilobase [kb]) form of pTn*5*-DR1Δ (Fig. 1) was used so that gel electrophoresis could distinguish descendants of the input recombinant plasmids from any recombinant (6-kb) plasmids that might arise and be selected during the period of mixed growth. Figure 3, lanes 1 and 14, shows that the mixture of cells used to start the competition experiment contained equal concentrations of 10-kb pTn*5*-DR1 (lowest band) and 12-kb pTn*5*-DR1Δ (dimer) (next lowest band). After 20 and 40 h of growth on ampicillin-containing medium (Fig. 3, lanes 3 and 4), pTn*5*-DR1Δ had been lost; the predominant plasmids were the sizes of input monomeric pTn*5*-DR1 (bottom band) and its dimeric derivative (top band), which arises by homologous, but *recA*-independent, recombination (12, 14). More important for the assessment of the role of selection in the accumulation of recombinant plasmids, there was a severalfold enrichment for pTn*5*-DR1Δ even during growth on antibiotic-free agar (Fig. 3, lanes 4 and 5). Growth with high levels of kanamycin caused more intense selection for pTn*5*-DR1Δ (Fig. 3, lanes 12 and 13). Thus, the high rate of accumulation of recombinant derivatives of pTn*5*-DR1 in RecA$^-$ cells reflects selection. There is no evidence for induction of a novel DNA exchange reaction specific to IS*50*.

## DISCUSSION

The suggestion that the transposase gene within IS*50* also encodes a recombination activity specific for direct repeats

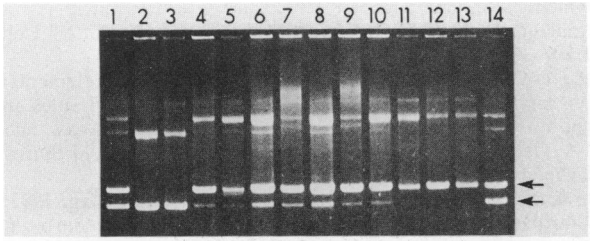


FIG. 3. Competition between pTn5-DR1 and pTn5-DR1Δ. Equal numbers of cells in young stationary-phase cultures harboring monomeric pTn5-DR1 (10 kb) and dimeric pTn5-DR1Δ (12 kb) were mixed to achieve an equimolar starting ratio of the two plasmid types (lanes 1 and 14). Samples of the starting mixtures ($10^8$ cells) were spread on media containing 250 μg of ampicillin per ml (lanes 2 and 3), no antibiotic (lanes 4 and 5), 10 μg of kanamycin per ml (lanes 6 and 7), 25 μg of kanamycin per ml (lanes 8 and 9), 60 μg of kanamycin per ml (lanes 10 and 11), and 200 μg of kanamycin per ml (lanes 12 and 13). The cell populations were incubated for 20 h (odd-numbered lanes) or 42 h (even-numbered lanes) before plasmid DNA extraction. The bacteria had undergone about eight doublings in cell number and had reached stationary phase during the first 20 h of incubation. The arrows on the right indicate the positions of dimeric pTn5-DR1Δ and monomeric pTn5-DR1 (upper and lower arrows, respectively). The top bands correspond to higher oligomeric forms of monomeric pTn5-DR1 and dimeric pTn5-DR1Δ.

of IS50 (27) was intriguing. If correct it could have led to a straightforward in vitro assay for transposase based on intramolecular recombination, and it would have required a reassessment of our hypothesis (4, 5) that Tn5 transposition is conservative. The $Kan^r$ $Amp^s$ plasmid that led Zupancic and co-workers (27) to this suggestion, like our $Kan^r$ $Amp^r$ pTn5-DR1 (17), impairs growth on kanamycin-containing media, whereas the recombinant derivatives of these plasmids do not. It was assumed, however (27), that (i) the speed of recombinant accumulation depended solely on the rate of crossing over, (ii) the increased yield of recombinants after transformation reflected an induction of recombination, and (iii) stable plasmids with mutations in the transposase gene had, in fact, been stabilized by loss of the transposase function.

The results presented here show that (i) cells containing recombinant (pTn5-DR1-Δ) plasmids grow better than those containing pTn5-DR1, especially in the presence of kanamycin, (ii) recombinants do not accumulate after transformation when plasmid-carrying cells are selected with ampicillin instead of kanamycin, and (iii) simple inactivation of the transposase gene does not alleviate the growth inhibition or instability characteristic of pTn5-DR1, but that other mutations in the plasmid can reverse the inhibition. Our studies, along with characterizations of unstable Hfr strains (10) and of auxotroph accumulation in DNA polymerase I-deficient *E. coli* (3) and numerous studies of selection in bacterial chemostats (13) illustrate how differences in growth rate can lead to the proliferation of rare variants in a population. If the effects of selection are not considered, measurements of frequencies of these variants can lead to erroneous estimates of the rates of their formation.

The detrimental effects caused by pTn5-DR1 and pZ4 are unusual and not generally seen when other pBR322::Tn5-related plasmids are examined. The instability of pTn5-DR1 derivatives with mutations at the *Hin*dIII and at the *Xho*I sites of $IS50_R$ showed that selection is independent of transposase. Selection is most intense in kanamycin-containing medium, but it is also evident in antibiotic-free medium. The Tn5-encoded aminoglycoside phosphotransferase responsible for kanamycin resistance is periplasmic (11); thus the selection might be caused by a cell membrane alteration, e.g., a fusion protein whose amino- and carboxy-terminal regions come from the *tet* gene and the central segment of Tn5, respectively. Although the mechanism by which pTn5-DR1 inhibits growth remains unknown, it is the difference in growth rates, not an extraordinarily high frequency of recombination, that is primarily responsible for instability. We ascribe the rare crossovers between the direct repeats of IS50 to the pathway of *recA*-independent recombination (12, 14), shown previously to operate on bacterial plasmids (in studies which did not involve transposable elements). There is no reason to postulate that instability involves any special characteristic of the sequence of IS50 or that IS50 encodes a function that causes recombination between IS50 elements.

## ACKNOWLEDGMENTS

We are grateful to G. Adelt and B. Khursheed for expert technical assistance and to C. M. Berg for critical readings of this manuscript.

This work was supported by Public Health Service research grants 5 RO1 AI 14267 and 1 RO1 AI 18980 to D.B. from the National Institutes of Health.

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